RESPONSE OF WHEAT PLANTS (*TRITICUM AESTIVUM* L) TO STRESS AND SYNTHETIC ELICITORS OF SYSTEMIC ACQUIRED RESISTANCE AS EXPRESSED BY PHENOLIC LEVELS IN FOLIAGE AND MATURE GRAIN

by

OSCAR F RAMOS

B.S., Zamorano, 2004
M.S., Purdue University, 2009

AN ABSTRACT OF A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Abstract

Producers of whole wheat products are interested in marketing the health-promoting benefits of wheat antioxidants. However, they need a steady crop supply with consistent levels of antioxidants. The variable phenolic content in wheat crops is a problem. The objectives of this research were to 1) identify the factor (s) that contribute the most to the variability in phenolic content, 2) understand the mechanism (s) responsible for phenolic synthesis, and 3) artificially trigger that mechanism (s). Phenolics are hypothesized to be part of the defense response of hard red winter wheat (*Triticum aestivum* L) to stress. The effect of insect feeding, pathogen infection, and heat stress on phenolics in grains from wheat plants cv. Karl 92 was evaluated. Bird-cherry oat aphid (*Rhopalosiphum padi*) feeding stress significantly explained the variation in phenolic content. Furthermore, the relative allocation of carbon resources to grain yield/phenolic content was influenced by the stage of the plant at which aphid feeding started to occur. Based on these findings, phenolics were hypothesized to be an active defense response acting through a mechanism known as systemic acquired resistance (SAR). In order to prove this hypothesis, several synthetic elicitors of SAR were tested for their effectiveness at inducing *de novo* phenolic synthesis in wheat foliage and in mature grains. Elicitors that acted through the salicylic- and jasmonic acid signaling pathways were effective at inducing phenolic synthesis by 49% and 177%, respectively, in the leaves 36 hours post spray application. They also elicited a phenolic response in mature grains of up to 21% induction. Enhancement of the levels of naturally occurring phenolic compounds with antioxidant activity in wheat grains through SAR activation is a value addition strategy that can potentially increase the profitability of hard red winter wheat crops. It can also provide manufacturers of whole wheat with natural antioxidants that can potentially be used to substitute their synthetic counterparts in wheat based products.

RESPONSE OF WHEAT PLANTS (*TRITICUM AESTIVUM* L) TO STRESS AND SYNTHETIC ELICITORS OF SYSTEMIC ACQUIRED RESISTANCE AS EXPRESSED BY PHENOLIC LEVELS IN LEAVES AND MATURE GRAIN

by

OSCAR F RAMOS

B.S., Zamorano, 2004
M.S., Purdue University, 2009

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Approved by:

Co-Major Professor
Praveen Vadlani, PhD, MBA

Approved by:

Co-Major Professor
Ronald L. Madl, PhD

Copyright

OSCAR F RAMOS

2016

Abstract

Producers of whole wheat products are interested in marketing the health-promoting benefits of wheat antioxidants. However, they need a steady crop supply with consistent levels of antioxidants. The variable phenolic content in wheat crops is a problem. The objectives of this research were to 1) identify the factor (s) that contribute the most to the variability in phenolic content, 2) understand the mechanism (s) responsible for phenolic synthesis, and 3) artificially trigger that mechanism (s). Phenolics are hypothesized to be part of the defense response of hard red winter wheat (*Triticum aestivum* L) to stress. The effect of insect feeding, pathogen infection, and heat stress on phenolics in grains from wheat plants cv. Karl 92 was evaluated. Bird-cherry oat aphid (*Rhopalosiphum padi*) feeding stress significantly explained the variation in phenolic content. Furthermore, the relative allocation of carbon resources to grain yield/phenolic content was influenced by the stage of the plant at which aphid feeding started to occur. Based on these findings, phenolics were hypothesized to be an active defense response acting through a mechanism known as systemic acquired resistance (SAR). In order to prove this hypothesis, several synthetic elicitors of SAR were tested for their effectiveness at inducing *de novo* phenolic synthesis in wheat foliage and in mature grains. Elicitors that acted through the salicylic- and jasmonic acid signaling pathways were effective at inducing phenolic synthesis by 49% and 177%, respectively, in the leaves 36 hours post spray application. They also elicited a phenolic response in mature grains of up to 21% induction. Enhancement of the levels of naturally occurring phenolic compounds with antioxidant activity in wheat grains through SAR activation is a value addition strategy that can potentially increase the profitability of hard red winter wheat crops. It can also provide manufacturers of whole wheat with natural antioxidants that can potentially be used to substitute their synthetic counterparts in wheat based products.

Table of Contents

List of Figures xi

List of Tables xiii

Acknowledgements xxi

Dedication xxii

Chapter 1 - Introduction 1

References 4

Chapter 2 - Hypothesis and objectives 7

References 10

Chapter 3 - Effect of insect feeding, pathogen infection, and heat stress on antioxidant properties of wheat bran 12

Abstract 12

Introduction 13

Materials and methods 14

Materials. 14

Experimental Design 15

Insect feeding: 15

Rust infection: 16

Heat stress: 16

Sample Preparation. 16

Extraction Procedure. 17

Free phenolic fraction: 18

Bound fraction: 18
Determination of Total Phenolic Content. 18
Determination of Antioxidant Capacity. 19
Statistical Analysis. 20
Results and discussion 20
Comparison of the phenolic concentrations observed in these studies with those found in the literature 21
Effects of stress on plant performance 23
Heat stress. 23
Insect feeding stress. 23
Rust infection. 24
Effect of stress on phenolic concentration and antioxidant capacity 24
Insect feeding stress. 24
Heat Stress. 25
Rust Infection Stress. 26
Abbreviations used 28
Figures 29
Tables 34
References 39
Chapter 4 - Bird-cherry oat aphid (*Rhopalosiphum padi*) feeding stress induces enhanced levels of phenolics in mature wheat grains 44
Abstract 44
Introduction 45

Materials and methods **47**
Plant materials. 47
Aphid colony. 47
Aphid feeding stress. 47
Experimental design. 48
Grain sample preparation. 49
Extraction procedure. 49
Free fraction: 49
Free and conjugated fraction: 50
Bound fraction: 50
Determination of total phenolic content. 50
Determination of antioxidant potential. 51
Analytical testing. 51
Statistical analysis. 51
Results **52**
Discussion **53**
Comparison of phenolic concentration in the free, free and conjugated and bound fractions. 53
R. padi feeding treatments. 53
Fitness cost. 55
Potential mechanism. 55
Tables **57**
References **60**

Chapter 5 - Synthetic elicitors of systemic acquired resistance promote *de novo* synthesis of phenolics in foliage of hard red winter wheat (*Triticum aestivum* L) 63

Abstract... 63

Materials and methods 66

Plant material. 66

Elicitor treatments. 66

Sample collection and storage.. 67

Experimental design and statistical analyses. 67

Phenolic extraction procedure.. 68

Determination of total phenolic content (TPC). 68

Statistical analysis. 69

Results 69

Effect of elicitor treatments over time. 69

Two- and three-way interactions.. 70

Discussion 70

Abbreviations used 75

Tables 76

Figures... 78

References.. 80

CHAPTER 6 - Synthetic elicitors of systemic acquired resistance administered to wheat (*Triticum aestivum* L) plants at the jointing stage induced phenolics levels in mature grains 85

Abstract... 85

Introduction **86**

Materials and methods **87**

Plant Materials. 87

Elicitor Treatments. 87

Experimental Design. 88

Grain sample preparation. 89

Extraction Procedure. 89

Determination of Total Phenolic Content. 89

Statistical analysis. 90

Results **90**

Covariance and ANOVA analysis. 90

Effect of elicitors on yield. 91

Effect of elicitor treatments. 91

Cultivar effects. 91

Discussion **91**

Abbreviations used **94**

Tables **95**

Figures **96**

References **97**

Chapter 7 - Summary and Conclusions 99

Chapter 8 - Future Research 101

Chapter 9 - Appendix list 103

Appendix A - Statistical analysis 103

List of Figures

Figure 3-1 Left: wheat plants exposed to elevated temperatures (30/25 degrees Celsius day/night and 84-90% RH). Right: control plants were kept at 20/15 degrees Celsius day/night and 50-70% RH. 29

Figure 3-2 Top: Distribution of kernel minor diameter (mm) from heat stressed and control grains. Bottom: Representative grain sample from heat stressed and control plants.......... 30

Figure 3-3 Insect feeding. From left to right: insect infested (covered with nets) and control plants (uncovered), close-up of insect colonies feeding on the stem of the wheat plant, close-up of Bird cherry oat aphid.......... 31

Figure 3-4 Plants infested with an avirulent strain of rust (Puccinia triticinia).......... 32

Figure 3-5 Resistant and susceptible phenotypes to leaf rust (Puccinia triticinia), from left to right: Thatcher+Lr34, Thatcher+Lr12, Thatcher+Lr13, Thatcher (Susceptible). Source: Kolmer (2013).......... 33

Figure 5-1 Least Square Means +/- 95% confidence interval total phenolic content (mg ferulic acid equivalents /g dry leaf tissue), in leaves of Karl 92 and Ike wheat plants treated with SAR elicitors at 24-, 36- and 48 hours post application. Red lines indicate TPC levels 24 h before treatment application of 50 mM thiamine hydrochloride (B1), 5mM riboflavin (B2), 1mM benzo (1,2,3) thiadiazole (BTH), 0.7 mM 2,6-dichloropyridine-4-carboxylic acid (DCPCA), ethanol control (EtOH), distilled deionized water (H_2O), 44 mM methyl jasmonate (MeJa), unsprayed control (null), and 50 mM sodium salicylate (SS)………....78

Figure 5-2 Total phenolic content (mg ferulic acid equivalent/g dry leaf) in leaves of two Ike replicate plants at 24-, 36-, and 48 hours post application of the following SAR elicitor treatments and controls: 50 mM thiamine hydrochloride (B1), 5mM riboflavin (B2), 1mM

benzo (1,2,3) thiadiazole (BTH), 0.7 mM 2,6-dichloropyridine-4-carboxylic acid (DCPCA), ethanol control (EtOH), distilled deionized water (H_2O), 44 mM methyl jasmonate (MeJa), not sprayed control (Null), and 50 mM sodium salicylate (SS)…………………………..79

Figure 6-1 Total phenolic content of wheat bran extracts from not sprayed, solvent-only, and plants treated with thiamine (B1), riboflavin (B2), 2,6-dichloropyridine-4 carboxylic acid (INA), sodium salicylate (SS), methyl jasmonate (MJ), and benzo(1,2,3)thiadiazole-7 carbothioic acid S-methyl ester (BTH). Means and standard errors are shown (n=3). Bars with "*" indicates significant difference from not-sprayed control, and bars with "**" indicates significant difference from not-sprayed and solvent-only treated plants (Bonferroni adjusted P<0.004167)………………………………………………………………….96

List of Tables

Table 3-1. Mean±SE spikes/pot, grain yield/pot, and grain yield/spike (3 plants/pot). Number of replications (N) is shown in parenthesis. 34

Table 3-2. Single kernel moisture, weight, and minor diameter characterization (n=300 kernels), and bran milling yield of composited grain samples from plants exposed to different stresses. Means and standard deviations are shown. 35

Table 3-3. Total phenolic content (TPC) and % DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenged by free and bound fractions extracted from wheat bran of plants fed on by *R. padi* for 45 days. Means and standard errors from six grain subsamples are shown. 36

Table 3-4. Total phenolic content (TPC) and % DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenged by free and bound fractions extracted from wheat bran of plants grown at 30/25°C day/night from 21 days after first spike until physiological maturity. Means and standard errors from six grain subsamples are shown. 37

Table 3-5. Total phenolic content (TPC) and % DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenged by free and bound fractions from wheat bran of plants infected with a compatible race of *Puccinia triticina* for 21 days. Means and standard errors from six grain subsamples are shown. 38

Table 4-1. Number of spikes and grain yield from Karl 92 plants in *R. padi* feeding treatments and *R. padi*-free control. 57

Table 4-2. Mean ± SE phenolic concentration (µg FAE/g bran) in the free, free and conjugated, and bound fraction of bran extracts from Karl 92 wheat plants stressed by bird-cherry oat aphid (Rhopalosiphum padi) feeding at 5-tiller, 7 or 21 days post-anthesis (DPA) for 14 days, or moderate feeding at 5-tiller through 35 DPA. 58

Table 4-3. Mean ± SE percentage of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenged after 30 minutes by free, free and conjugated, and bound phenolics in bran extracts from Karl 92 wheat plants stressed by bird-cherry oat aphid (Rhopalosiphum padi) feeding at the 5-tiller, 7 or 21 days post-anthesis (DPA) for 14 days, or moderate feeding at 5-tiller through 35 DPA. 59

Table 5-1. Least Square Means ± SE total phenolic content (mg ferulic acid equivalent/g dry leaf) of hard red winter wheat leaves at 24-, 36-, and 48 h post application of SAR elicitors and controls. Average across evaluation times is shown. P-values for the test $\mu_{elicitor}$-$\mu_{control}$>0 are shown. ns = not significant at (P > 0.1) 76

Table 5-2. Least Square Means ± SE total phenolic content (mg ferulic acid equivalent/g dry leaf) of hard red winter wheat cv. Ike leaves at 24-, 36- and 48 h post application of SAR elicitors and controls 77

Table 6-1. Mean±SD (g) grain yield per pot (3 plant/pot) in grams. P values shown for the difference between elicitors and control. Number in parenthesis is number of pots 95

Table 6-2 Mean ± standard error total phenolic content (µg ferulic acid equivalent/g bran) of wheat bran extract from both Karl 92 and Ike wheat non-sprayed (Null), solvent-only (H2O or EtOH), and plants treated with thiamine (B1), riboflavin (B2), 2,6-dichloropyridine-4 carboxylic acid (INA), sodium salicylate (SS), methyl jasmonate (MJ), and benzo(1,2,3)thiadiazole-7 carbothioic acid S-methyl ester (BTH). 95

Table 9-1. Two sample T-tests for unequal variances for the effect of insect feeding on grain yield per pot (grams) 103

Table 9-2. Two sample T-tests with unequal variances for the effect of insect feeding on number of spikes per pot 103

Table 9-3. Two sample T-tests with unequal variances for the effect of insect feeding on grain yield per spike (grams) 103
Table 9-4. Two sample T-test with unequal variances for the effect of heat stress on grain yield per pot (grams) 104
Table 9-5. Two sample T-test with unequal variances for the effect of heat stress on number of spikes per pot 104
Table 9-6. Two sample T-test with unequal variances for the effect of heat stress on grain yield per spike (grams) 105
Table 9-7. Two sample T-tests with unequal variances for the effect of rust infection on grain yield per pot (grams) 105
Table 9-8. Two sample T-test with unequal variances for the effect of rust infection on number of spikes per pot 105
Table 9-9. Two sample T-tests with unequal variances for the effect of rust infection on grain yield per spike (grams) 106
Table 9-10. Proc Glimmix model information for the effect of insect feeding on free and bound total phenolic content (TPC) and % DPPH scavenged after 30 min (DPPH) 106
Table 9-11. Type III of fixed effects for the effect of insect feeding stress, extraction, and milling on free TPC 106
Table 9-12. Type III of fixed effects for the effect of insect feeding, extraction, and milling on bound TPC 107
Table 9-13. Type III of fixed effects on the effect of insect feeding, extraction, and milling on DPPH scavenged by the free phenolics 107

Table 9-14. Type III of fixed effects on the effect of insect feeding, extraction, and milling on DPPH scavenged by the bound phenolics 107

Table 9-15. Proc Glimmix model information for the effect of heat stress on free and bound total phenolic content (TPC) and % DPPH scavenged after 30 min (DPPH) 108

Table 9-16. Type III test of fixed effects for the effect of heat stress, extraction, and milling on free TPC 108

Table 9-17. Type III of fixed effects for the effect of heat stress, extraction, and milling on bound TPC 108

Table 9-18. Type III of fixed effects on the effect of heat stress, extraction, and milling on DPPH scavenged by the free phenolics 109

Table 9-19. Type III test of fixed effects on the effect of heat stress, extraction, and milling on DPPH scavenged by the bound phenolics 109

Table 9-20. Proc Glimmix model information for the effect of rust infection on free and bound total phenolic content (TPC) and % DPPH scavenged after 30 min (DPPH) 109

Table 9-21. Type III tests of fixed effects for the effect of rust infection, extraction, and milling on free TPC 110

Table 9-22. Type III tests of fixed effects for the effect of rust infection, extraction, and milling on the bound TPC 110

Table 9-23. Type III tests of fixed effects for the effect of rust infection, extraction, and milling on the DPPH scavenged by the free phenolics 110

Table 9-24. Type III test of fixed effects for the effect of rust infection, extraction, and milling on DPPH scavenged by the bound phenolics 111

Table 9-25. Proc Mixed model information for the effect of stress by bird-cherry oat aphid feeding at several growth stages on free total phenolic content (TPC) of wheat grains..... 111

Table 9-26. Sources of variation and levels for each treatment used in ANOVA..................... 111

Table 9-27. Type III test of fixed effects for the effect of *R. padi* feeding on free, free and conjugated, and bound TPC 112

Table 9-28. Mean and SE estimates of free TPC for each level of *R. padi* feeding treatments 112

Table 9-29. Differences of LSM for free TPC among *R. padi* feeding treatments, Bonferroni adjusted (P<0.0125) 112

Table 9-30. Mean and SE estimates of free and conjugated TPC for each level of *R. padi* feeding treatments 113

Table 9-31. Differences of LSM for free and conjugated TPC among *R. padi* feeding treatments, Bonferroni adjusted (P<0.0125).. 113

Table 9-32. Mean and SE estimates of bound TPC for each level of *R. padi* feeding treatments 113

Table 9-33. Differences of LSM for bound TPC among *R. padi* feeding treatments, Bonferroni adjusted (P<0.0125) 114

Table 9-34. Test III tests of fixed effects for the effect of *R. padi* feeding on free, free and conjugated, and bound DPPH 114

Table 9-35. Mean and SE estimates of free DPPH for each level of *R. padi* feeding treatment 114

Table 9-36. Differences of LSM for free DPPH among *R. padi* feeding treatments, Bonferroni adjusted (P<0.0125) 115

Table 9-37. Mean and SE estimates of free and conjugated DPPH for each level of *R. padi* feeding treatment.. 115

Table 9-38. Differences of LSM for free and conjugated DPPH among *R. padi* feeding treatments, Bonferroni adjusted (P<0.0125) 115

Table 9-39. Mean and SE estimates of bound DPPH for each level of *R. padi* feeding treatment 116

Table 9-40. Differences of LSM for bound DPPH among *R. padi* feeding treatments, Bonferroni adjusted (P<0.0125) 116

Table 9-41. Processing plan for extraction and analysis of wheat leaf samples 117

Table 9-42. Proc Glimmix model information for the effect of synthetic elicitors of SAR on total phenolic content in wheat leaves 126

Table 9-43. Sources of variation and levels for each source used in ANOVA 126

Table 9-44. Test III test of fixed effects for the effect of cultivar, elicitor treatment, and post application time on total phenolic content in wheat leaves 127

Table 9-45. Least Squares Means and SE estimates of total phenolic content for each cultivar127

Table 9-46. Least Square Means and SE estimates of total phenolic content for elicitor treatments 127

Table 9-47. Elicitor treatment effect sliced by cultivar*collection time 128

Table 9-48. Least Square Means and SE estimates of total phenolic content for cultivar*elicitor treatment combinations 129

Table 9-49. Least Square Means and SE estimates for the total phenolic content of elicitor treatment * post application time combinations 130

Table 9-50. Least Square Mean and SE estimates for the total phenolic content of elicitor treatment * post application time combinations in Ike wheat 131

Table 9-51. Least Square Means and SE estimates for total phenolic content of elicitor treatment * Post application time combinations in Karl 92 wheat 132

Table 9-52. Proc MIXED model information for the effect of synthetic SAR elicitor on the total phenolic content of mature wheat grains 133

Table 9-53. Sources of variation, covariance parameters, and levels of each source used in ANOVA 133

Table 9-54. Covariance parameters estimates on the effect of block and residuals on the fixed effects 133

Table 9-55. Type III tests of fixed effects for the effect of synthetic elicitors of SAR on total phenolic content in mature wheat grains.. 134

Table 9-56. Least Square Means and SE estimates for the total phenolic content in mature grains of wheat cultivars 134

Table 9-57. Least Square Means and SE estimates for the total phenolic content in mature grains of elicitor-treated plants 134

Table 9-58. Least Square Means and SE estimates for the total phenolic content in mature grains of cultivar*elicitor treatment combinations 135

Table 9-59. Estimates of the differences between total phenolic content in mature grains of elicitor-treated plants and controls. Significant differences were determined at P<0.004167, Bonferroni adjusted.. 136

Table 9-60. Estimates of the differences between total phenolic content in mature grains of elicitor treated and control Ike plants. Significant differences were determined at P<0.00208, Bonferroni adjustment 136

Table 9-61. Estimates of the differences between total phenolic content in mature grains of elicitor-treated and control Karl 92 plants. Significant differences were determined at P<0.00208, Bonferroni adjustment 137

Acknowledgements

I would like to acknowledge the support from my advisors: Dr. Praveen Vadlani for always providing extra help and resources to complete my research project, Dr. Dirk Maier for encouraging me to join the program and his support throughout it, Dr. C. Michael Smith for his continual feedback on my manuscripts and for providing me with a greenhouse, the tools and advice to conduct this investigation, Dr. Allan K. Fritz for his support early on this research as well as that of his team Andy Auld, Robert Steele, and Dr. Peter Nyori. Above all, I would like to acknowledge Dr. Ronald L. Madl for his wonderful guidance and advice from the beginning of my program until this very end. His gentleness and scholarship has taught me a lot.

I also would like to acknowledge the following professors at KSU: Dr. Doug Jardine, Dr. Brian McCornack, Dr. John Fellers, Dr. Anna Whitfield, Dr. Leigh Murray, and the instructors Nicholas Bloedow and Carlos and Keyla Campabadal.

The greenhouse and laboratory research work could not have been completed without the help from: Gengjun Chen, Jerry Sullivan, Jithma Abeykoon, Phillip Gollom, Dan Carlson, Jiayi Xu, Katrina Fox, Yichen Zhang, Arthur Cannon, Terri Braden, and Steve Kramer.

I am appreciative of the administrative and technical support lent by the following: Jim Hodgson, Ron Stevenson (RIP), Terri Mangiaracino, Susan Kelly, Liz Savage, and Beverly McGee and the rest of the students/staff at the Bioprocessing and Renewable Energy Lab and Biomaterials and Technology Lab in BIVAP.

This research was funded by the Kansas Wheat Commission. Also, the following agencies provided me with financial support to complete my doctoral program in Grain Science: Johnson Cancer Research Center, Department of Grain Science and Department of Entomology at KSU. I would also like to acknowledge the financial support from Louise and Vance Ehmke.

Dedication

To the hard working wheat grower

Chapter 1 - Introduction

Antioxidants are substances that delay the kinetics of oxidation reactions. A dietary antioxidant is a substance in foods that significantly decreases the adverse effects of reactive oxygen species, reactive nitrogen species, or both, on normal physiological functions in humans (Food and Nutrition Board, Institute of Medicine 1998).

The main sources of antioxidants in human diets varied by region. Mediterranean diets are rich in cereals, legumes, dried and fresh fruits, tubers, vegetables, olive oil, and fish (Ferris-Tortajada et al, 2012). The sources of antioxidants found in the Western diet are mostly vitamin A, C, E, and polyphenols found in many fruits and vegetables, nutritional supplements, or as additives in processed foods and beverages (Landete, 2013).

Cereal grains have potential to become an important dietary source of antioxidants in the U.S. The latest edition of the USDA Dietary Guidelines for Americans (2015-2020) recommend consumers to eat at least 48 g of whole grains per day for a healthy U.S.-style eating pattern at the 2000 calorie level. Dietary fiber, minerals and vitamins found in whole grains are the key nutrients that contribute health-promoting benefits. Furthermore, it is becoming more evident that phytochemicals, which are specific compounds in plants with biological activity, found in whole grains are responsible for these health promoting benefits. For example, ferulic acid effectively prevented the oxidative damage to 1) proteins in neuronal cells that causes Alzheimer's disease (Kanski et al, 2002), 2) low density lipoprotein in plasma levels that lead to cardiovascular disease (Ohta et al, 1997; Schroeter et al, 2000), and 3) lipids in cell membranes

that help maintain cell integrity (Trombino et al, 2004). By following these guidelines, whole grains can have a major health promoting impact on American consumers because, on a per serving basis, the concentration of phytochemicals in whole grains and the total antioxidant activity is comparable to that found in some fruits and vegetables (Sun et al, 2002; Chu et al, 2002; Adom and Liu, 2002).

Wheat plays an important role among cereal grains in the human diet. It accounts for one-third of the total worldwide grain production. The phytochemicals in wheat are ferulic acid, simple phenolic acids, flavonoids, zeaxanthin, lutein, and cryptoxanthin (Adom et al, 2005). Most of the ferulic and other phenolic acids, flavonoids, and zeaxanthin are found in the bran/germ fraction, while the endosperm can have up to 50% of the total lutein and cryptoxanthin (Adom et al, 2005). Approximately 25% of these compounds can be found in free forms and 75% are bound to structural components in the grain (Adom and Liu, 2002). Whole wheat products can become an important source of dietary antioxidants in the U.S. diet. The health promoting potential of free and esterified phenolic acids has already been evaluated in whole wheat and wheat bran based ready to eat breakfast cereals (Baublis et al, 2000). Wheat ranked second among the major cereal crops for antioxidant content and activity on a whole grain weight basis (Adom and Liu, 2002). The radical scavenging properties and reducing capacity of wheat grains have been studied extensively (Adom et al., 2005; Kwami Adom et al., 2003; Okarter et al., 2010; Yu et al., 2002), and the health benefits of wheat antioxidants have been determined in experimental trials. Wheat bran extracts significantly reduced lipid peroxidation in human low density lipoprotein in vitro (Yu et al., 2005) and the incidence of colon tumors in mice (Carter et al., 2006). The anti-proliferative properties of wheat also have

been evaluated. Wheat bran extracts inhibited the growth of HT-29 and Caco-2 human colorectal cancer cells, and prostate adenocarcinoma cancer cells (Lei Liu et al., 2012; Lv et al., 2012; Whent et al., 2012).

Novel wheat-based products with enriched levels of antioxidants have been developed to take advantage of the health-promoting benefits of wheat. Milling fractions with varying levels of phenolics were produced when wheat grains were sequentially de-branned with a pearling technique (Beta et al., 2005). Phenolic-rich wheat brans with improved baking functionality were produced through alkaline hydrolysis, high pressure homogenization, and enzymatic modification (Guo et al., 2011), yeast fermentation (Katina et al., 2012; Moore et al., 2007), microbial fermentation and enzymatic modification (Coda et al., 2014), and enzyme modification alone (Moore et al., 2006). However, any approach to enrich wheat flour or bran with antioxidants must pass the scrutiny of a health-conscious consumer that is increasingly informed of the food industry practices.

U.S. consumer preferences for natural, minimally processed, non-GMO, natural foods, and fiber-, mineral-, vitamin-, and antioxidant-enriched foods has made the food industry reformulate their products in order to comply with consumer's demands (Sloan, 2015). This could create market opportunities for specialty food ingredients such as antioxidant-rich wheat.

The potential of using wheat for producing antioxidant-rich food crops has been considered before as a value-added strategy for dryland farmers (Yu et al., 2002). Wheat (*Triticum aestivum*, L) is the main food grain cultivated in the U.S. Winter wheat accounts for

70-80% of the total U.S. wheat production (ERS, 2015). Approximately half of the domestic wheat production is sold into export markets. The state of Kansas, which produces only winter wheat, ranked second in total U.S. wheat export value (NASS, 2012). The wheat sector has suffered many challenges in the past decade including a weak domestic market for wheat products and foreign competition. Wheat planted area has decreased because wheat lags behind in yield improvement as well as in overall returns compared to GM corn and soybean crops (Madl, R., *personal communication*). Although GM technology benefits farmers in the U.S. Northern plains and irrigated agricultural systems where there are several crops to choose from for cultivation, it is not the case in dryland farming areas of the Central Plains where winter wheat is one of the few crops adapted to that climate. Value addition of winter wheat crops represents an opportunity for dryland farmers and others to increase the profitability of their crops (Coltrain et al., 2000). Antioxidant-rich wheat crops can be sold in niche markets to avoid the volatility of commodity markets and capitalize on high value markets created by U.S. consumer preferences.

References

Adom, K.K., and R.H. Liu. 2002. Antioxidant activity of grains. J. Agric. Food Chem. 50:6182-6187.

Adom, K.K., M.E. Sorrells, and R.H. Liu. 2005. Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. J. Agric. Food Chem. 53:2297-2306.

Baublis, A.J., C.R. Lu, F.M. Clydesdale, and E.A. Decker. 2000. Potential of wheat-based breakfast cereals as a source of dietary antioxidants. J. Am. Coll. Nutr. 19:308S-311S.

Beta, T., S. Nam, J.E. Dexter, and H.D. Sapirstein. 2005. Phenolic content and antioxidant activity of pearled wheat and roller-milled fractions. Cereal Chem. 82:390-393.

Carter, J.W., R. Madl, and F. Padula. 2006. Wheat antioxidants suppress intestinal tumor activity in min mice. Nutr. Res. 26:33-38.

Chu, Y., J. Sun, X. Wu, and R.H. Liu. 2002. Antioxidant and antiproliferative activities of common vegetables. J. Agric. Food Chem. 50:6910-6916.

Coda, R., C.G. Rizzello, J.A. Curiel, K. Poutanen, and K. Katina. 2014. Effect of bioprocessing and particle size on the nutritional properties of wheat bran fractions. Innovative Food Science & Emerging Technologies 25:19-27.

Coltrain, D., D. Barton, and M. Boland. 2000. Value Added: Opportunities and Strategies.Retrieved October 20, 2003.

Economic Research Service, 2015. Wheat. USDA Economic Research Service, Washington, D.C. Available at: http://www.ers.usda.gov/topics/crops/wheat.aspx (Last accessed June 10th, 2015).

Ferris-Tortajada, J., O. Berbel-Tornero, J. Garcia-Castell, J.A. Ortega-Garcia, and J.A. Lopez-Andreu. 2012. Dietetic factors associated with prostate cancer. protective effects of mediterranean diet. Actas Urol. Esp. 36:239-245.

Food and Nutrition Board. 1998. Dietary reference intakes. Proposed definition and plan for review of dietary antioxidants and related compounds. National Academy Press, Washington, DC. Available at: www.nap.edu (Last accessed on March 14th, 2016)

Guo, M., K. Petrofsky, L. Zhang, P. Chen, A. Hohn, M. Youn, D. Gallaher, R. Liu, J. Faubion, M. Bunzel, L. Marquart, and R. Ruan. 2011. Improving the functionality and bioactivity in wheat bran. Faseb j. 25:.

Kanski, J., M. Aksenova, A. Stoyanova, and D.A. Butterfield. 2002. Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems in vitro: Structure-activity studies. J. Nutr. Biochem. 13:273-281.

Katina, K., R. Juvonen, A. Laitila, L. Flander, E. Nordlund, S. Kariluoto, V. Piironen, and K. Poutanen. 2012. Fermented wheat bran as a functional ingredient in baking. Cereal Chem. 89:126-134.

Landete, J.M. 2013. Dietary intake of natural antioxidants: Vitamins and polyphenols. Crit. Rev. Food Sci. Nutr. 53:706-721.

Lei Liu, K.M. Winter, L. Stevenson, C. Morris, and D.N. Leach. 2012. Wheat bran lipophilic compounds with in vitro anticancer effects. Food Chem. 130:156-164.

Lv, J., L. Yu, Y. Lu, Y. Niu, L. Liu, J. Costa, and L. Yu. 2012. Phytochemical compositions, and antioxidant properties, and antiproliferative activities of wheat flour. Food Chem. 135:325-331.

Moore, J., Z. Cheng, L. Su, and L.L. Yu. 2006. Effects of solid-state enzymatic treatments on the antioxidant properties of wheat bran. J. Agric. Food Chem. 54:9032-9045.

Moore, J., Z. Cheng, J. Hao, G. Guo, J. Liu, C. Lin, and L. Yu. 2007. Effects of solid-state yeast treatment on the antioxidant properties and protein and fiber compositions of common hard wheat bran. J. Agric. Food Chem. 55:10173-10182.

National Agricultural Statistics Service. 2012. Kansas Farm Facts 2012. USDA Natl. Agric. Statistics Serv., Kansas Field Office, Topeka, KS. Available at http://www.nass.usda.gov/Statistics_by_State/Kansas/Publications/Annual_Statistical_Bulletin/ff2012.pdf (Last accessed on June 10th, 2015).

Ohta, T., T. Nakano, Y. Egashira, and H. Sanada. 1997. Antioxidant activity of ferulic acid β-glucuronide in the LDL oxidation system. Biosci. Biotechnol. Biochem. 61:1942-1943.

Okarter, N., and R.H. Liu. 2010. Health benefits of whole grain phytochemicals. Crit. Rev. Food Sci. Nutr. 50:193-208.

Okarter, N., C. Liu, M.E. Sorrells, and R.H. Liu. 2010. Phytochemical content and antioxidant activity of six diverse varieties of whole wheat. Food Chem. 119:249-257.

Schroeter, H., R.J. Williams, R. Matin, L. Iversen, and C.A. Rice-Evans. 2000. Phenolic antioxidants attenuate neuronal cell death following uptake of oxidized low-density lipoprotein. Free Radical Biology and Medicine 29:1222-1233.

Sloan, E. 2015. The top ten food trends. Food Technology 69(4): 24-43.

Sun, J., Y. Chu, X. Wu, and R.H. Liu. 2002. Antioxidant and antiproliferative activities of common fruits. J. Agric. Food Chem. 50:7449-7454.

Trombino, S., S. Serini, F. Di Nicuolo, L. Celleno, S. Andò, N. Picci, G. Calviello, and P. Palozza. 2004. Antioxidant effect of ferulic acid in isolated membranes and intact cells: Synergistic interactions with α-tocopherol, β-carotene, and ascorbic acid. J. Agric. Food Chem. 52:2411-2420.

Whent, M., H. Huang, Z. Xie, H. Lutterodt, L. Yu, E.P. Fuerst, C.F. Morris, L. Yu, and D. Luthria. 2012. Phytochemical composition, anti-inflammatory, and antiproliferative activity of whole wheat flour. J. Agric. Food Chem. 60:2129-2135.

Yu, L., S. Haley, J. Perret, M. Harris, J. Wilson, and M. Qian. 2002. Free radical scavenging properties of wheat extracts. J. Agric. Food Chem. 50:1619-1624.

Yu, L.L., and K.Q. Zhou. 2005. Antioxidant properties of bran extracts from 'platte' wheat grown at different locations. Food Chem. 90:311-316.

Chapter 2 - Hypothesis and objectives

Phenolic compounds are secondary metabolites needed for pigmentation, growth, reproduction, and defense among other plant functions. Unlike primary metabolism compounds that are required for cell maintenance and proliferation, secondary metabolites are present in specialized cells and are not directly essential for basic photosynthesis or respiratory metabolism. However, they are thought to be required for the plant's survival in the environment. The phenolics compounds found in wheat play a protective or defense role. Flavonoids are known to protect against ultraviolet (UV) radiation (Li et al, 1993). Lutein, zeaxanthin, and cryptoxanthin have a role in the light-harvesting complex by preventing and partially compensating for oxidative damage (Jahns and Holzwarth, 2006). Simple phenolic acids act as signaling molecules and have been proposed to act as defense compounds against pathogens (Mandal et al, 2010).

The theories and mechanistic models that explain how secondary metabolites change after damage have been summarized by Karban and Baldwin (2007). While not perfect, they provided a framework to analyze correlational studies in the published literature, pose research questions, design experiments, and discuss results in this dissertation.

1. Carbon/nutrient (C/N) theory states that when resources exist in excess of growth requirements, they are routed into secondary metabolism (Hamilton et al, 2001). Although protein is not a secondary metabolism compound, the mechanism of the C/N theory can be exemplified by wheat crop yield and protein response to increasing rates of nitrogen applications: As nitrogen fertilization increases, yield and protein rise concurrently. Yield responses to nitrogen are greater than protein responses up to certain levels of application. As nitrogen is applied

beyond these levels the wheat plant will no longer use it to increase yield, but will utilize it to increase grain protein content (Bly and Woodard, 2003). Although it has been hypothesized that this theory could be useful to explain the synthesis of nitrogen-containing secondary metabolites such as nicotine in tobacco plants, its usefulness has not been proven. C/N imbalance in these experiments was artificially triggered by decreasing plant carbon through leaf removal (Baldwin et al, 1993), growing under high CO_2 conditions (Fajer et al, 1992), and also under high nitrogen conditions (Gonthier et al, 2011).

2. Substrate/enzyme imbalances occur when secondary metabolites accumulate as a result of overflow primary metabolism (Karban and Baldwin, 2007). In other words, the plant has no ability to regulate secondary metabolite production.
3. Growth/differentiation balance theory states that all secondary metabolites have an ontogenetically determined phenology and that their synthesis is emphasized during periods of plant differentiation (Karban and Baldwin, 2007). A shift from growth to differentiation may occur in response to suboptimal nutrient resources (Wilkens et al, 1996), pathogen attack (Schnee et al, 2010), or insect infestation (Lorio, 1988) depending on the plant species and type of secondary metabolites.

Although theories 1-3 did not successfully explain the production of secondary metabolites in several studies, an alternative hypothesis to theory 1 based on observed results points to leaf damage as a trigger for secondary metabolite production. Furthermore, according to theory 3, several factors can shift the plant's ontogenetic state from growth to defense. The

following theories differ from 1-3 in that plants do regulate the production of secondary metabolites and their concentration are dependent on the plant's need for defense.

4. The generalized stress response theory postulates that plants have a hormonally mediated, centralized system of physiological responses for coping with many diverse stresses (Chapin, 1991). Since some stress-related plant hormones affect the production of some induced defenses, these defenses may be part of the generalized stress response (Karban and Baldwin, 2007).
5. The active defense response theory postulates that endogenously-produced damage cues or cues specific to the invading organism activate specific defense responses (Karban and Baldwin, 2007). An example of this specific type of response is the production of the isoflavone "medicarpin" in alfalfa (*Medicago sativa*).
6. Optimal defense theory states that defense has a cost because resources allocated to it cannot simultaneously be allocated to other functions (Zangerl and Bazzaz, 1992).

Theory of generalized stress (No.4) was utilized in Chapter 3 of this dissertation to determine the effect of insect feeding, pathogen infection, and heat stress on antioxidant properties of wheat bran. This choice was based on 1) preliminary data that showed how phenolic content in hard red winter wheat varied by cultivar, location, and growing season, and 2) published studies that found correlation between specific stress factors and total phenolic content.

The notion that plants have ontogenetically determined stages to grow and to defend themselves was the basis for the experimental design in Chapter 4, which determined that bird-cherry oat aphid feeding stress enhanced levels of phenolics in mature wheat grains. The research question was, is there a wheat stage at which the plant is more sensitive to stress in terms of phenolic induction in mature grains? In addition to theory 2, the balance between plant performance and defense in terms of phenolic induction is discussed in light of the optimal defense theory (No. 6).

The theory of active defense response (No. 5) is explored in Chapters 5 and 6. This theory was used to prove how simple phenolic acids are synthesized as part of the signaling of the systemic acquired resistance (SAR) defense response in wheat foliage and mature grains.

The objectives of this research were to: 1) identify the factor (s) that contribute the most to the variability in wheat grain phenolic content; 2) understand the mechanism (s) responsible for phenolic synthesis, and 3) find artificial factors that trigger that mechanism (s).

References

Baldwin, I.T., R.C. Oesch, P.M. Merhige, and K. Hayes. 1993. Damage-induced root nitrogen metabolism in Nicotiana sylvestris: Testing C/N predictions for alkaloid production. J. Chem. Ecol. 19:3029-3043.

Bly, A.G., and H.J. Woodard. 2003. Foliar nitrogen application timing influence on grain yield and protein concentration of hard red winter and spring wheat. Agron. J. 95:335-338.

Chapin, F.S. 1991. Integrated responses of plants to stress. Bioscience 41:29-36.

Dalkin, K., J. Jorrin, and R.A. Dixon. 1990. Stress responses in alfalfa (medicago sativa L.) VII. induction of defence related mRNAs in elicitor-treated cell suspension cultures. Physiol. Mol. Plant Pathol. 37:293-307.

Fajer, E., M. Bowers, and F. Bazzaz. 1992. The effect of nutrients and enriched CO2 environments on production of carbon-based allelochemicals in plantago: A test of the carbon/nutrient balance hypothesis. Am. Nat. 140:707-723.

Gonthier, D.J., J.D. Witter, A.L. Spongberg, and S.M. Philpott. 2011. Effect of nitrogen fertilization on caffeine production in coffee (coffea arabica). Chemoecology 21:123-130.

Hamilton, J., A. Zangerl, E. DeLucia, and M. Berenbaum. 2001. The carbon–nutrient balance hypothesis: Its rise and fall. Ecol. Lett. 4:86-95.

Jahns, P., and A.R. Holzwarth. 2012. The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. Biochimica Et Biophysica Acta (BBA)-Bioenergetics 1817:182-193.

Karban, R., and I.T. Baldwin. 2007. Induced Responses to Herbivory. University of Chicago Press. Chicago.

Li, J., T.M. Ou-Lee, R. Raba, R.G. Amundson, and R.L. Last. 1993. Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell 5:171-179.

Lorio Jr, P.L. 1988. Growth differentiation-balance relationships in pines affect their resistance to bark beetles (coleoptera: Scolytidae). *In* Mechanisms of woody plant defenses against insects. Springer, New York, p. 73-92.

Mandal, S.M., D. Chakraborty, and S. Dey. 2010. Phenolic acids act as signaling molecules in plant-microbe symbioses. Plant Signaling & Behavior 5:359-368.

Schnee, S.; Jolivet, J.; Calonnec, A. 2010. Dynamics of ontogenic resistance and growth variation in the interaction powdery mildew-grapevine. In: Proceedings of the 6th International workshop on grapevine downy and powdery mildew. Bordeaux, France, p. 54-56

Wilkens, R.T., J.M. Spoerke, and N.E. Stamp. 1996. Differential responses of growth and two soluble phenolics of tomato to resource availability. Ecology 77:247-258.

Zangerl, A.R., and F.A. Bazzaz. 1992. Theory and pattern in plant defense allocation. Plant Resistance to Herbivores and Pathogens. University of Chicago Press, Chicago, p. 363-391.

Chapter 3 - Effect of insect feeding, pathogen infection, and heat stress on antioxidant properties of wheat bran

Abstract

The potential of hard red winter wheat (*Triticum aestivum* L) to produce antioxidant rich crops has been considered as a value addition strategy to increase farm profitability, but the inherent variability in phenolics levels in wheat crops is a barrier. This problem also makes marketing the health promoting benefits of whole wheat products difficult. Although some variability is explained by genetic diversity, a significant portion is owing to stress factors such as elevated temperatures, fungal attack, and insect damage. Limited information is available on formal trials designed to investigate these relationships. In this study, wheat (*Triticum aestivum* L) cultivar Karl 92 was stressed by bird-cherry oat aphid (*Rhopalosiphum padi*) feeding, leaf rust (*Puccinia triticina*) infection, and post-anthesis high temperature stress. Total phenolic content (TPC) and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (%DPPH) of the resulting wheat bran and those of non-stressed plants were measured. Plant fitness parameters such as number of spikes and grain yield were also measured. The number of spikes was low for heat-stressed plants ($P<0.0151$) and the kernels were shriveled compared to control. Grain yield was high for rust-infected plants relative to control ($P<0.0821$). Aphid feeding and heat stress explained some of the variation in TPC ($P<0.0719$ and $P<0.0633$, respectively) and %DPPH ($P<0.0038$ and $P<0.0048$, respectively) of free phenolics on a bran weight basis, but rust infection did not. None of the stress factors had a significant induction effect on bound phenolics.

KEYWORDS: antioxidants, phenolics, aphid feeding, rust infection, heat stress

Introduction

The commercial success of health-promoting wheat-based products depends on the quality and steady supply of wheat crops with consistently high antioxidant content. However, wheat crops are a heterogeneous mixture of cultivars with varying antioxidant contents. For example, total phenolic content of 10 commonly grown hard red winter wheat cultivars varied by 2.87-fold, and the antioxidant content of Ike wheat, a cultivar grown in Western Kansas, varied by 1.55 across locations and growing seasons (Madl, R. *Unpublished*). The variability in antioxidant content of some wheat market classes has been measured (Li et al., 2008; Verma et al., 2008). Although some of the variability is a result of genetics, the majority is owing to environment as indicated by analysis of variance of total phenolics measured for hard winter wheat (Moore et al., 2006), soft wheat (Yingjian Lu et al., 2015), and durum wheat (Bellato et al., 2013).

Information about the growing conditions or biotic factors that most influence the levels of phenolics in wheat crops can be helpful to understand what triggers their production in plants. Specific factors that have been linked to the variability in phenolics in wheat crops include temperatures at or above 30°C and duration of heat stress (Heimler et al., 2010; Yu and Zhou, 2005), fungal attack (Zhou et al., 2007), and insect feeding damage (Boyko et al., 2006; Smith et al., 2010). In some of these studies, induction of total phenolics and individual phenolic compounds are hypothesized to be part of a specific defense response from wheat cultivars resistant to pathogens or insects. Specific defense responses are triggered by molecular interactions between resistance genes in resistant cultivars and gene products from avirulent pathogens or insects. On the other hand, general responses to stress are also triggered by molecular interactions between abiotic and biotic factors and plants but do not necessarily

involve the expression of resistance genes. A general stress response to biotic factors is preceded by the following events: plant recognition of damage, changes in plant chemistry, and production of plant signaling molecules (Smith and Boyko, 2007).

Experimental trials designed to investigate whether these inducible responses affect grain phenolics levels in wheat plants have not been published. The stress factors in this study were chosen based on their potential to trigger a general stress response in hard red winter wheat cv. Karl 92. The objective of this study was to determine the effect of insect feeding, pathogen infection, and heat stress on the antioxidant properties of wheat bran extracts.

Materials and methods

This study was conducted in controlled-environment facilities in the Department of Agronomy, Kansas State University, Manhattan, KS, USA. The experiments were conducted in 2010.

Materials. The hard red winter wheat cultivar Karl 92, a well-adapted semi-dwarf and early maturing Kansas cultivar (Narasimhamoorthy et al., 2006; Sears et al., 1997) was used in this study because it has been a popular cultivar grown in the U.S. Midwest. This cultivar is heat-susceptible and has lost its resistance to rust, which makes it an ideal genotype for the study of general plant stress responses. Seeds were sown in 4-cm-deep trays containing commercial Sunshine Metro Mix 200 potting soil (Hummert International, Topeka, KS). Seedlings were raised in a greenhouse at 21/16ºC day/night and a photoperiod of 14:10 h (light:dark) with supplemental light from high pressure sodium lamps. Fourteen day-old seedlings were kept in a

vernalization chamber for 6 weeks at 4°C and subsequently, transplanted into 15-cm diameter pots (3 plants/pot). Potting medium was commercial Sunshine Metro Mix 200 potting soil fertilized with Osmocote (Scotts, Marysville, OH), a controlled-release fertilizer with 19:6:12 $N:P_2O_5:K_2O$, at 5 g per pot. Pots were kept in a greenhouse room under the constant environment conditions stated above. They were watered every day for 2 hours through a capillary matting system (Hummert International, Earth City, MO).

Experimental Design. The following experiments were conducted in controlled greenhouse environments. The time of application of each stress was set to reflect actual field conditions as closely as possible. In each experiment, stress type was the only variable, with other growing conditions kept constant for both stressed and non-stressed plants.

Insect feeding: Karl 92 plants were infested with third or fourth instar non-viruliferous bird cherry oat aphids (*Rhopalosiphum padi*) at the five-tiller growth stage (Zadoks scale=25). A heavily infested leaf of a plant from an *R. padi* colony was placed in each pot, and plants were entirely covered with 30 x 70 cm insect sleeves (149 x 149 microns/ 6.5 cm^2) to prevent aphid escape (Figure 3-3). The *R. padi* colony was started from a natural population of spring migrant aphids. *R. padi* individuals were allowed to feed and reproduce undisturbed inside of the insect sleeve-covered pot to reflect field infestation levels (Whitworth and Ahmad, 2008). The infestation was stopped at the late milk stage (Zadoks scale=77) with the systemic insecticide Marathon 1G® (OHP, Inc; Mainland, PA), active ingredient: imidacloprid, which was applied to the base of the plants at a dose of 1.5 g per pot. A separate set of control plants was

preemptively treated with Marathon 1G® to protect them from aphid infestation and their grains were used as control for this experiment.

Rust infection: Karl 92 plants were inoculated with spores of a compatible race of leaf rust (*Puccinia triticina*) 21 days after the first spike appeared (Zadoks scale=71). Inoculum was obtained from the Rust and Wheat Genomics, USDA-ARS Hard Winter Wheat Genetics Unit, Manhattan, KS. Plants were sprayed with an atomized suspension of urenidiospores mixed with the isoparaffinic light oil Soltrol 170 (Chevron Phillips Chemical Company, The Woodlands, TX). Inoculated plants were placed in a dark mist chamber (Percival scientific, INC, Perry, IA) for 16 h at 20°C and close to 100% RH. Inoculated and un-inoculated control plants were kept in a growth chamber (Conviron, Model PGR15, Winnipeg, Manitoba, Canada) set at 20/15ºC day/night, 16-h photoperiod, and 50-70% relative humidity until they reached physiological maturity (Figure 3-4).

Heat stress: Karl 92 plants were exposed to chronic heat stress as described by Yang et al (2002) in a Conviron growth chamber set at 30/25°C day/night and 84-90% relative humidity 21 days after the first spike appeared (Zadoks scale=71) and until physiological maturity. A set of control plants was placed in a separate Conviron growth chamber set at 20/15°C day/night, 50-70% relative humidity, and 16-h photoperiod. Light intensity was 420 $\mu mol/m^2/s$ at top of the plant canopy in both chambers (Figure 3-1).

Sample Preparation. Plants were harvested when they reached physiological maturity. The heads were cut, bundled, and placed on trays. Bundles were dried further in a

room at 15°C, hand-threshed and stored at 4°C until milling. Composited grain samples were cleaned and sorted by size using dockage test sieves with 5.16 mm round holes and a 1.6 x 9.5 mm slotted sieve. The sound and healthy grains (overs from the slotted sieve) were tempered to 15.6±0.22% moisture content and analyzed by a Single Kernel Characterization System (SKCS 4100, Perten Instruments, Sweden). The grain samples were milled on a Quadrumat Jr. Mill (Brabender, South Hackensack, NJ). The bran was collected from the mill and sifted in a plansifter. Particles equal or larger than 0.16 mm^2 were kept for extraction and analysis.

Extraction Procedure. Phenolics were extracted from the bran in two fractions, free and bound, with a modified version of the procedure reported by Krygier et al. (1982). This modified version was developed to eliminate non-phenolic compounds from the extracts that interfere with the Folin reagent. These non-phenolic compounds have been identified by Everette et al. (2010). One gram of wheat bran was weighed and placed in a 50-ml centrifuge tube. 30 ml of petroleum ether was added and the tube was shaken in a wrist shaker for 1 hour to extract lipids. The petroleum ether was decanted and discarded, and the bran was transferred into a Petri dish to allow evaporation of residual ether. The dry bran was transferred to an Omni Mixer-Homogenizer holding tube (Omni International, Kennesaw, GA), and was homogenized for 2 minutes with 10 ml of methanol:acetone:water solution (7:7:6 v/v/v, adjusted to pH 2 with concentrated hydrochloric acid). The supernatant was decanted into a graduated cylinder and made up to 35 ml with fresh solution. This mixture was used to wash the bran from the homogenizer holding tube into a centrifuge tube. The tube was shaken for 2 hours and centrifuged at 5000 x *g* for 10 minutes at 10°C. The supernatant was decanted into a new 50-ml centrifuge tube, and the bran pellet was saved for alkaline hydrolysis. The supernatant was

poured into a round-bottomed flask and concentrated under vacuum at 40°C. Final volume was recorded. This supernatant was used to obtain the free phenolic fraction, and the bran pellet was used to obtain the bound phenolic fraction.

Free phenolic fraction: An aliquot of 5-10 ml of supernatant was mixed with 30 ml of ethyl ether/ethyl acetate solvent (1:1 v/v). The mixture was hand-shaken for 1 minute and poured into a separatory funnel. The upper and lower phases were collected separately. This procedure was repeated twice on the lower phase using fresh solvent each time. The three aliquots of solvent were pooled together. Magnesium sulfate was added to remove any remaining water. The mixture was concentrated under vacuum at 40°C. The concentrated solution containing free and conjugated phenolic compounds was reconstituted with 10 ml methanol and saved for subsequent analysis.

Bound fraction: The bran pellet was hydrolyzed with 5 ml of 4M NaOH at 60°C for 2.5 hours. The hydrolysate was acidified to pH 2 with hydrochloric acid and defatted with 5 ml of hexane. The hexane was decanted, and the residue was mixed with 4 ml of ethyl ether/ethyl acetate solvent (1:1 v/v). The mixture was hand-shaken for 1 minute. The tube was centrifuged at 5000 x *g* for 5 minutes, and the supernatant was drawn out with a pipette. This procedure was repeated six times. The supernatants were pooled and diluted with an equal amount of methanol (1:1 v/v).

Determination of Total Phenolic Content. The free and bound phenolic fractions obtained in the extraction procedure were analyzed for total phenolic content (TPC). The

procedure was a modified version of the Folin Ciocalteau assay described by Singleton and Rossi (1965). The Folin Ciocalteau assay determines the reducing capacity of a sample by measuring the color change in the Folin reagent (Huang et al., 2005). An aliquot of 200 µL of the extracts was pipetted into a test tube and the following reagents were added: 1.5 ml of 0.2 N Folin-Ciocalteau reagent and 1.5 ml of 6% sodium carbonate (w/v). This mixture was vortexed and allowed to incubate in the dark at 23°C for 90 minutes. Ferulic acid solutions with known concentrations and a methanol blank were also tested and incubated with the samples. After incubation, absorbance was measured at 725 nm with a UV-VIS Spectrophotometer (Shimadzu, model UV-1650, Columbia, MD). Absorbance vs. concentration was plotted for the ferulic acid standard solutions. The linear equation obtained was used to calculate concentration from the absorbance of the samples, and these values were reported as ferulic acid equivalents (FAE).

Determination of Antioxidant Capacity. The free and bound fractions used to determine TPC were also analyzed for antioxidant capacity with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay. This assay measures how likely the sample is to donate a hydrogen atom and reduce the DPPH molecule's unpaired electron (Huang et al., 2005). The procedure was as follows: 3.9 ml of DPPH solution in methanol (25 mg/ml) was mixed with 0.1 ml of sample. The absorbance of the reaction was measured at 515 nm after a 30 minute incubation period in the dark at 23°C. Absorbance (ABS) is correlated to concentration of DPPH. The percentage of DPPH scavenged (% DPPH) was calculated as follows: $(1-[ABS_{sample}\ (t=30)/ABS_{control}\ (t=0)])*100$ where "t" is time in minutes.

Statistical Analysis. TPC and % DPPH data obtained from these experiments were analyzed with a PROC GLIMMIX procedure in SAS 9.4 statistical software (SAS Institute, Cary, NC) appropriate for a completely randomized design with unequal numbers of replications. The effect of each stress factor on TPC and % DPPH scavenged by free and bound wheat bran extracts was analyzed using one-way ANOVA at an alpha=0.1 due to the variability of phenolics due to environment (Yu and Zhou, 2005), plant to plant variability (Riedell et al, 2003 and references therein), and the inherent variability of the induced-defense response approach (Bruce, 2014). Six grain subsamples for each stress factor were analyzed in duplicate in the lab, and the means and standard errors were reported. Plant parameters (spikes/pot, grain yield/pot, and grain yield/spike) were analyzed with a two-sample t-Test with unequal variances.

Results and discussion

Phenolics are plant secondary metabolites involved in defense responses to insect or fungal attack and general stress responses to environmental factors such as heat (Lattanzio et al., 2006). Specific phenolics compounds that are produced and accumulated in some plant species can act as feeding deterrents to insects, i.e. chlorogenic acid in chrysanthemum is a resistance factor to western flower thrips (*Frankliniella occidentalis)* (Leiss et al., 2009). In wheat, recombinant inbred lines of resistant background to specific pathogens often have relatively high total phenolic content compared to their susceptible counterparts (Eisa et al., 2013). Although published studies have reported the levels of foliar phenolics in specific plant/pathogen interactions, few have studied the effect on the levels of phenolics in the grain. In this study,

experimental trials were designed to trigger general defense or stress responses between *R. padi, P. triticina*, or elevated temperatures, and Karl 92 wheat plants.

Comparison of the phenolic concentrations observed in these studies with those found in the literature

Comparison of the total phenolics content found in this study with those found in other studies is difficult because the extraction methods for phenolics used are not the same. However, some comparisons can be made with some studies. Zhou et al (2004) reported that the range in phenolics content of bran of 7 wheat varieties was 2.2-2.9 mg GAE/g bran. This is about 4-5 times higher than the levels of free phenolics reported in this study, but did not separate the phenolics from other compounds present in the bran that can potentially interfere with the Folin ciocaltau reagent (Everette et al, 2010). Some of these compounds are, in order of increasing reactivity, potassium iodide, copper and zinc complexes, iron chloride, thiamine (Vitamin B1), and pyridoxine (Vitamin B6). The reactivity of vitamin B6 to the Folin ciocalteu reagent was almost 21% that of gallic acid (Everette et al, 2010), but its total amount in wheat bran is relatively low (0.176 mg/100 g bran) (USDA ARS, 2016). On the other hand, the relative reactivity of potassium salt is low (0.02% that of gallic acid) but its total amount in the bran is high (227 mg/100 g bran), which can make it a significant contributor to the total antioxidant activity of wheat bran as measured by the Folin ciocalteu assay if it is not removed during the extraction procedure (Everette et al, 2010; USDA ARS, 2016). The total phenolics content may also be over- or underestimated by the choice of extraction solvent. According to Julkunen-Tiitto (1985), 50% acetone protects conjugated phenolics from degradation while 50% methanol solutions breaks down salicortin, a labile phenolic glycoside, into salicin. Zhou et al (2004)

utilized a 50% acetone:water (v/v) extraction solvent, while a mixture of methanol, acetone, and water (7:6:1 v/v/v) was used in this study. Therefore, the levels of phenolics shown here were a reflection of, mostly, the free simple phenolic acids, and that may explain the lower values compared to those reported by Zhou et al (2004). These authors studied several winter wheat cultivars from different wheat producing regions in the world. These were grouped according to total phenolic content as follows: U.S. hard white winter wheat ‘Avalanche’ and Canadian durum> U.S. hard red winter wheat ‘Akron’ and Swiss red> Illinois red soft, Canadian white, and Australian general purpose. Some white winter market class cultivars also had a higher cancer cell killing ability than red winter wheat (Drankhan et al., 2003), which is an indication that coat color has no correlation with the amount of free soluble phenolics.

The free and bound phenolic contents shown here were similar to those obtained by Kim et al (2006), who utilized a step-wise, exhaustive extraction procedure to obtain phenolic fractions of varying degrees of solubility and size. The phenolics extractable in 80% methanol totaled 0.63 and 0.57 mg GAE/g bran for two hard red winter wheat varieties in that study. On the other hand, those phenolics hydrolyzed with alkaline and acidic conditions from the 80% methanol extract residue were 3.2 and 3.4 mg GAE/g bran for the same.

The total phenolics content, simple sum of free and bound phenolics, ranged from 4.95 to 6.15 mg FAE/g bran in this study. These values are 2-3 times higher than those obtained by Jonnala et al (2010) for regular bread wheat and 7 waxy wheat samples (2-2.5 mg GAE/g bran). Contributions of the free phenolics to the total phenolics content ranged from 12-18%, whereas that of the bound phenolics was 84-89%. These ranges are narrower than those reported by Adom et al (2003) for 11 diverse wheat classes and experimental lines (16-28% for free vs. 72-84% for bound).

Effects of stress on plant performance

Grain yield and number of spikes per pot from stressed and non-stressed plants are shown in Table 3-1. Table 3-2 shows the moisture content, kernel weight, kernel minor diameter, and bran yield for each stress type and control.

Heat stress. The number of spikes per pot of heat stressed plants was significantly lower than control plants (Table 3-1). Kernels were shriveled. The minor diameter and kernel weight were lower than control grains (Table 3-2, Figure 3-2). In contrast, bran yield after milling was high compared to control grains (Table 3-2). Heat stress reduces the performance of wheat cultivars regardless of their level of tolerance/susceptibility (Nawaz et al, 2013). According to the same researchers, the severity of this reduction is higher at booting or heading stages compared to anthesis and grain filling stages. Spike number and single kernel weight were not significantly affected by high temperature when Narayanan et al (2015) stressed Karl 92 plants for 7 days starting at the onset of anthesis. Grain yield per spike is a function of grain number and single kernel weight. Narayanan et al (2015) observed decreased seed set that led to low grain yield per spike. In this study, kernel weight and minor diameter were lower than control, a reflection of the poor grain fill that heat-stressed plants experienced. These kernels were not aborted in spite of the heat stress because this started when the plant was past the risk of kernel abortion (Hays et al, 2007; Spiertz, et al, 2006). On the other hand, grain yield per spike expressed as total weight of grain per spike was similar to control (Table 3-1), which indicates that the density of seeds from heat-stressed plants was high. .

Insect feeding stress. This factor did not significantly affect the yield parameters measured in this study (Table 3-1), which is opposite to the observations by Riedell et al. (2003). A possible explanation for this lack of fitness cost on grain yield is the priming effect of defense

responses caused by Marathon, the neonicotinoid and systemic insecticide applied to plants (Ford et al, 2010).

Rust infection. Grain yield per pot and per spike was significantly high (Table 3-1), perhaps due to a higher number of grains per spike since both number of spikes and kernel weight were similar to control (Tables 3-1 and 3-2). Figure 3-4 shows the susceptible Karl 92 phenotype infected with *P. triticina*. The high density of the pustules and loss of chlorophyll probably compromised photosynthesis, so an explanation for higher grain yield is carbon mobilization from stem reserves, which is a tolerance response to late developing leaf diseases (Blum, 1998).

Effect of stress on phenolic concentration and antioxidant capacity

Insect feeding stress. *R. padi* feeding explained some of the variation in TPC ($P<0.0719$) and %DPPH ($P<0.0038$) in the free fraction of wheat bran extracts but not in the bound fraction ($P>0.1662$ and $P>0.5004$, respectively) (Table 3-3). The significant change of TPC and %DPPH in the free fraction of wheat bran extracts shows that plants responded to *R. padi* feeding damage. The effector salivary proteins responsible for these changes are not known, but they likely do not involve a wheat plant R-gene interaction because Karl 92 has not known resistance to *R. padi*. Unlike other aphids, *R. padi* does not inflict visual damage to the plants by its phloem-sucking feeding habit (Franzen et al., 2008), which can mask the susceptibility of wheat genotypes. The *R. padi*-Karl 92 combination used in this experimental trial did elicit a phenolic response that, along with the fact that wheat plants survived the infestation, adds evidence to the hypothesis postulated by Smith and Boyko (2007) that "plant recognition of damage inflicted by aphids leads to changes in plant chemistry, followed by the

production of plant signaling molecules that trigger a general stress response." One of the main changes in plant chemistry that is observed after feeding by phloem-sucking insects is a shift in carbon/nitrogen metabolism (Zhu et al., 2008). This metabolic shift has direct consequences to plant fitness and other parameters. For example, Riedell et al. (2003) observed 8% reduction in kernel weight and Ni et al. (2001) reported a significant increase in total protein content in fresh wheat leaves in response to *R. padi* feeding stress. Although the exact defense mechanism in this trial is not known, a general defense response such as salicylic acid-mediated systemic acquired resistance (SAR) was likely at work because it has been shown that molecular markers for SAR are expressed in response to aphid feeding as well as other phloem-sucking insects (de Ilarduya et al., 2003; Kaloshian et al., 2005). If this is the case, further experiments are needed to separate the potentially confounding effects of aphid feeding cues and imidacloprid, the active ingredient in Marathon® and synthetic elicitor of SAR (Ford et al., 2010), on phenolics.

Heat Stress. TPC in the free and bound fractions of heat-stressed grains were 20% and 22% lower than the control, respectively (Table 3-4). The % DPPH scavenged by phenolics in the free and bound fractions were 2.52 and 4.21% lower than the control, respectively (Table 3-4). The effect of heat stress on the plants was a consistent and significant reduction of the levels of phenolics and the antioxidant capacity measured in the bran as well as the number of spikes per pot. Published studies have reported that some weather conditions negatively affect the levels of phenolics in wheat crops. For instance, TPC was negatively correlated with the number of hours that atmosphere temperature exceeded 32°C during the grain filling period in hard red winter wheat crops (Moore et al., 2006). Temperature and rainfall were the main factors influencing the levels of free phenolics, flavonoids, and anti-radical activity in several varieties

of durum and soft wheat (Heimler et al., 2010). The nature of that study prevented authors from separating the effects of water deprivation and high temperatures on phenolics. In this study, both heat-stressed and non-stressed plants were watered with the same periodicity to avoid confounding effects. Narayanan et al. (2015) found that wheat plants maintained chlorophyll concentration, but the photosynthetic capacity was reduced in response to high temperature stress during anthesis and grain-fill developmental stages. Heat stress may have caused a suppression of the phenylalanine pathway, a key biochemical pathway responsible for growth and defense (Tohge et al. 2013), because phenolics in the bound fraction, those covalently linked to lignin and other polymers, were significantly reduced as well as the number of spikes.

Rust Infection Stress. Wheat bran showed no significant changes in phenolic content or antioxidant capacity in response to rust infection in this study (Table 3-5). Published studies have shown that the biochemical base for resistance to various fungi diseases in wheat consists of induction of phenolic compounds (Eisa et al., 2013; Gogoi et al., 2001). Phenolics are part of the localized and rapid hypersensitive response in the leaves of wheat cultivars resistant to rust pathogens (Beardmore et al., 1983). The ongoing process of lignification is also a resistance response that these cultivars use to stop further pathogen invasion (Menden et al., 2007). These hypersensitive and lignin responses are typical of incompatible interactions between avirulent pathogens and resistant cultivars (Bolton et al., 2008a; Gachomo et al., 2003). These interactions are characterized by a high degree of specificity between the pathogen race and the host genotype (Bogdanove, 2002). On the other hand, compatible interactions between avirulent pathogens and cultivars that lack the corresponding resistance gene determines disease susceptibility. In this study, a compatible *P. triticina* race was chosen because of its mild

virulence in Karl 92. This virulence was shown by the development of the characteristic pustules of *P. triticina* in the leaves of the plants as shown in Figure 3-4. The susceptible phenotype shown in that figure is different than those shown in wheat genotypes that express leaf rust resistance genes (*Lr*) or the hypersensitive response shown in Figure 3-5. The *P. triticina* race–Karl 92 interaction was expected to elicit a defense response similar to basal defense or SAR. Bolton et al (2008b) found that the gene encoding shikimate kinase, an enzyme in the shikimate pathway leading to phenylpropanoid production, was downregulated in a compatible interaction, while several general stress-related genes were upregulated. Taken together with the findings in this study, simple phenolics are not part of the general defense response to leaf rust pathogens.

In summary, the experiments conducted and presented in this study were, to our knowledge, the first attempt to formally test the relationships found in the published literature between insect feeding, pathogen infection, and heat stress, and the levels of grain phenolics using a single wheat cultivar and factors that induce general defense or stress responses. There were some effects on plant fitness as a response to stress. The number of spikes per pot at physiological maturity was significantly lower for heat-stressed plants compared to control, and kernels were shriveled as shown by the relatively low kernel weight and minor diameter. Grains from heat stressed plants yielded a relatively high percentage of bran. Grain yield per spike was significantly higher in rust-infected plants compared to control, but kernel weight and minor diameters are similar to control. There were no significant changes in number of spikes, grain yield, or individual kernel measurements in response to insect feeding. The antioxidant properties of wheat bran from some of these stressed plants were affected. Aphid feeding and heat stress explained some of the variation in antioxidant properties of wheat bran significantly at

P<0.1. Aphid feeding increased TPC and %DPPH of free phenolic extracts, but heat stressed decreased them both in the free and bound extracts. Rust-infection did not significantly change the phenolic concentration or antioxidant capacity in wheat bran, in spite of the high grain yield. Although there was an effect on plant parameters associated with the defense response to pathogen infection and heat stress, phenolic induction in the grains was not part of that defense response. This does not conform to the hypothesis that phenolics are part of a general stress defense response. In the next chapter, phenolic induction in wheat grains will be studied as part of an active defense response to insect feeding.

Abbreviations used

SAR, systemic acquired resistance

TPC, total phenolic content

FAE, ferulic acid equivalent

DPPH, 2,2-diphenyl-1-picrylhydrazyl

ANOVA, Analysis of variance

Figures

Figure 3-1 Left: wheat plants exposed to elevated temperatures (30/25° Celsius day/night and 84-90% RH). Right: control plants were kept at 20/15° Celsius day/night and 50-70% RH.

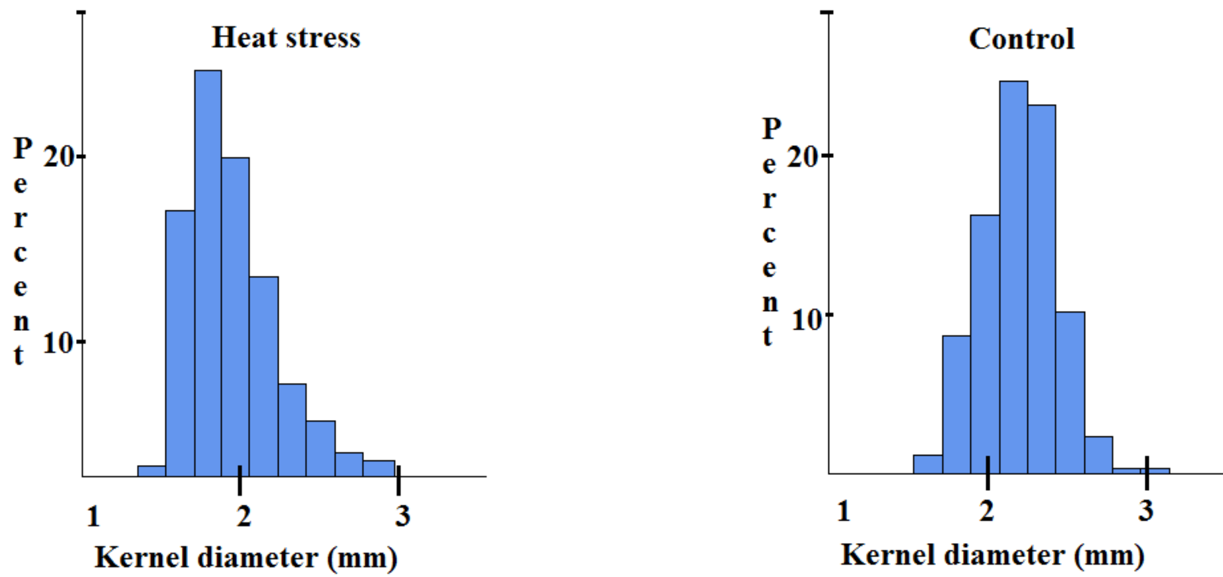

Figure 3-2 Top: Distribution of kernel minor diameter (mm) from heat stressed and control grains. Bottom: Representative grain sample from heat stressed and control plants.

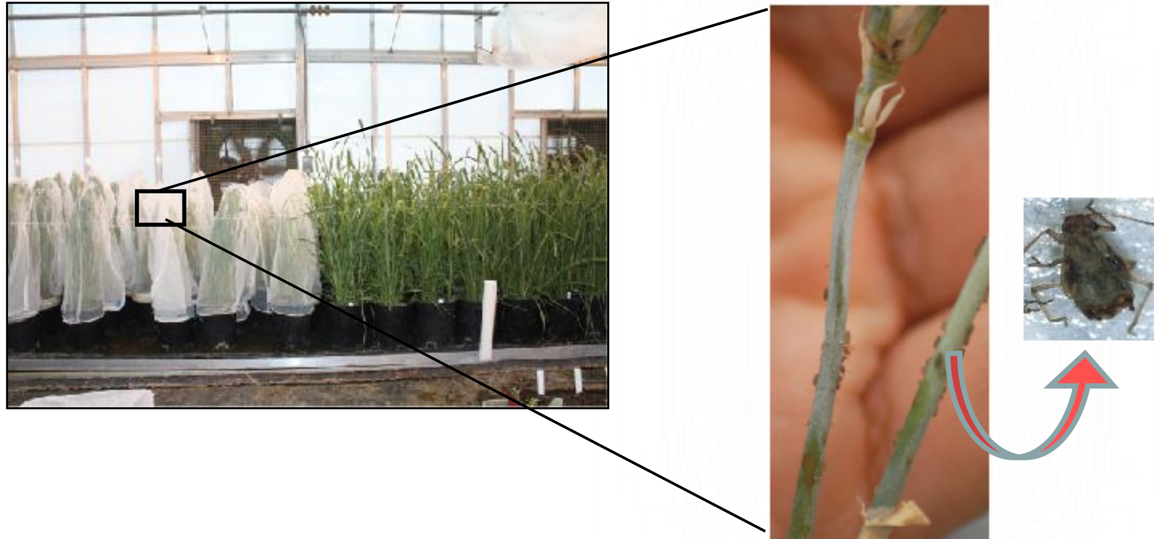

Figure 3-3 Insect feeding. From left to right: insect infested (covered with nets) and control plants (uncovered), close-up of insect colonies feeding on the stem of the wheat plant, close-up of bird cherry oat aphid (*Rhopalosiphum padi*).

Figure 3-4 Karl 92 wheat plants infested with a compatible race of leaf rust (*Puccinia triticinia*)

Figure 3-5 Resistant and susceptible wheat phenotypes to leaf rust (*Puccinia triticinia*), from left to right: Thatcher+Lr34, Thatcher+Lr12, Thatcher+Lr13, Thatcher (Susceptible). Source: Kolmer (2013)

Tables

Table 3-1. Mean±SE spikes/pot, grain yield/pot, and grain yield/spike (3 plants/pot). Number of replications (N) is shown in parenthesis.

Stress type/control	Spikes/pot	Grain yield/pot (g)	Grain yield/spike (g)
Heat stress	14±4 (22)	4.11±1.35 (26)	0.325±0.081 (17)
P>F[†]	*0.0151*	*0.1645*	*0.8758*
Rust infection	23±4 (16)	12.31±3.87 (16)	0.571±0.221 (16)
P>F	*0.6045*	*0.0821*	*0.0819*
Insect feeding	25±6 (26)	7.07±2.21 (26)	0.307±0.135 (26)
P>F	*0.4701*	*0.2964*	*0.4888*
Heat/Rust control	24±3 (3)	7.65±3.83 (3)	0.307±0.165 (3)
Insect feeding control	26±4 (7)	9.68±4.06 (7)	0.384±0.188 (4)

[†]Probability of a larger F due to chance for the hypothesis $\mu_{stress}=\mu_{control}$

Table 3-2. Single kernel moisture, weight, and minor diameter characterization (n=300 kernels), and bran milling yield of composited grain samples from plants exposed to different stresses. Means and standard deviations are shown.

Stress type/control	Moisture content (%, wet basis)	Kernel weight (mg)	Kernel minor diameter (mm)	Bran yield (%)
Heat stress	15.97±0.92	22.00±8.64	2.32±0.31	49.60
Rust infection	15.51±0.53	33.85±8.84	2.72±0.32	38.50
Heat/Rust control	15.37±0.41	36.07±8.90	2.82±0.32	30.90
Insect feeding	15.58±0.57	33.73±8.69	2.76±0.28	34.20
Insect feeding control	15.71±0.58	29.87±8.16	2.60±0.28	33.80

Table 3-3. Total phenolic content (TPC) and % DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenged by free and bound fractions extracted from wheat bran of plants fed on by *R. padi* for 45 days. Means and standard errors from six grain subsamples are shown.

***R. padi* feeding**	**TPC**			
	mg FAE/g bran (Mean±SE)		**% Change relative to control**	
	Free	**Bound**	**Free**	**Bound**
Stressed	0.885±0.059	4.938±0.157	+25.66	-6.59
Non-stressed	0.704±0.059	5.287±0.157		
***P>F*†**	*0.0719*	*0.1662*		
	% DPPH scavenged after 30 min			
	% DPPH scavenged (Mean±SE)		**Increase (+) or decrease (-) from control**	
	Free	**Bound**	**Free**	**Bound**
Stressed	9.698±0.575	19.96±0.573	+3.71	-0.58
Non-stressed	5.988±0.575	20.54±0.573		
P>F	*0.0038*	*0.5004*		

†Probability of a larger F due to chance for the hypothesis $\mu_{stress}=\mu_{control}$

Table 3-4. Total phenolic content (TPC) and % DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenged by free and bound fractions extracted from wheat bran of plants grown at 30/25°C day/night from 21 days after first spike until physiological maturity. Means and standard errors from six grain subsamples are shown.

Heat stress	TPC			
	mg FAE/g bran (Mean±SE)		% Change relative to control	
	Free	Bound	Free	Bound
Stressed	0.574±0.045	3.974±0.110	-20.11	-22.15
Non-stressed	0.719±0.045	5.104±0.110		
P>F [†]	*0.0633*	*0.0003*		
	%DPPH scavenged after 30 min			
	% DPPH scavenged (Mean±SE)		Increase (+) or decrease (-) from control	
	Free	Bound	Free	Bound
Stressed	3.534±0.410	15.61±0.486	-2.53	-4.21
Non-stressed	6.060±0.410	19.83±0.486		
P>F	*0.0048*	*0.0009*		

[†] Probability of a larger F due to chance for the hypothesis $\mu_{stress}=\mu_{control}$

Table 3-5. Total phenolic content (TPC) and % DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenged by free and bound fractions from wheat bran of plants infected with a compatible race of *Puccinia triticina* for 21 days. Means and standard errors from six grain subsamples are shown.

Rust infection	**TPC**			
	mg FAE/g bran (Mean±SE)		**% Change relative to control**	
	Free	**Bound**	**Free**	**Bound**
Stressed	0.716±0.058	5.207±0.241	-0.40	+2.01
Non-stressed	0.719±0.058	5.104±0.241		
P>F [†]	*0.9724*	*0.7731*		
	%DPPH scavenged after 30 min			
	% DPPH scavenged (Mean±SE)		**Increase (+) or decrease (-) from control**	
	Free	**Bound**	**Free**	**Bound**
Stressed	6.096±0.444	20.44±0.610	+0.03	+0.61
Non-stressed	6.060±0.444	19.83±0.610		
P>F	*0.9560*	*0.5030*		

[†]Probability of a larger F due to chance for the hypothesis $\mu_{stress}=\mu_{control}$

References

Adom, K.K., M.E. Sorrells, and Rui Hai Liu. 2003. Phytochemical profiles and antioxidant activity of wheat varieties. J. Agric. Food Chem. 51:7825-7834.

Beardmore, J., J.P. Ride, and J.W. Granger. 1983. Cellular lignification as a factor in the hypersensitive resistance of wheat to stem rust. Physiological Plant Pathology 22:209-&.

Bellato, S., R. Ciccoritti, V. Del Frate, D. Sgrulletta, and K. Carbone. 2013. Influence of genotype and environment on the content of 5-n alkylresorcinols, total phenols and on the antiradical activity of whole durum wheat grains. J. Cereal Sci. 57:162-169.

Blum, A. 1998. Improving wheat grain filling under stress by stem reserve mobilisation. Euphytica 100:77-83.

Bogdanove, A.J. 2002. Protein-protein interactions in pathogen recognition by plants. Plant Mol. Biol. 50:981-989.

Bolton, M.D., J.A. Kolmer, and D.F. Garvin. 2008a. Wheat leaf rust caused by *Puccinia triticina*. Molecular Plant Pathology 9:563-575.

Bolton, M.D., J.A. Kolmer, W.W. Xu, and D.F. Garvin. 2008b. Lr34-mediated leaf rust resistance in wheat: Transcript profiling reveals a high energetic demand supported by transient recruitment of multiple metabolic pathways. Mol. Plant-Microbe Interact. 21:1515-1527.

Boyko, E.V., C.M. Smith, V.K. Thara, J.M. Bruno, Y. Deng, S.R. Starkey, and D.L. Klaahsen. 2006. Molecular basis of plant gene expression during aphid invasion: Wheat pto-and pti-like sequences are involved in interactions between wheat and russian wheat aphid (homoptera: Aphididae). J. Econ. Entomol. 99:1430-1445.

Bruce, T. J. 2014. Variation in plant responsiveness to defense elicitors caused by genotype and environment. *Frontiers in plant science*, *5*.

De Ilarduya, O.M., Q. Xie, and I. Kaloshian. 2003. Aphid-induced defense responses in mi-1-mediated compatible and incompatible tomato interactions. Mol. Plant-Microbe Interact. 16:699-708.

Dixon, A. 1971. The life-cycle and host preferences of the bird cherry-oat aphid, *Rhopalosiphum padi* L., and their bearing on the theories of host alternation in aphids. Ann. Appl. Biol. 68:135-147.

Eisa, M., R. Chand, and A.K. Joshi. 2013. Biochemical and histochemical traits: A promising way to screen resistance against spot blotch (*Bipolaris sorokiniana*) of wheat. Eur. J. Plant Pathol. 137:805-820.

Everette, J., Q. Everette, A. Bryant, Y. Green, G. Abbey, R. Wangila, and Walker. 2010. Thorough study of reactivity of various compound classes toward the folin-ciocalteu reagent. J. Agric. Food Chem. 58:8139-8144.

Ford, K.A., J.E. Casida, D. Chandran, A.G. Gulevich, R.A. Okrent, K.A. Durkin, R. Sarpong, E.M. Bunnelle, and M.C. Wildermuth. 2010. Neonicotinoid insecticides induce salicylate-associated plant defense responses. Proc. Natl. Acad. Sci. U. S. A. 107:17527-17532.

Franzen, L.D., A.R. Gutsche, T.M. Heng-Moss, L.G. Higley, and T.B. Macedo. 2008. Physiological responses of wheat and barley to Russian wheat aphid, *Diuraphis noxia* (Mordvilko) and bird cherry-oat aphid, *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae). Arthropod-Plant Interactions 2:227-235.

Gachomo, E.W., O.O. Shonukan, and S.O. Kotchoni. 2003. The molecular initiation and subsequent acquisition of disease resistance in plants. African Journal of Biotechnology 2:26-32.

Gogoi, R., D.V. Singh, and K.D. Srivastava. 2001. Phenols as a biochemical basis of resistance in wheat against karnal bunt. Plant Pathol. 50:470-476.

Hays, D.B., J.H. Do, R.E. Mason, G. Morgan, and S.A. Finlayson. 2007. Heat stress induced ethylene production in developing wheat grains induces kernel abortion and increased maturation in a susceptible cultivar. Plant Science 172:1113-1123.

Heimler, D., P. Vignolini, L. Isolani, P. Arfaioli, L. Ghiselli, and A. Romani. 2010. Polyphenol content of modern and old varieties of *Triticum aestivum* L. and T. durum desf. grains in two years of production. J. Agric. Food Chem. 58:7329-7334.

Huang, D., B. Ou, and R.L. Prior. 2005. The chemistry behind antioxidant capacity assays. J. Agric. Food Chem. 53:1841-1856.

Jonnala, R.S., S. Irmak, F. MacRitchie, and S.R. Bean. 2010. Phenolics in the bran of waxy wheat and triticale lines. J. Cereal Sci. 52:509-515.

Julkunen-Tiitto, R. 1985. Phenolic constituents in the leaves of northern willows: Methods for the analysis of certain phenolics. J. Agric. Food Chem. 33:213-217.

Kaloshian, I., L. Kaloshian, and Walling. 2005. Hemipterans as plant pathogens. Annu. Rev. Phytopathol. 43:491-521.

Kim, K., R. Tsao, R. Yang, and S.W. Cui. 2006. Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. Food Chem. 95:466-473.

Kolmer, J. 2013. Leaf rust of wheat: Pathogen biology, variation and host resistance. Forests 4:70-84.

Krygier, K., F. Sosulski, and L. Hogge. 1982. Free, esterified, and insoluble-bound phenolic acids. 1. extraction and purification procedure. J. Agric. Food Chem. 30:330-334.

Kwami Adom, K., M.E. Sorrells, and Rui Hai Liu. 2003. Phytochemical profiles and antioxidant activity of wheat varieties. J. Agric. Food Chem. 51:7825-7834.

Lattanzio, V., V.M. Lattanzio, and A. Cardinali. 2006. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. Phytochemistry: Advances in Research 661:23-67.

Leiss, K.A., F. Maltese, Y.H. Choi, R. Verpoorte, and P.G.L. Klinkhamer. 2009. Identification of chlorogenic acid as a resistance factor for thrips in chrysanthemum. Plant Physiol. 150:1567-1575.

Li, L., P.R. Shewry, and J.L. Ward. 2008. Phenolic acids in wheat varieties in the HEALTHGRAIN diversity screen. J. Agric. Food Chem. 56:9732-9739.

Menden, B., M. Kohlhoff, and B.M. Moerschbacher. 2007. Wheat cells accumulate a syringyl-rich lignin during the hypersensitive resistance response. Phytochemistry 68:513-520.

Moore, J., J. Liu, K. Zhou, and L. Yu. 2006. Effects of genotype and environment on the antioxidant properties of hard winter wheat bran. J. Agric. Food Chem. 54:5313-5322.

Narasimhamoorthy, B., B. Gill, A. Fritz, J. Nelson, and G. Brown-Guedira. 2006. Advanced backcross QTL analysis of a hard winter wheat× synthetic wheat population. Theor. Appl. Genet. 112:787-796.

Narayanan, S., P. Prasad, A. Fritz, D. Boyle, and B. Gill. 2015. Impact of high Night-Time and high daytime temperature stress on winter wheat. Journal of Agronomy and Crop Science 201:206-218.

Nawaz, Muhammad Farooq, S.A. Cheema, and Abdul Wahid. 2013. Differential response of wheat cultivars to terminal heat stress. International Journal of Agriculture and Biology 15:1354-1358.

Ni, X.Z., S.S. Quisenberry, T. Heng-Moss, J. Markwell, G. Sarath, R. Klucas, and F. Baxendale. 2001. Oxidative responses of resistant and susceptible cereal leaves to symptomatic and nonsymptomatic cereal aphid (hemiptera : Aphididae) feeding. J. Econ. Entomol. 94:743-751.

Riedell, W.E., R.W. Kieckhefer, M.A.C. Langham, and L.S. Hesler. 2003. Root and shoot responses to bird cherry-oat aphids and barley yellow dwarf virus in spring wheat. Crop Sci. 43:1380-1386.

Sears, R.G., T.J. Martin, T.S. Cox, O.K. Chung, S.P. Curran, W.F. Heer, and M.D. Witt. 1997. Registration of 'Karl 92' wheat. Crop Sci. 37:628-628.

Singleton, V.L., and J.A. Rossi. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16:144-158.

Smith, C.M., and E.V. Boyko. 2007. The molecular bases of plant resistance and defense responses to aphid feeding: Current status. Entomol. Exp. Appl. 122:1-16.

Smith, C.M., X. Liu, L.J. Wang, X. Liu, M. Chen, S. Starkey, and J. Bai. 2010. Aphid feeding activates expression of a transcriptome of oxylipin-based defense signals in wheat involved in resistance to herbivory. J. Chem. Ecol. 36:260-276.

Spiertz, J., R. Hamer, H. Xu, C. Primo-Martin, C. Don, and P. Van Der Putten. 2006. Heat stress in wheat (triticum aestivum L.): Effects on grain growth and quality traits. Eur. J. Agron. 25:89-95.

Tohge, T., M. Watanabe, R. Hoefgen, and A.R. Fernie. 2013. Shikimate and phenylalanine biosynthesis in the green lineage. Front. Plant. Sci. 4:62.

USDA ARS. 2016. National nutrient database for standard reference release. Available at: https://ndb.nal.usda.gov/ndb/foods/show/5613?manu=&fgcd= (last accessed on April 2016)

Verma, B., P. Hucl, and R.N. Chibbar. 2008. Phenolic content and antioxidant properties of bran in 51 wheat cultivars. Cereal Chem. 85:544-549.

Whitworth, R., and J. Ahmad. 2008. Kansas crop pests: bird-cherry oat aphid. Kansas State University Agricultural Experiment Station and Cooperative Extension Service. Available at: http://www.bookstore.ksre.ksu.edu/pubs/MF2823.pdf (Last accessed on September 28th, 2015).

Yang, J., R. Sears, B. Gill, and G. Paulsen. 2002. Genotypic differences in utilization of assimilate sources during maturation of wheat under chronic heat and heat shock stresses. Euphytica 125:179-188.

Yingjian Lu, Junli Lv, Junjie Hao, Yuge Niu, M. Whent, J. Costa, and Liangli Yu. 2015. Genotype, environment, and their interactions on the phytochemical compositions and radical scavenging properties of soft winter wheat bran. LWT -- Food Science and Technology 60:277-283.

Yu, L.L., and K.Q. Zhou. 2005. Antioxidant properties of bran extracts from 'platte' wheat grown at different locations. Food Chem. 90:311-316.

Zhou, K., L. Su, and L.L. Yu. 2004. Phytochemicals and antioxidant properties in wheat bran. J. Agric. Food Chem. 52:6108-6114.

Zhou, K., J. Hao, C. Griffey, H. Chung, S.F. O'Keefe, J. Chen, and S. Hogan. 2007. Antioxidant properties of fusarium head blight-resistant and-susceptible soft red winter wheat grains grown in virginia. J. Agric. Food Chem. 55:3729-3736.

Zhu, L., X. Liu, X. Liu, R. Jeannotte, J.C. Reese, M. Harris, J.J. Stuart, and M. Chen. 2008. Hessian fly (*Mayetiola destructor*) attack causes a dramatic shift in carbon and nitrogen metabolism in wheat. Mol. Plant-Microbe Interact. 21:70-78.

Chapter 4 - Bird-cherry oat aphid (*Rhopalosiphum padi*) feeding stress induces enhanced levels of phenolics in mature wheat grains

Abstract

Enhancement of naturally occurring phenolic compounds with antioxidant activity in hard red winter wheat grains is a value addition strategy that can potentially increase the profitability of wheat crops. Phenolics are plant secondary metabolites known to be involved in defense against arthropods and pathogen attack. In this study, we investigated the effect of bird-cherry oat aphid (*Rhopalosiphum padi* L) feeding in wheat (*Triticum aestivum* L) at different phenological stages on phenolic concentration in mature grains. Aphids were allowed to feed and reproduce for 14 days on wheat plants at the 5-tiller, 7 or 21 days post-anthesis (DPA) stages of development. Plants infested at 5-tiller and 7 DPA stages had higher free phenolic concentration than aphid-free control, and those moderately infested at 5-tiller through 35 DPA had significantly higher concentration of free and free and conjugated phenolics and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity than aphid-free control. Although there were no significant differences among phenological stages, more resources were allocated to defense, i.e. free phenolic concentration, and less to growth, i.e. grain yield, when infestation started at early stages. The phenolic response was long-lasting and systemic, so systemic acquired resistance was hypothesized to be the mechanism of induction. This information will aid in developing wheat crops with consistently high antioxidant levels.

KEYWORDS: antioxidant enhancement, phenolics, *R. padi* feeding, phenological stages

Introduction

Hard red winter wheat (*Triticum aestivum* L) has traditionally been the main crop cultivated in dry land farming areas, where farmers often depend on unpredictable environmental conditions to grow a crop and on the volatility of market prices to obtain a profit. The potential of hard red winter wheat for antioxidant rich crops has been considered as a value-added strategy that would allow farmers to increase the profitability of their crops (Yu et al., 2002). Enhancement of antioxidant levels in wheat bran has been achieved through the application of post-harvest techniques (Beta et al., 2005; Guo et al., 2011), but the inherent variability of antioxidant levels in wheat crops is still a constraint. Although some of this variability is the result of genetic differences, the majority is due to environmental factors (Moore et al., 2006).

Phenolics, the main compounds with antioxidant activity in wheat, are plant secondary metabolites involved in crop resistance to insect pests (Abdel-Aal et al., 2001; Berner and van der Westhuizen, 2010). Insects of the order Hemiptera and family Aphididae are among the most economically important pests that occur in wheat fields across the U.S (Qureshi and Michaud, 2005). Bird-cherry oat aphid (*Rhopalosiphum padi* L) is one of the three most common aphid species that colonize winter wheat fields in the spring season (Whitworth and Ahmad, 2008). Unlike other aphids, *R. padi* does not elicit leaf chlorosis, but it does trigger physiological changes on wheat plants (Franzen et al., 2008). Aphid feeding damage caused by *R. padi* induced the total phenolic content in leaves of susceptible and resistant winter wheat cultivars as well as the activities of phenylalanine ammonia-lyase and tyrosine ammonia-lyase, key enzymes in the shikimate and phenylalanine pathways responsible for phenolic synthesis

(Leszczyński, 1985). Biochemical changes in the leaves of other crops have also been reported as a direct effect of *R. padi* feeding damage (Eleftherianos et al., 2006). These changes in plant chemistry are in agreement with the hypothesis of elicitation of a general stress response to aphid feeding. Aphids probe the sieve element sap with their stylet and secrete digestive saliva which contains components able to trigger defense responses (Smith and Boyko, 2007). Some of the inducible defense responses in plants are systemic acquired resistance and induced resistance, which depend on salicylic and jasmonic acid, respectively (Shah and Zeier, 2014).

The response of wild wheat (*Triticum uniaristatum* L) to *R. padi* aphid feeding damage has been characterized in terms of phenolic induction on the leaves. It required a minimum of 25 individual aphids and a 48-hour feeding period (Gianoli and Niemeyer, 1997). This response was transient, restricted to the feeding site, and more individual aphids (up to 40) did not significantly affect the phenolic induction in the leaves (Gianoli and Niemeyer, 1998). Based on this knowledge, this study was designed to investigate how hard red winter wheat cv. Karl 92 at 5-tiller, 7 or 21 days post-anthesis (DPA) responds to *R. padi* feeding stress in terms of phenolic induction in mature grains. Since the phenolic response is transient, and restricted to the feeding site, the hypothesis is that *R. padi* feeding stress will induce a greater phenolic response in mature grains when it occurs at late (7 or 21 DPA) vs. early (5-tiller) phenological stages.

Materials and methods

Plant materials. Hard red winter wheat cultivar Karl 92 was grown from seeds in a greenhouse set at 21±5°C /16±5°C (day/night air temperature) and a photoperiod of 14:10 h (light:dark) with supplemental light from high pressure sodium lamps. Two week-old seedlings were kept in a vernalization chamber set at 4°C for six weeks. Subsequently, the seedlings were transplanted into pots (15 cm diameter) at a density of three plants per pot. Potting medium was commercial Sunshine Metro Mix 200 (Hummert International, Earth City, MO) fertilized with 5 g/pot Osmocote (19:6:12 N:P_2O_5:K_2O) (Scotts, Marysville, OH). These pots were placed in a greenhouse at the conditions stated above.

Aphid colony. A colony of *R. padi* aphids was started with a few apterous females obtained from USDA-ARS (Stillwater, Oklahoma). Aphids were kept on 10 day-old barley cultivar "Sundance" under 14:10 (light:dark) h photoperiod with supplemental light and 22±1°C temperature.

Aphid feeding stress. Sets of Karl 92 plants (3 plants per pot) were infested with *R. padi* at the following phenological stages: 5-tiller (Zadoks scale 26-30), 7 DPA (Zadoks scale 71-77), and 21 DPA (Zadoks scale 83-87). Anthesis was defined as half of main panicle flowering. A heavily infested leaf from the *R. padi* colony was placed on each pot and the aphids were allowed to feed and reproduce freely on the plants for 14 days. *R. padi* populations reached 14±5, 73±23, and 38±1 apterous individuals per tiller for the 5-tiller, 7 DPA, and 21 DPA stages, respectively. There were two replications per treatment. Each replication was

conducted inside of a rectangular-shaped 2.3 x 1.2 x 1.2 m (length x width x height) compartment made with insect-proof screen (81 x 81 mesh) of opening size 0.15 x 0.15 mm and 66% light transmission (Hummert International, Earth City, MO). At day 15th of the aphid infestation, 4500 *Hippodamia convergens* young adults were released into the compartments. These insects were very effective aphid predators and usually killed the *R. padi* population overnight. Two additional sets of plants were treated as controls. One was moderately infested starting at 5-tiller through 35 DPA stages of development (Zadoks scale 26-87). A moderate infestation was achieved by keeping the number of aphids to 7±4 per tiller. Another set was kept *R. padi*-free during the entire growing season by releasing 4,500 *H. convergens* adult individuals every 14 days. Adult thrip-predatory mites (*Neoseiulus cucumeris)* were also released periodically on all plants to control the western flower thrip (*Frankliniella occidentalis*). Predatory insects were used instead of synthetic insecticides to avoid the potentially confounding effects of crop protection products on plant defense responses (Ford et al., 2010). Plants were watered every day for 2 h through a capillary matting system (Hummert International, Earth City, MO).

Experimental design. The experimental design was a randomized complete block (RCBD) where blocks (repetitions) were the following growing seasons: March-July 2012, September 2012-February 2013, and March-July 2013. The RCBD was chosen to account for 1) any existing gradient in growing conditions throughout the greenhouse room by using replications and 2) environmental or seasonal effects by using of repetitions. Additionally, seasonal effects on phenolic concentration in *R. padi* feeding treatments should also be observed

in *R. padi*-free control plants which were grown in each repetition. Grains from plants in each replication were pooled, except for those in block March-July 2012, which were subdivided into 4 subsamples. Grain samples from each repetition were kept separate.

Grain sample preparation. Grain samples were cleaned and sorted by size using dockage test sieves with 5.16 mm-diameter holes and a 1.6 x 9.5 mm slotted sieve. The sound and healthy grains (overs from the slotted sieve) were tempered to 15±0.5% moisture content before milling on a Quadrumat Jr. Mill (Brabender, South Hackensack, NJ). The bran was collected from the mill and sifted in a plansifter. Bran particles equal or larger than 400 microns (overs from U.S. sieve no. 40) were kept for extraction and analysis because wheat grain phenolics are largely concentrated in the bran (Adom et al., 2005).

Extraction procedure. Phenolics were extracted from the bran in three fractions: free, free and conjugated, and bound, with the procedure reported in Ramos et al (2015, *revised*). Briefly, one gram of wheat bran was defatted, extracted with methanol:acetone:water solvent (7:7:6 v/v/v, adjusted to pH 2 with 12 M hydrochloric acid), incubated for 2 hours at 23°C with shaking motion, and centrifuged at 5000 *g* for 10 minutes at 10°C. The supernatant was concentrated under vacuum at 40°C and used to obtain the free, and free and conjugated phenolic fractions. The bran pellet was used to obtain the bound phenolic fraction.

Free fraction: An aliquot of 5-10 ml of supernatant was liquid-liquid extracted with 30 ml of ethyl ether/ethyl acetate solvent (1:1 v/v) three times. The non-polar layer was recovered each time. The three aliquots of non-polar layer were pooled together and magnesium sulfate

was added to remove any remaining water. The mixture was concentrated under vacuum at 40°C. The concentrated solution containing free phenolic compounds was reconstituted with 10 ml methanol and saved for subsequent analysis.

Free and conjugated fraction: An aliquot of 5-10 ml of supernatant was hydrolyzed with 5 ml of 2M NaOH at 60°C for 2 h. The hydrolysate was acidified to pH 2 with 12 M hydrochloric acid (HCl). Subsequently, it was liquid-liquid extracted, concentrated, and reconstituted in methanol for analysis as described for the free fraction.

Bound fraction: The bran pellet was hydrolyzed with 5 ml of 4M NaOH at 60°C for 2.5 hours. The hydrolysate was acidified to pH 2 with 12M HCl and defatted with hexane. The residue was liquid-liquid extracted with 4 ml of ethyl ether/ethyl acetate solvent (1:1 v/v) and centrifuged at 5000 x *g* for 5 minutes six times. The supernatants were pooled together and diluted with an equal amount of methanol (1:1 v/v).

Determination of total phenolic content. The phenolic concentration in the free, free and conjugated, and bound fractions were determined with the Folin Ciocalteau assay. The procedure is briefly summarized here. An aliquot of 200 μL of the extracts was pipetted into a test tube and the following reagents were added: 1.5 ml of 0.2 N Folin-Ciocalteau reagent and 1.5 ml of 6% sodium carbonate (w/v). This mixture was vortexed and allowed to incubate in the dark at 23°C for 90 minutes. Ferulic acid solutions of known concentrations and a methanol blank were also tested and incubated with the samples. After incubation, absorbance was measured at 725 nm with a UV-VIS Spectrophotometer (Shimadzu, model UV-1650, Columbia,

MD). Absorbance vs. concentration was plotted for the ferulic acid standard solutions. The linear equation obtained was used to calculate concentration from the absorbance of the samples, and these values were reported as ferulic acid equivalents (FAE).

Determination of antioxidant potential. The antioxidant potential was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay (Huang et al., 2005). The procedure was as follows: 3.9 ml of DPPH solution in methanol (25 mg/ml) was mixed with 0.1 ml of sample. The absorbance of the reaction was measured at 515 nm after a 30-minute incubation period in the dark at 23°C. Absorbance (ABS) is correlated to concentration of DPPH. The percentage of DPPH scavenged (% DPPH) was calculated as follows: $(1-[ABS_{sample}\ (t=30)/ABS_{control}\ (t=0)])*100$ where "t" is time in minutes and the $ABS_{control}$ is absorbance of methanol blank.

Analytical testing. Grain samples were milled, extracted and analyzed in 13 rounds of testing chronologically spanning from shortly after the harvest of first repetition until after harvest of the third one. The test rounds were used as blocks in the ANOVA because they were a reflection of the experimental repetitions.

Statistical analysis. Plant parameters (spikes/pot, grain yield/pot, and grain yield/spike) were analyzed with a two-sample t-Test with unequal variances. Phenolic concentration and % DPPH data of free, free and conjugated, and bound fractions were analyzed using the PROC MIXED procedure in SAS 9.4 statistical software (The SAS institute, Cary, NC). The sources of variation and the degrees of freedom approximated with the Kenward-Roger method for unbalanced designs (Spilke et al., 2005) were: *R. padi* feeding treatments (t=5, df= 4), analytical test rounds (b=13, df=12), treatment*rounds (df=25), rep(treatment*rounds)

(df=23). Statistical significance was declared at P<0.05 and separation of means was done with the Least Square Means test (Bonferroni adjusted P<0.0125).

Results

Yield components of the *R. padi* feeding treatments and control are shown in Table 4-1. *R. padi* feeding damage at the 5-tiller stage caused a 414% increase in the number of spikes, but grain yield/spike was reduced by 69% compared to *R. padi*-free plants. *R. padi* feeding stress starting at 5-tiller through 35 DPA caused a 56% grain yield reduction and a reduction of the number of spikes of 52% (Table 4-1).

R. padi feeding treatments had a significant effect on phenolic concentration in the free (F=5.04; DF=4, 78; P=0.0012) and free and conjugated (F=3.23, DF=4, 70; P=0.0172) fractions of wheat bran extracts (Table 4-2). Similarly, *R. padi* feeding treatments explained some of the variation observed in % DPPH scavenged by the free (F=5.11; DF=4, 59; P=0.0013) and the free and conjugated (F=3.38; DF=4, 60; P=0.0148) fractions of wheat bran extracts (Table 4-3). No differences were detected in phenolic concentration or % DPPH of wheat bran extracts from plants infested for 14 days at the 5-tiller, 7 or 21 DPA stages of development (Tables 4-2 and 4-3). However, phenolic concentration in the free fraction of wheat bran from plants infested at 5-tiller, 7 DPA, and 5-tiller through 35 DPA were significantly higher than *R-padi*-free control (Table 4-2). Additionally, phenolic concentration and %DPPH in the free and conjugated fraction of wheat bran from plants infested at 5 tillers through 35 DPA were significantly higher than control (Tables 4-2 and 4-3). *R. padi* feeding treatments did not explain any variation in phenolic concentration or %DPPH in the bound fraction of wheat bran extracts (Tables 4-2 and 4-3).

Discussion

Comparison of phenolic concentration in the free, free and conjugated and bound fractions. Phenolic acids, the most common form of phenolic compounds found in whole cereal grains, exist in three forms: free soluble, esterified to sugars and other low molecular mass compounds, and insoluble covalently linked forms (Li et al., 2008). These three phenolic forms are obtained from the extraction procedure used in this study in the free, free and conjugated, and bound fractions, respectively. The range of phenolic concentrations found in the free, free and conjugated, and bound fractions of 130 winter wheat cultivars commonly grown in Europe were 3-30, 81-276, and 208-878 μg/g dry whole flour (Li et al., 2008). The rank of the fractions was similar to that observed in this study (free<free-conjugated<bound), but the phenolic concentrations were approximately one order of magnitude lower than those observed for the phenolic-rich bran used in this study. The range of phenolic concentrations in the free fraction shown in Table 4-2 was lower than that obtained for 51 Canadian wheat cultivars, but the range of concentrations in the bound fraction was in the high end of the range of bound phenolics for the same cultivars (Verma et al., 2008).

***R. padi* feeding treatments.** In this study, phenolics in the free fraction, and to some extent free and conjugated, were more responsive to *R. padi* feeding than bound phenolics. Phenolic acids in the free and free and conjugated fraction may be synthesized to act as feeding deterrents or signaling molecules in response to aphid feeding (Leiss et al., 2009; Mandal et al., 2010). On the other hand, bound phenolics are linked to cell wall structural components such as lignin and arabinoxylans through ester bonds (Stalikas, 2007). These structural characteristics are usually constitutive and therefore, under tight genetic control, which helps explains the lack of induction of bound phenolic synthesis in response to *R. padi* feeding.

No differences were observed in phenolic concentration or % DPPH in the different fractions among the phenological stages studied, contrary to our hypothesis. However, *R. padi* feeding at the 5-tiller and 7 DPA stages for 14 days significantly induced the phenolic concentration in the free fraction of wheat bran extracts by 12.64% and 6.8% compared to *R. padi*-free control (Table 4-2). *R. padi* feeding from 5 tillers through 35 DPA also significantly increased phenolic concentration in the free and free and conjugated fractions of wheat bran extracts by 8.71% and 5.84% compared to *R. padi*-free plants (Table 4-2). The DPPH scavenged by these extracts were 19% and 15% higher than control (Table 4-3). These results show that *R. padi* feeding stress enhances the phenolic concentration and antioxidant activity in mature grains of infested plants in agreement with our previous investigation (Ramos et al 2015, *revised*). The phenolic induction may be mediated by the salicylic acid or jasmonic acid-dependent defense responses (De Ilarduya et al., 2013). Giordanengo et al. (2010) proposed that aphids down-regulate the jasmonic acid-dependent defense genes, but up-regulate the salicylic acid-signaling pathway because it is an inefficient defense against the feeding of aphids. *R. padi* aphids fed on and reproduced successfully in these experiments as shown especially in plants infested at 5-tillers through 35 DPA, which indicates that the phenolic defense response observed was inefficient against their feeding and reproduction as suggested by Giordanengo et al. (2010). Additionally, the salicylic acid defense pathway is characterized by a significant increase in the production of free and conjugated salicylic acid (Vernooji et al., 1995) in agreement with the significant and consistent increase in free and free and conjugated phenolic concentration observed in this study in response to *R. padi* feeding at 5-tiller-35 DPA.

Fitness cost. The fitness cost exacted to the plant in terms of grain yield per spike is in agreement with the optimal defense theory (Zangerl and Bazzaz, 1992), and also consistent with the 13-24% grain yield reduction observed in winter wheat infested by *R. padi* at the tillering stage (Savaris et al., 2013). There was an unusually high number of spikes in response to *R. padi* feeding at the 5-tiller stage. This phenomenon was similar to that observed in winter wheat plants that do not complete the vernalization process. However, lack of complete vernalization as a cause for the high tillering phenomenon observed was refuted because the set of control plants, which were exposed to the same vernalization process, did not show this high number of tillers. An alternative explanation is that this phenomenon was possibly the result of either, a rapid compensatory effect for the reduced or stunted growth experienced during 14 days due to the aphid infestation, and/or additional stress brought about by an unknown factor post-aphid infestation. These plants allocated more resources to defense in terms of phenolic concentration, even after developing a high number of tillers, which indicates that phenolics as a defense response was a priority to the plant during carbon allocation. Although the phenolic response was not contingent on the phenological stage of the plant as hypothesized, the relative allocation of resources to grain yield/phenolics was influenced by the plant stage when *R. padi* feeding stress starts. More resources were allocated to defense and less to yield when stress started at early stages (5-tillers, 7 DPA, 5-tillers through 35 DPA), while less resources were allocated to defense and more to yield when stress started at late stages (21 DPA).

Potential mechanism. The defense response observed was long lasting and systemic because *R. padi* feeding starting at the 5-tiller stage affected the phenolic concentration in mature grains, i.e. parts of the plants that are distal in terms of time and space to the feeding site. We

propose that systemic acquired resistance (SAR) was the mechanism at work in this study. SAR is an immune defense mechanism in plants mediated by the salicylic acid signaling pathway (Fu and Dong, 2013). Phenolic induction as part of a more active defense response will be the topic of study in the next two chapters.

Tables

Table 4-1. Number of spikes and grain yield from Karl 92 plants in *R. padi* feeding treatments and *R. padi*-free control.

***R. padi* feeding treatments**	**Spikes/pot**	**Grain yield/pot (g)**	**Grain yield/spike (g)**
5-tiller (N=7)	107.86±17.03	40.02±7.68	0.37±0.05
P(T≤t)	*0.001*	*0.001*	*0.001*
7 DPA (N=5)	16.20±4.49	17.79±7.74	1.07±0.28
P(T≤t)	*0.078*	*0.084*	*0.346*
21 DPA (N=2)	17.00±5.66	18.39±4.96	1.20±0.69
P(T≤t)	*0.501*	*0.310*	*0.98*
5-tiller through 35 DPA (N=9)	10.11±5.99	5.31±4.78	0.52±0.32
P(T≤t)	*0.001*	*0.001*	*0.001*
R. padi-free control (N=5)	21.20±3.03	25.61±3.47	1.21±0.11

Table 4-2. Mean ± SE phenolic concentration (µg FAE/g bran) in the free, free and conjugated, and bound fraction of bran extracts from Karl 92 wheat plants stressed by bird-cherry oat aphid (Rhopalosiphum padi) feeding at 5-tiller, 7 or 21 days post-anthesis (DPA) for 14 days, or moderate feeding at 5-tiller through 35 DPA.

***R. padi* feeding treatments**	**Free**	**Free and conjugated**	**Bound**
5-tiller	566.7 ± 15.920 a‡	1181.9 ± 31.28 ab	4148.2±40.63a
7 DPA	537.3 ± 8.173 a	1163 ± 23 ab	4132.2±25.42a
21 DPA	524.1 ± 28.86 ab	1161.6 ± 48.29 ab	4164.9±68.56a
5-tiller through 35 DPA	546.9 ± 9.621 a	1196.8 ± 24.36 a	4155.6±28.09a
***R. padi*-free control**	503.1 ± 9.488 b	1130.8 ± 23.88 b	4157.5±27.34a
P>F†	0.0012	0.0172	0.8706

†Probability of a larger F due to chance among levels of *R. padi* treatments
‡LSM means followed by a different letter in a column significantly differ (Bonferroni adjusted P<0.0125)

Table 4-3. Mean ± SE percentage of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenged after 30 minutes by free, free and conjugated, and bound phenolics in bran extracts from Karl 92 wheat plants stressed by bird-cherry oat aphid (Rhopalosiphum padi) feeding at the 5-tiller, 7 or 21 days post-anthesis (DPA) for 14 days, or moderate feeding at 5-tiller through 35 DPA.

***R. padi* feeding treatments**	**Free**	**Free and conjugated**	**Bound**
5-tiller	9.21 ± 0.84 ab‡	13.19 ± 0.92 ab	25.07 ± 1.06 a
7 DPA	9.15 ± 0.76 ab	13.72 ± 0.73 a	24.80 ± 0.98 a
21 DPA	8.45 ± 1.05 ab	12.52 ± 1.38 ab	25.93 ± 1.28 a
5-tiller through 35 DPA	9.93 ± 0.78 a	14.04 ± 0.77 a	25.82 ± 1.00 a
***R. padi*-free control**	8.34 ± 0.77 b	12.17 ± 0.76 b	25.18 ± 0.99 a
P>F†	0.0013	0.0148	0.2531

†Probability of a larger F due to chance among levels of *R. padi* treatments
‡LSM means followed by a different letter in a column significantly differ (Bonferroni adjusted P<0.0125)

References

Abdel-Aal, E.S.M., P. Hucl, F.W. Sosulski, R. Graf, C. Gillott, and L. Pietrzak. 2001. Screening spring wheat for midge resistance in relation to ferulic acid content. J. Agric. Food Chem. 49:3559-3566.

Adom, K.K., M.E. Sorrells, and R.H. Liu. 2005. Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. J. Agric. Food Chem. 53:2297-2306.

Berner, J.M., and A.J. van der Westhuizen. 2010. The selective induction of the phenylalanine ammonia-lyase pathway in the resistance response of wheat to the Russian wheat aphid. Cereal Research Communications 38:506-513.

Beta, T., S. Nam, J.E. Dexter, and H.D. Sapirstein. 2005. Phenolic content and antioxidant activity of pearled wheat and roller-milled fractions. Cereal Chem. 82:390-393.

De Ilarduya, O.M., Q. Xie, and I. Kaloshian. 2003. Aphid-induced defense responses in mi-1-mediated compatible and incompatible tomato interactions. Mol. Plant-Microbe Interact. 16:699-708.

Eleftherianos, I., P. Vamvatsikos, D. Ward, and F. Gravanis. 2006. Changes in the levels of plant total phenols and free amino acids induced by two cereal aphids and effects on aphid fecundity. J. Appl. Entomol. 130:15-19.

Ford, K.A., J.E. Casida, D. Chandran, A.G. Gulevich, R.A. Okrent, K.A. Durkin, R. Sarpong, E.M. Bunnelle, and M.C. Wildermuth. 2010. Neonicotinoid insecticides induce salicylate-associated plant defense responses. Proc. Natl. Acad. Sci. U. S. A. 107:17527-17532.

Franzen, L.D., A.R. Gutsche, T.M. Heng-Moss, L.G. Higley, and T.B. Macedo. 2008. Physiological responses of wheat and barley to Russian wheat aphid, *Diuraphis noxia* (Mordvilko) and bird cherry-oat aphid, *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae). Arthropod-Plant Interactions 2:227-235.

Fu, Z.Q., and X. Dong. 2013. Systemic acquired resistance: Turning local infection into global defense. Annual Review of Plant Biology 64:839-863.

Gianoli, E., and H.M. Niemeyer. 1998. Allocation of herbivory-induced hydroxamic acids in the wild wheat *Triticum uniaristatum*. Chemoecology 8:19-23.

Gianoli, E., and H.M. Niemeyer. 1997. Characteristics of hydroxamic acid induction in wheat triggered by aphid infestation. J. Chem. Ecol. 23:2695-2705.

Giordanengo, P., L. Brunissen, C. Rusterucci, C. Vincent, A. Van Bel, S. Dinant, C. Girousse, M. Faucher, and J. Bonnemain. 2010. Compatible plant-aphid interactions: How aphids manipulate plant responses. Comptes Rendus Biologies 333:516-523.

Guo, M., K. Petrofsky, L. Zhang, P. Chen, A. Hohn, M. Youn, D. Gallaher, R. Liu, J. Faubion, M. Bunzel, L. Marquart, and R. Ruan. 2011. Improving the functionality and bioactivity in wheat bran. IFT 2010 Annual Meeting, Chicago, IL.

Huang, D., B. Ou, and R.L. Prior. 2005. The chemistry behind antioxidant capacity assays. J. Agric. Food Chem. 53:1841-1856.

Leiss, K.A., F. Maltese, Y.H. Choi, R. Verpoorte, and P.G.L. Klinkhamer. 2009. Identification of chlorogenic acid as a resistance factor for thrips in chrysanthemum. Plant Physiol. 150:1567-1575.

Leszczyński, B. 1985. Changes in phenols content and metabolism in leaves of susceptible and resistant winter wheat cultivars infested by *Rhopalosiphum padi* (L.)(Family: Aphididae). Zeitschrift Für Angewandte Entomologie 100:343-348.

Li, L., P.R. Shewry, and J.L. Ward. 2008. Phenolic acids in wheat varieties in the HEALTHGRAIN diversity screen. J. Agric. Food Chem. 56:9732-9739.

Mandal, S.M., D. Chakraborty, and S. Dey. 2010. Phenolic acids act as signaling molecules in plant-microbe symbioses. Plant Signaling & Behavior 5:359-368.

Moore, J., J. Liu, K. Zhou, and L. Yu. 2006. Effects of genotype and environment on the antioxidant properties of hard winter wheat bran. J. Agric. Food Chem. 54:5313-5322.

Qureshi, J.A., and J.P. Michaud. 2005. Interactions among three species of cereal aphids simultaneously infesting wheat. J. Insect Sci. 5:13. Available online: http://insectscience.org/5.13

Ramos, O.; Smith, C.M.; Fritz, A.K.; Madl, R.L. 2015. Effect of insect feeding, pathogen infection, and heat stress on wheat bran antioxidant properties. Crop Science. *In review*

Savaris, M., S. Lampert, J. Salvadori, D. Lau, P.d.S. Pereira, and M. Smaniotto. 2013. Population growth and damage caused by rhopalosiphum padi (L.)(hemiptera, aphididae) on different cultivars and phenological stages of wheat. Neotropical Entomology 42:539-543.

Shah, J., and J. Zeier. 2014. Long-distance communication and signal amplification in systemic acquired resistance. Induced Plant Responses to Microbes and Insects 4:23-38

Smith, C.M., and E.V. Boyko. 2007. The molecular bases of plant resistance and defense responses to aphid feeding: Current status. Entomol. Exp. Appl. 122:1-16.

Spilke, J., H. Piepho, and X. Hu. 2005. Analysis of unbalanced data by mixed linear models using the MIXED procedure of the SAS system. Journal of Agronomy and Crop Science 191:47-54.

Stalikas,C.D. 2007. Extraction, separation, and detection methods for phenolic acids and flavonoids. Journal of Separation Science 30:3268-3295.

Verma, B., P. Hucl, and R.N. Chibbar. 2008. Phenolic content and antioxidant properties of bran in 51 wheat cultivars. Cereal Chem. 85:544-549.

Vernooij, B., L. Friedrich, P. Ahl Goy, T. Staub, H. Kessmann, and J. Ryals. 1995. 2, 6-dichloroisonicotinic acid-induced resistance to pathogens without the accumulation of salicylic acid. MPMI-Molecular Plant Microbe Interactions 8:228-234.

Whitworth, J.R. and A. Ahmad. 2008. Kansas crop pest bird-cherry oat aphid. Kansas State Research and Extension. Manhattan, KS. Available at: https://www.bookstore.ksre.ksu.edu/pubs/MF2823.pdf. Last accessed: May 13th, 2016

Yu, L., S. Haley, J. Perret, M. Harris, J. Wilson, and M. Qian. 2002. Free radical scavenging properties of wheat extracts. J. Agric. Food Chem. 50:1619-1624.

Zangerl, A.R., and F.A. Bazzaz. 1992. Theory and pattern in plant defense allocation. In Plant Resistance to Herbivores and Pathogens. University of Chicago Press, Chicago. 363-391p.

Chapter 5 - Synthetic elicitors of systemic acquired resistance promote *de novo* synthesis of phenolics in foliage of hard red winter wheat (*Triticum aestivum* L)

Abstract

Systemic acquired resistance (SAR) is an inducible plant defense response against pathogen and insect attack. Since phenolic compounds have a role in defense responses, activation of pathways involved in the SAR response can be a feasible strategy to produce wheat crops with enriched levels of grain phenolics. In addition to pathogens and insects, SAR is elicited by synthetic compounds that may reduce the biological variability in activating the defense response. In this study, six synthetic SAR elicitors were tested for their effectiveness to induce *de novo* phenolic synthesis in the foliage of hard red winter wheat cultivars Karl 92 and Ike. Solutions of thiamine (B1), riboflavin (B2), benzo (1,2,3)-thiadiazole (BTH), 2,6-dichloropyridine-4-carboxylic acid (DCPCA), methyl jasmonate (MeJa), and sodium salicylate (SS) at concentrations known to express transcripts from SAR genes were sprayed on wheat foliage at the tillering stage. Total leaf phenolic content (TLPC) (measured as ferulic acid equivalents) was determined at 24-, 36- and 48 hours post application (hpa). Wheat foliage reacted to SAR elicitors at the post-translational level by *de novo* phenolic synthesis. The phenolic induction was short-lived, peaking at 36 hpa and disappearing or reaching a plateau at 48 hpa. Ike treated with MeJa had 177% higher TLPC than not-sprayed control, while plants treated with DCPCA had 49% higher TLPC than control. Since most elicitors worked through either SA- or MeJa signaling and the induction was short, the role of the newly synthesized phenolics must be signaling and not directly phytotoxic.

Keywords: Systemic acquired resistance, defense response, synthetic elicitors, *de novo* phenolic synthesis, signaling

Introduction

Phenolic induction in mature grains is part of the defense response of wheat to bird cherry oat aphid (*Rhopalosiphum padi)* feeding stress, presumably working through systemic acquired resistance (Ramos et al, 2015, in review). Systemic acquired resistance is one of several inducible defense responses (Shah and Zeier, 2013). It is an immune defense mechanism, in which a local primary infection or infestation induces a general resistance state in distal (systemic) parts of the plant (Fu and Dong, 2013).

SAR can also be triggered by synthetic elicitors such as salicylic acid (Ward et al, 1991), 2,6-dichloroisonicotinic acid (Vernooji et al, 1995), or benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Gorlach et al, 1996). The expression of SAR depends on, at least, the hormone salicylic acid (SA), and the transcription factor, NPR1. One of the initial triggers of SAR is the cellular imbalance in phenylalanine levels caused by the pathogen attack (Pajerowska-Mukhtar et al, 2012). Phenylalanine is one of the key precursors of phenolic acids in the phenylpropanoid pathway. Therefore, phenolics may be directly or indirectly involved in SAR playing a role in plant recognition of damage and/or signaling.

In this study, the effectiveness of synthetic elicitors of SAR at inducing *de novo* phenolic synthesis in wheat foliage (*Triticum aestivum* L) was evaluated. The chemical compounds studied have been proposed as elicitors of SAR based on their ability to induce PR-gene related proteins such as PR1, a widely recognized molecular marker for SAR. These are: sodium salicylate (Ward et al, 1991), methyl jasmonate (Xu et al, 1994; Wang et al, 2005), 2,6-dichloropyridine-4-carboxylic acid (Vernooji et al, 1995), thiamine (Ahn et al, 2005), riboflavin (Dong and Beer, 2000), and BTH (Gorlach et al, 1996). Solutions of these SAR elicitors were exogenously applied to wheat plants cv. Karl 92 and Ike at the tillering stage at concentrations

known to induce expression of PR-gene transcripts in order to 1) determine whether phenolic compounds are produced in green leaf tissue as a direct response to elicitor treatment, 2) evaluate the timing and duration of the response, and 3) determine whether this response is observed across varieties.

Materials and methods

Plant material. Experiments were conducted in a Department of Entomology greenhouse at the Kansas State University Throckmorton Plant Science Center from April through August, 2014. Plants of the hard red winter wheat cultivars Karl 92 and Ike were grown from seeds in a greenhouse at 21/16°C day/night and a photoperiod of 14:10 (light:dark) h with supplemental light from high pressure sodium lamps. Fourteen day-old seedlings were kept in a vernalization chamber for 6 weeks, and subsequently transplanted into 15-cm diameter pots (3 plants/pot) and placed in a greenhouse room. Individual plants were used as experimental units. Karl 92 is a semi-dwarf, heat-susceptible cultivar that demonstrated potential for production of wheat crops with relatively high antioxidant levels, as expressed by total phenolic content (ferulic acid equivalent) and orthophenolics content (Vijayalakshmi et al, 2009, Drankhan et al 2003). Ike is a double null high amylose starch material that was ranked second among cultivars from different wheat classes for its antioxidant properties (Carter et al, 2006).

Elicitor treatments. Solutions of the following elicitors were sprayed on Karl 92 and Ike plants at the tillering stage (Zadoks scale=29): 50 mM thiamine hydrochloride (B1), 0.5 mM riboflavin (B2), 0.7 mM 2,6-dichloropyridine-4-carboxylic acid (DCPCA), 50 mM sodium salicylate (SS), 44 mM methyl jasmonate (MeJA), and 1 mM benzo (1,2,3) thiadiazole-7-

carbothioic acid *S*-methyl ester (BTH). All elicitors were obtained from Sigma-Aldrich (St. Louis, MO) except BTH, which was procured as Actigard 50WG® from Syngenta (Greensboro, NC). Two sets of Ike and Karl 92 plants were sprayed with either ethanol, the solvent used for MeJa, or distilled deionized water, the solvent for all other elicitors. A set of Ike and Karl 92 plants were kept intact without elicitor or solvent spray and their leaves were used as not-sprayed controls (null). Spray applications were made in the afternoon of July 9, 2014, in the hall outside of the greenhouse. The plants were transferred to the greenhouse after 12-h post-spray quarantine period.

Sample collection and storage. Two replicate plants per cultivar*elicitor combination were uprooted from pots at 24-, 36- and 48 h post application (hpa), taken to a -10°C walk-in cold room and dipped in a liquid nitrogen bath. Frozen leaves were clipped from the plants and stored in a 150 g plastic sample bag at -80°C until analysis.

Experimental design and statistical analyses. After the quarantine period, elicitor-treated and control plants were placed in 2.3 x 1.2 x 1.2 m rectangle-shaped insect-proof compartments and arranged in a completely randomized design. Each compartment had at least one experimental unit from each cultivar*elicitor*hpa combination. Two plant-replicates per treatment combination were collected. Composite leaf samples from each replicate were divided into three subsamples. The subsamples were analyzed according to a processing plan based on the 3-sample throughput of the extraction heating equipment (Model EMEA31000/CE, Electrothermal, Staffordshire, UK). Each extraction set included at least one subsample from control plants. The processing plan specified the order and sample position in the extraction

heating equipment to block analytical variability. This plan was generated with the statistical software SAS 9.4 (SAS Institute 2009).

Phenolic extraction procedure. Leaves were dried in a convection oven at 48°C for 8 h, and the resulting 0.5-1 g samples were extracted in a Soxhlet extractor containing 60ml acetone-60ml water for 24 h. The 120 ml solvent mixture was changed three times during extraction. The round-bottom flask of each Soxhlet extractor unit was rinsed three times with 5 ml acetone when extraction was finished. Combined extracts were concentrated at 85°C to less than 100 ml in a rotary evaporator and a final collection volume was recorded.

Determination of total phenolic content (TPC). The Folin reagent (10 M) was diluted in water by a factor of 10 in a volumetric flask. Extracts were vortexed. A 200 µl aliquot of each extract was pipetted into a glass test tube and combined with 1500 µl of Folin reagent. The test tube was vortexed and allowed to sit for 5 min. Sodium carbonate (1500 µL) was added. The samples were incubated in the dark for 90 min. Ferulic acid standard solutions at concentrations of 0.01, 0.025, 0.05, 0.075, and 0.1 mg/ml were prepared with ferulic acid reagent (Sigma-Aldrich, St. Louis, MO) and methanol. Absorbance of the samples, standard solutions and a methanol control at 725nm was read after the 90 min dark incubation period with a UV-VIS Spectrophotometer (Shimadzu, Model UV-1650, Columbia, MD). Absorbance vs. concentration was plotted for the ferulic acid standard solutions. The linear equation obtained was used to calculate concentration from the absorbance of the samples and these values, reported as ferulic acid equivalents (FAE) per gram of dry leaf tissue, were used to define TLPC.

Statistical analysis. The dataset consisted of TLPC values from 2 cultivars x 9 treatments x 3 post application times x 2 plant-replicates x 3 leaf subsamples combinations. Data were log-transformed due to heterogeneity of variances resulting from high variability and analyzed with the PROC GLIMMIX procedure (SAS Institute 2009). Cultivar, elicitor treatment, post application times and all two- and three-way interactions were fixed effects. The degrees of freedom were calculated with the Kenward-Roger method (Spilke et al 2005). Least Square Means (LS-Means) were estimated and differences between elicitors were obtained with the "slicediff" function in SAS to focus on relevant comparisons. A confidence level of 90% was used to declare significance. Confidence intervals (95%) for the LS-Means were plotted with back transformed data.

Results

Effect of elicitor treatments over time. In general, DCPCA, SS, and to a lesser extent, BTH, had an increasing effect on TLPC over time (Table 5-1 and Figure 5-1). Plants treated with DCPCA had on average 49% more TLPC than null control (Table 5-1). MeJa reached its highest TLPC 36 hpa when it showed 57% and 50% increase compared to EtOH and null controls, but declined at 48 hpa. Thiamine (B1) treated plants followed a similar pattern to MeJa over time, but TLPC was not different than controls (Table 5-1). Plants treated with B2 and EtOH had consistently the lowest TLPC. Total leaf phenolic content in null control plants fluctuated over the period of time evaluated (Table 5-1).

Two- and three-way interactions. The relative effectiveness of elicitor treatments was similar for both cultivars (B2<B1<MeJa<BTH<SS<DCPCA, cultivar-elicitor interaction was not significant at P<0.5172). The three-way interactions were significant between elicitor-cultivar-hpa for Ike at 36 hours (P<0.0448). Ike wheat treated with MeJa had 349% and 177% higher TLPC at 36 hpa compared to EtOH and null controls, respectively (Table 5-2 and Figure 5-1). Ike control plants sprayed with H2O had 166% higher TLPC at 36 hpa compared to null control. This response was shown consistently in Ike plant replicates A and B (Figure 5-2).

Discussion

The SAR synthetic elicitors tested were assessed at concentrations sufficient to activate PR1 or PR-related genes at 24 hours, a post-application time related to strong gene transcript expression. Methyl jasmonate and DCPCA affected the plants at the post-translational level by inducing *de novo* synthesis of leaf phenolics at 36 hours. The varying degrees of effectiveness and the duration of the response are linked to their mode of action. DCPCA, SS and BTH share structural similarities as salicylic acid (SA) analogues and the SAR response from them was expected to be similar. In general, the effect of DCPCA on TLPC averaged over cultivars and time was higher than that of other elicitors. Because of their structural similarity to SA, this elicitor acts at or shortly before the site of SA production in the SA-dependent signaling pathway (Ford et al, 2010). PR-1 transcript levels in tobacco plants were induced in response to 2,6-dichloroisonicotinic acid (INA), a chemical compound similar to DCPCA between 8- and 16 hpa (Vernooji et al, 1995). The maximum expression of PR-1 genes in wheat was observed at 24 hpa

in response to INA in tobacco (Vernooji et al, 1995). These researchers indicated that free- and conjugated SA levels significantly increase in response to INA treatment 32 hpa. In the present study, TLPC levels increased 36 hpa after DCPCA. This indicates these plants not only express PR-gene transcripts in response to this elicitor, but also induced *de novo* phenolic synthesis. The phenolic induction observed here probably corresponds to salicylic acid accumulation typical of SAR. Sodium salicylate is an SA analogue, but it did not induce significant levels of leaf phenolics in this study. Since the ability of SS to induce PR-1 gene transcripts and establish SAR was shown by Ward et al (1991), then either SS acts downstream of SA production and accumulation, i.e., *de novo* phenolic synthesis, or the phenolic response in the leaves occurs after 48 hpa, much later than that of DCPCA. Based on its chemical structure, SS should act as a SAR activator at or relatively closer to the site of SA production compared and DCPCA (Ford et al, 2010). Therefore, a phenolic response to SS is not likely to occur after that of DCPCA. Alternatively, SS acts downstream of SA accumulation and a significant phenolic response in the leaves is not to be expected. Métraux et al. (1990) found that approximately 98.3% of SS exogenously applied to cucumber plants was either distributed and sequestered or metabolized 24 hpa. Free SA undergoes rapid conjugation in the plant due to its phytotoxicity (Wildermuth, 2006). Therefore, a similar rapid metabolic response may be expected of a SA functional analogue like SS.

The rest of the SAR elicitors studied have different and distinctive structural characteristics and biological functions compared to the SA analogues. B1 is composed of a pyrimidine and a sulfur-containing thiazole ring and is a cofactor of many enzymes, including transketolase which feeds the shikimate pathway with carbon substrates (Henkes et al., 2001).

B2 is the precursor of the ubiquitous oxidoreductase cofactors FMN and FAD (Gerdes et al, 2012), and MeJa is the volatile methyl ester of jasmonic acid (Karban and Baldwin, 1997).

Similar to DCPCA and BTH, B1 also induces expression of PR-1 transcripts at 24 hpa (Ahn et al., 2007). This expression was short-lived compared to DCPCA and BTH because it disappeared at 48 hpa (Gorlach et al, 1996; Vernooji et al, 1995), but it was not enough to induce a significant phenolic response in this study. The fact that thiamine action is not SA-dependent (Tunc-Ozdemir et al, 2009), but B1-treated plants infected with virulent pathogens showed a longer and stronger PR-1 expression compared to thiamine alone indicates it boosts SAR activation (Ahn et al, 2007). Rice blight resistance genes are located close to and co-express with a thiazole biosynthetic gene responsible for the synthesis of a thiamine precursor (Wen et al, 2003). Rice mutants compromised on this resistance gene have low levels of thiamine, but exogenous applications restore resistance (Wang et al, 2006). Thiamine induced a phenylalanine ammonia lyase (PAL) gene in tobacco plants at 24 hpa (Ahn et al, 2005). Taken together with the data shown here, the weak phenolic response observed probably corresponds to a transient production of phenolics via the PAL gene.

Riboflavin has been reported to induce SAR in Arabidopsis (Dong and Beer, 2000). Although it induces the expression of PR-1 transcripts, the SAR response did not depend on SA-signaling pathway or SA accumulation in the leaves. Furthermore, flavin-dependent monooxygenase 1 (FMO1) is a transcriptional regulator of the SAR response (Gruner et al, 2013), but it acts in systemic leaves as a supporting signal to the SAR response not as an initial trigger. Therefore, the lack of phenolic response in B2-treated wheat plants in this study was due to the fact that riboflavin-SAR activation acts as a supporting signal downstream of SA accumulation.

Like salicylic acid and its analogues, MeJa is a synthetic elicitor of SAR in Arabidopsis (Li et al., 2014), pineapple (Soler et al., 2013), and wheat (Akbari-Vafaii et al., 2014). Methyl jasmonate induces the expression of NPR1 genes (Yang et al, 2013) and increases the levels of total chlorophyll, carotenoids, and defense-related enzymes, peroxidases (POD) and PAL in wheat (Akbari-Vafaii et al., 2014). MeJa can block the SA-dependent signaling pathway (Pena-Cortes et al, 2003; Spoel et al, 2003; Niki et al, 1998), which means MeJa must use an alternative signaling pathway that activates the same molecular marker of SAR to trigger an activation cascade of genes from the phenylpropanoid pathway (Akbari-Vafaii et al., 2014). This suggests that the phenolic response observed in Ike wheat must have used a signaling pathway different than that exerted by the SA analogue, DCPCA. This was also shown by the distinctive time pattern in the phenolic response shown by MeJa, which spiked after 36 hpa but plummeted to low levels at 48 hpa. The role of jasmonic acid and MeJa as an early defense response relative to salicylic acid has been documented by Zhu et al. (2014). Furthermore, the antagonistic effect of MeJa on SA-dependent signaling may explain why this phenolic response is not sustained after 36 hpa.

The phenolic response observed consistently in H2O-control Ike at 36 hpa was another indication of the distinct sensitivity of this cultivar compared to Karl 92. Volatiles components from adjacent elicitor-sprayed plants could have been readily absorbed by dissolving into the water on the surface of H2O-sprayed plants, thereby contributing to the effect observed. Alternatively, methyl jasmonate and methyl salicylate are two compounds that have been proposed as airborne signal molecules (Tamogami et al., 2012; Attaran et al, 2009; Park et al, 2007). These airborne signals facilitate communication between infected tissues and distal parts

of the same plant, as well as between plants undergoing insect attack and other plants around them. The significant response from Ike to MeJa treatment is an indication that this molecule may be involved in the phenolic response observed in H2O treated Ike plants. However, more investigation is warranted in order to rule out the activity of methyl salicylate or other signals.

In conclusion, the synthetic SAR elicitors methyl jasmonate and to a limited extent 2,6-dichloropyridine-4-carboxylic acid induce *de novo* phenolic synthesis in the foliage of hard red winter wheat plants. Hard red winter wheat not only reacts to synthetic SAR elicitors at the transcription level as suggested in the published literature, but they produce the enzymes from the phenylpropanoid pathway and allocate resources for the phenolic response. The transient nature and timing of the phenolics response by methyl jasmonate is indication that it corresponds to a transient expression of phenylalanine ammonia lyase gene, while the phenolic response from DCPCA is steady and may correspond to the salicylic acid accumulation typical of SAR activation. The fact that wheat responded to elicitors that work through the jasmonic acid and salicylic acid signaling pathways is an indication of the allelic diversity in enzymes responsible for phenolic production. We proposed that the role of the newly synthesized phenolics was signaling and not phytotoxic. Our next study will investigate the effect of these synthetic elicitors on *de novo* synthesis of phenolics in the grain of these hard red winter wheat cultivars.

Abbreviations used

SAR, systemic acquired resistance

PR, pathogenesis-related

BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester

DCPCA,2,6-dichloropyridine-4-carboxylic acid

SA, salicylic acid

B1, thiamine

B2, riboflavin

MeJa, methyl jasmonate

SS, sodium salicylate

H2O, water

EtOH, ethanol

Hpa, hours post application

mM, milimolar

TLPC, total leaf phenolic content

FAE, ferulic acid equivalent

INA, dichloroisonicotinic acid

FMN, flavin mononucleotide

FAD, flavin adenine dinucleotide

POD, peroxidases

PAL, phenylalanine lyase

NPR1, non-expresser of PR genes

Tables

Table 5-1. Least Square Means ± SE total phenolic content (mg ferulic acid equivalent/g dry leaf) of hard red winter wheat leaves at 24-, 36-, and 48 h post application of SAR elicitors and controls. Average across evaluation times is shown. P-values for the test $\mu_{elicitor}$-$\mu_{control}$>0 are shown. ns = not significant at (P > 0.1)

Elicitor	***Hours Post Application (Hpa)***			***Average***
	24 hpa	**36 hpa**	**48 hpa**	
Riboflavin (B2)	59.41±1.27	50.59±1.27	51.91±1.32	53.84±1.16
$\mu_{elicitor}$-μ_{H2O}>0	*ns*	*ns*	*ns*	*ns*
$\mu_{elicitor}$-μ_{Null}>0	*ns*	*ns*	*ns*	*ns*
Thiamine (B1)	56.66±1.28	79.62±1.27	45.06±1.28	58.8±1.15
$\mu_{elicitor}$-μ_{H2O}>0	*ns*	*ns*	*ns*	*ns*
$\mu_{elicitor}$-μ_{Null}>0	*ns*	*ns*	*ns*	*ns*
Methyl jasmonate	60.78±1.28	92.36±1.28	50.02±1.31	65.48±1.16
$\mu_{elicitor}$-μ_{EtOH}>0	*ns*	*P=0.020*	*ns*	*ns*
$\mu_{elicitor}$-μ_{Null}>0	*ns*	*P=0.051*	*ns*	*ns*
Benzothiadiazole (BTH)	51.81±1.27	76.23±1.27	82.02±1.33	68.68±1.16
$\mu_{elicitor}$-μ_{H2O}>0	*ns*	*ns*	*ns*	*ns*
$\mu_{elicitor}$-μ_{Null}>0	*ns*	*ns*	*ns*	*ns*
Sodium salicylate (SS)	63.88±1.27	70.13±1.27	98.91±1.32	76.24±1.16
$\mu_{elicitor}$-μ_{H2O}>0	*ns*	*ns*	*ns*	*ns*
$\mu_{elicitor}$-μ_{Null}>0	*ns*	*ns*	*ns*	*ns*
DCPCA	68.01±1.32	87.32±1.27	162.26±1.27	98.77±1.16
$\mu_{elicitor}$-μ_{H2O}>0	*ns*	*ns*	*ns*	*ns*
$\mu_{elicitor}$-μ_{Null}>0	*ns*	*P=0.071*	*ns*	*P=0.058*
H2O	55.7±1.27	104.50±1.27	100.12±1.32	83.53±1.16
μ_{H2O}-μ_{Null}>0	*ns*	*P=0.022*	*ns*	*ns*
μ_{H2O}-μ_{B2}>0	*ns*	*P=0.039*	*ns*	*P=0.040*
μ_{H2O}-μ_{B1}>0	*ns*	*ns*	*P=0.037*	*P=0.092*
Null	60.41±1.28	46.44±1.27	104.76±1.27	66.49±1.16
μ_{Null}-μ_{B1}>0	*ns*	*ns*	*P=0.019*	*ns*
μ_{Null}-μ_{B2}>0	*ns*	*ns*	*P=0.0635*	*ns*
EtOH	55.25±1.27	40.04±1.27	46.67±1.31	49.92±1.16

Table 5-2. Least Square Means ± SE total phenolic content (mg ferulic acid equivalent/g dry leaf) of hard red winter wheat cv. Ike leaves at 24-, 36- and 48 h post application of SAR elicitors and controls

	Ike			
Elicitor	**t=24 h**	**t=36 h**	**t=48 h**	**Average**
Riboflavin (B2)	48.32±1.41	56.85±1.41	50.69±1.41	51.83±1.22
$\mu_{elicitor}-\mu_{control}>0$	*ns*	*ns*	*ns*	*ns*
Thiamine (B1)	72.01±1.41	49.62±1.41	42.09±1.41	53.18±1.22
$\mu_{elicitor}-\mu_{control}>0$	*ns*	*ns*	*ns*	*ns*
Methyl jasmonate	57.04±1.41	135.82±1.41‡†	53.65±1.41	74.63±1.22‡
$\mu_{elicitor}-\mu_{control}>0$	*ns*	*P=0.0036, P=0.0416*	*ns*	*P=0.0441*
Benzothiadiazole (BTH)	63.28±1.41	38.65±1.41	52.89±1.43	50.57±1.22
$\mu_{elicitor}-\mu_{control}>0$	*ns*	*ns*	*ns*	*ns*
Sodium salicylate (SS)	45.69±1.41	73.03±1.41	113.4±1.41	72.33±1.22
$\mu_{elicitor}-\mu_{control}>0$	*ns*	*ns*	*ns*	*ns*
DCPCA	49.96±1.41	76.36±1.41	140.63±1.41	81.25±1.22
$\mu_{elicitor}-\mu_{control}>0$	*ns*	*ns*	*ns*	*ns*
EtOH	57.47±1.41	30.24±1.41	41.78±1.41	41.72±1.22
$\mu_{elicitor}-\mu_{control}>0$	*ns*	*ns*	*ns*	*ns*
H2O	58.15±1.41	130.28±1.41†	105.7±1.41	92.86±1.22*
$\mu_{elicitor}-\mu_{control}>0$	*ns*	*P=0.0487*	*ns*	*P<0.05*
Null	55.78±1.43	48.99±1.41	117.41±1.41	68.46±1.22

†Significantly different than null control
‡Significantly different than solvent control
*Significantly different than B1, B2, and BTH.

Figures

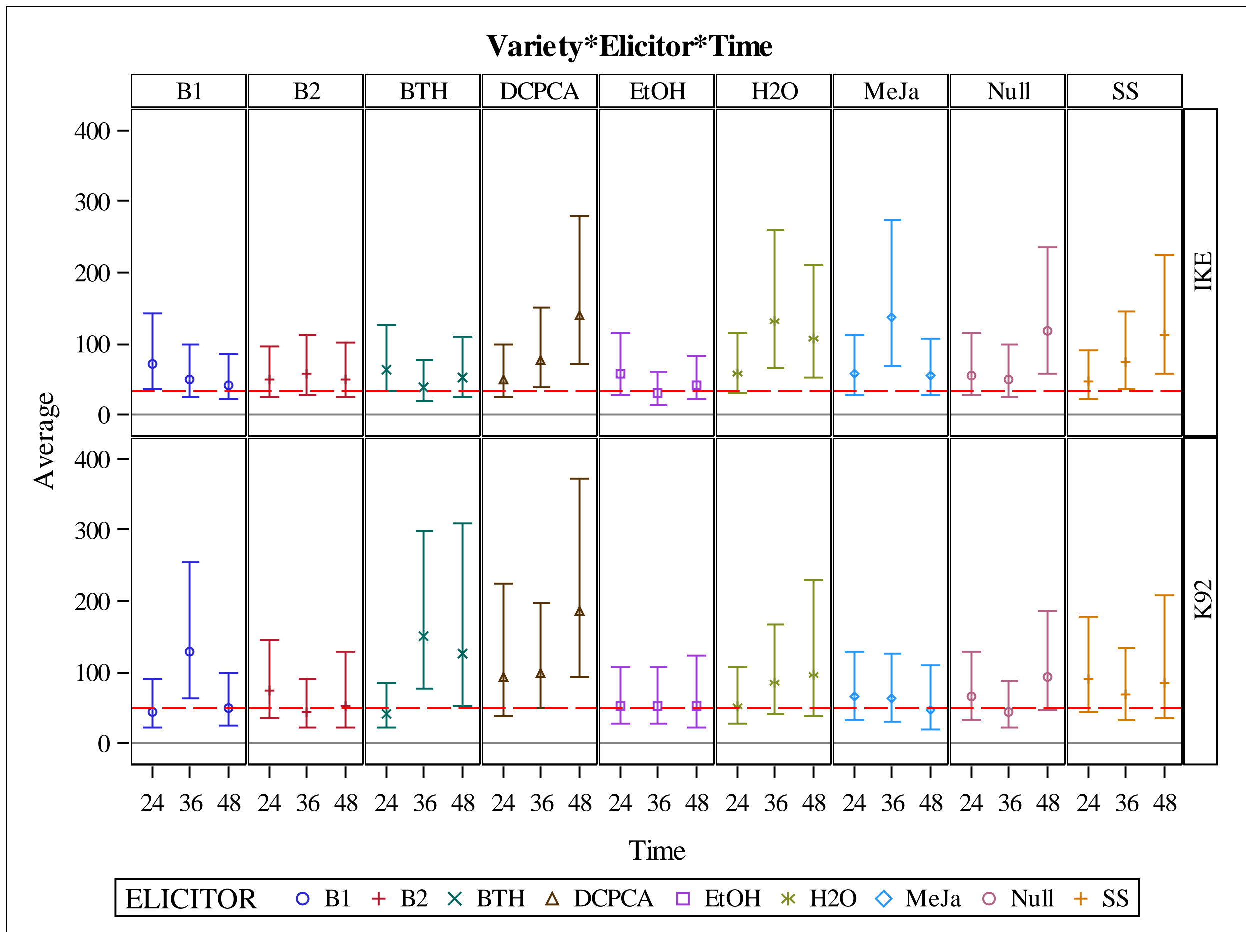

Figure 5-1. Least Square Means +/- 95% confidence interval total phenolic content (mg ferulic acid equivalents /g dry leaf tissue), in leaves of Karl 92 and Ike wheat plants treated with SAR elicitors at 24-, 36- and 48 hours post application. Red lines indicate TPC levels 24 h before treatment application of 50 mM thiamine hydrochloride (B1), 5mM riboflavin (B2), 1mM benzo (1,2,3) thiadiazole (BTH), 0.7 mM 2,6-dichloropyridine-4-carboxylic acid (DCPCA), ethanol control (EtOH), distilled deionized water (H_2O), 44 mM methyl jasmonate (MeJa), unsprayed control (null), and 50 mM sodium salicylate (SS).

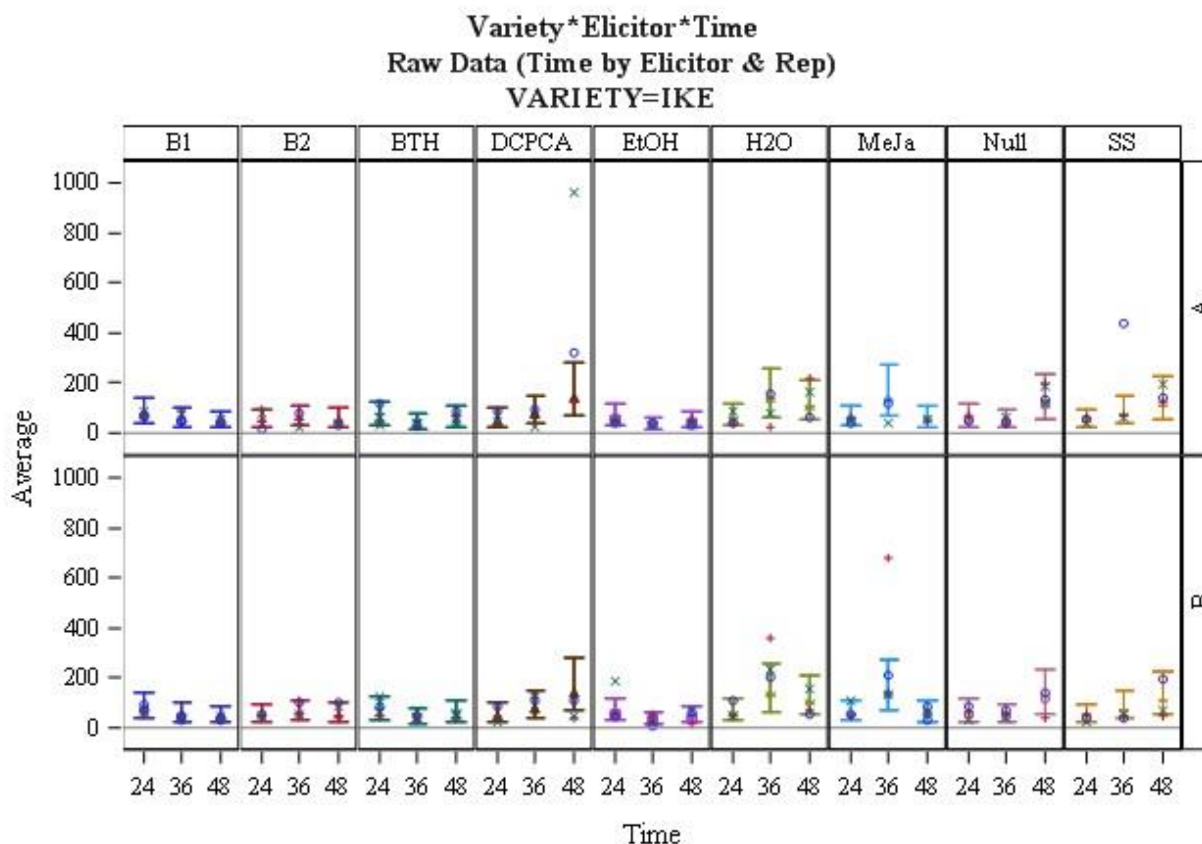

Figure 5-2. Total phenolic content (mg ferulic acid equivalent/g dry leaf) in leaves of two Ike replicate plants at 24-, 36-, and 48 hours post application of the following SAR elicitor treatments and controls: 50 mM thiamine hydrochloride (B1), 5mM riboflavin (B2), 1mM benzo (1,2,3) thiadiazole (BTH), 0.7 mM 2,6-dichloropyridine-4-carboxylic acid (DCPCA), ethanol control (EtOH), distilled deionized water (H_2O), 44 mM methyl jasmonate (MeJa), not sprayed control (Null), and 50 mM sodium salicylate (SS).

References

Ahn, I.P., S. Kim, and Y.H. Lee. 2005. Vitamin B-1 functions as an activator of plant disease resistance. Plant Physiol. 138:1505-1515.

Ahn, I., S. Kim, Y. Lee, and S. Suh. 2007. Vitamin B-1-induced priming is dependent on hydrogen peroxide and the NPR1 gene in arabidopsis. Plant Physiol. 143:838-848.

Akbari-Vafaii, A., S. Ketabchi, and A. Moradshahi. 2014. Effect of methyl jasmonate (MeJA) on biochemical responses of wheat seedlings infected by fusarium culmorum. Archives of Phytopathology and Plant Protection 47:1893-1904.

Akbari-Vafaii, A., S. Ketabchi, and A. Moradshahi. 2014. Effect of methyl jasmonate (MeJA) on biochemical responses of wheat seedlings infected by fusarium culmorum. Archives of Phytopathology and Plant Protection 47:1893-1904.

Attaran, E., T.E. Zeier, T. Griebel, and J. Zeier. 2009. Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in arabidopsis. Plant Cell 21:954-971.

Buonaurio, R., L. Scarponi, M. Ferrara, P. Sidoti, and A. Bertona. 2002. Induction of systemic acquired resistance in pepper plants by acibenzolar-S-methyl against bacterial spot disease. Eur. J. Plant Pathol. 108:41-49.

Chanda, B., Y. Xia, M.K. Mandal, K. Yu, K. Sekine, Q. Gao, D. Selote, Y. Hu, A. Stromberg, and D. Navarre. 2011. Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants. Nat. Genet. 43:421-427.

Chaturvedi, R., B. Venables, R.A. Petros, V. Nalam, M. Li, X. Wang, L.J. Takemoto, and J. Shah. 2012. An abietane diterpenoid is a potent activator of systemic acquired resistance. The Plant Journal 71:161-172.

Colson-Hanks, E., S. Allen, and B. Deverall. 2000. Effect of 2, 6-dichloroisonicotinic acid or benzothiadiazole on alternaria leaf spot, bacterial blight and verticillium wilt in cotton under field conditions. Australas. Plant Pathol. 29:170-177.

Dann, E., and B. Deverall. 1996. 2, 6-dichloro-isonicotinic acid (INA) induces resistance in green beans to the rust pathogen, uromyces appendiculatus, under field conditions. Australas. Plant Pathol. 25:199-204.

Dann, E., B. Diers, J. Byrum, and R. Hammerschmidt. 1998. Effect of treating soybean with 2, 6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) on seed yields and the level of disease caused by sclerotinia sclerotiorum in field and greenhouse studies. Eur. J. Plant Pathol. 104:271-278.

Drankhan, K., J. Carter, R. Madl, C. Klopfenstein, F. Padula, Y. Lu, T. Warren, N. Schmitz, and D.J. Takemoto. 2003. Antitumor activity of wheats with high orthophenolic content. Nutr. Cancer 47:188-194.

El-Shetehy, M., C. Wang, M. Shine, K. Yu, A. Kachroo, and P. Kachroo. 2015. Nitric oxide and reactive oxygen species are required for systemic acquired resistance in plants. Plant Signaling & Behavior 10:e998544.

Gao, Q., K. Yu, Y. Xia, M. Shine, C. Wang, D. Navarre, A. Kachroo, and P. Kachroo. 2014. Mono-and digalactosyldiacylglycerol lipids function nonredundantly to regulate systemic acquired resistance in plants. Cell Reports 9:1681-1691.

Gorlach, J., S. Volrath, G. KnaufBeiter, G. Hengy, U. Beckhove, K.H. Kogel, M. Oostendorp, T. Staub, E. Ward, H. Kessmann, and J. Ryals. 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8:629-643.

Ishii, H., Y. Tomita, T. Horio, Y. Narusaka, Y. Nakazawa, K. Nishimura, and S. Iwamoto. 1999. Induced resistance of acibenzolar-S-methyl (CGA 245704) to cucumber and japanese pear diseases. Eur. J. Plant Pathol. 105:77-85.

Kunkel, B.N., and D.M. Brooks. 2002. Cross talk between signaling pathways in pathogen defense. Curr. Opin. Plant Biol. 5:325-331.

Leskovar, D.I., and K. Kolenda. 2002. Strobilurin acibenzolar-S-methyl controls white rust without inducing leaf chlorosis in spinach. Ann. Appl. Biol. 140:171-175.

Li, B., C. Zhang, Q. Chai, Y. Han, X. Wang, M. Liu, H. Feng, and Z. Xu. 2014. Plasmalemma localisation of DOUBLE HYBRID PROLINE-RICH PROTEIN 1 and its function in systemic acquired resistance of arabidopsis thaliana. Functional Plant Biology 41:768-779.

Li, B., C. Zhang, Q. Chai, Y. Han, X. Wang, M. Liu, H. Feng, and Z. Xu. 2014. Plasmalemma localisation of DOUBLE HYBRID PROLINE-RICH PROTEIN 1 and its function in systemic acquired resistance of arabidopsis thaliana. Functional Plant Biology 41:768-779.

Liu, P., C.C. von Dahl, and D.F. Klessig. 2011. The extent to which methyl salicylate is required for signaling systemic acquired resistance is dependent on exposure to light after infection. Plant Physiol. 157:2216-2226.

Louws, F., M. Wilson, H. Campbell, D. Cuppels, J. Jones, P. Shoemaker, F. Sahin, and S. Miller. 2001. Field control of bacterial spot and bacterial speck of tomato using a plant activator. Plant Dis. 85:481-488.

Luzzatto-Knaan, T., Z. Kerem, A. Lipsky, and I. Yedidia. 2013. A systemic response of geophytes is demonstrated by patterns of protein expression and the accumulation of signal molecules in zantedeschia aethiopica. Plant Physiology and Biochemistry 71:218-225.

Malamy, J., J.P. Carr, D.F. Klessig, and I. Raskin. 1990. Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. Science 250:1002-1004.

Maldonado, A.M., P. Doerner, R.A. Dixon, C.J. Lamb, and R.K. Cameron. 2002. A putative lipid transfer protein involved in systemic resistance signalling in arabidopsis. Nature 419:399-403.

Maxson-Stein, K., S. He, R. Hammerschmidt, and A.L. Jones. 2002. Effect of treating apple trees with acibenzolar-S-methyl on fire blight and expression of pathogenesis-related protein genes. Plant Dis. 86:785-790.

Metraux, J.P., H. Signer, J. Ryals, E. Ward, M. Wyss-Benz, J. Gaudin, K. Raschdorf, E. Schmid, W. Blum, and B. Inverardi. 1990. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science 250:1004-1006.

Morris, S.W., B. Vernooij, S. Titatarn, M. Starrett, S. Thomas, C.C. Wiltse, R.A. Frederiksen, A. Bhandhufalck, S. Hulbert, and S. Uknes. 1998. Induced resistance responses in maize. Mol. Plant-Microbe Interact. 11:643-658.

Navarova, H., F. Bernsdorff, A.C. Doring, and J. Zeier. 2012. Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. Plant Cell 24:5123-5141.

Park, S.W., E. Kaimoyo, D. Kumar, S. Mosher, and D.F. Klessig. 2007. Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science 318:113-116.

Park, S., E. Kaimoyo, D. Kumar, S. Mosher, and D.F. Klessig. 2007. Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science 318:113-116.

Perez, L., M.E. Rodriguez, F. Rodriguez, and C. Roson. 2003. Efficacy of acibenzolar-S-methyl, an inducer of systemic acquired resistance against tobacco blue mould caused by peronospora hyoscyami f. sp. tabacina. Crop Protection 22:405-413.

Rasmussen, J.B., R. Hammerschmidt, and M.N. Zook. 1991. Systemic induction of salicylic acid accumulation in cucumber after inoculation with pseudomonas syringae pv syringae. Plant Physiol. 97:1342-1347.

SAS Institute. 2009. The GLIMMIX procedure, SAS/STAT user’s guide, version 9.2. SAS Institute, Cary, North Carolina.

Shah, J., and J. Zeier. 2014. Long-distance communication and signal amplification in systemic acquired resistance. Induced Plant Responses to Microbes and Insects23.

Shah, J., R. Chaturvedi, Z. Chowdhury, B. Venables, and R.A. Petros. 2014. Signaling by small metabolites in systemic acquired resistance. The Plant Journal 79:645-658.

Soler, A., P. Marie-Alphonsine, C. Corbion, and P. Queneherve. 2013. Differential response of two pineapple cultivars (ananas comosus (L.) merr.) to SAR and ISR inducers against the nematode rotylenchulus reniformis. Crop Protection 54:48-54.

Spilke, J., H. Piepho, and X. Hu. 2005. Analysis of unbalanced data by mixed linear models using the MIXED procedure of the SAS system. Journal of Agronomy and Crop Science 191:47-54.

Sun, J., H. Jiang, and C. Li. 2011. Systemin/Jasmonate-mediated systemic defense signaling in tomato. Molecular Plant 4:607-615.

Tamogami, S., K. Noge, M. Abe, G.K. Agrawal, and R. Rakwal. 2012. Methyl jasmonate is transported to distal leaves via vascular process metabolizing itself into JA-ile and triggering VOCs emission as defensive metabolites. Plant Signaling & Behavior 7:1378-81.

Truman, W.M., M.H. Bennett, C.G. Turnbull, and M.R. Grant. 2010. Arabidopsis auxin mutants are compromised in systemic acquired resistance and exhibit aberrant accumulation of various indolic compounds. Plant Physiol. 152:1562-1573.

Tunc-Ozdemir, M., G. Miller, L. Song, J. Kim, A. Sodek, S. Koussevitzky, A.N. Misra, R. Mittler, and D. Shintani. 2009. Thiamin confers enhanced tolerance to oxidative stress in arabidopsis. Plant Physiol. 151:421-432.

Vernooij, B., L. Friedrich, P. Ahl Goy, T. Staub, H. Kessmann, and J. Ryals. 1995. 2, 6-dichloroisonicotinic acid-induced resistance to pathogens without the accumulation of salicylic acid. MPMI-Molecular Plant Microbe Interactions 8:228-234.

Vijayalakshmi, K., A.K. Fritz, G.M. Paulsen, G. Bai, S. Pandravada, and B.S. Gill. 2010. Modeling and mapping QTL for senescence-related traits in winter wheat under high temperature. Mol. Breed. 26:163-175.

Wang, C., M. El-Shetehy, M. Shine, K. Yu, D. Navarre, D. Wendehenne, A. Kachroo, and P. Kachroo. 2014. Free radicals mediate systemic acquired resistance. Cell Reports 7:348-355.

Wang, J.W., and J.Y. Wu. 2005. Nitric oxide is involved in methyl jasmonate-induced defense responses and secondary metabolism activities of taxus cells. Plant Cell Physiol. 46:923-930.

Ward, E.R., S.J. Uknes, S.C. Williams, S.S. Dincher, D.L. Wiederhold, D.C. Alexander, P. Ahl-Goy, J.P. Metraux, and J.A. Ryals. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3:1085-1094.

Wildermuth, M.C. 2006. Variations on a theme: Synthesis and modification of plant benzoic acids. Curr. Opin. Plant Biol. 9:288-296.

Xu, Y., P. Chang, D. Liu, M.L. Narasimhan, K.G. Raghothama, P.M. Hasegawa, and R.A. Bressan. 1994. Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell 6:1077-1085.

Zhang, S., M. Reddy, N. Kokalis-Burelle, L.W. Wells, S.P. Nightengale, and J.W. Kloepper. 2001. Lack of induced systemic resistance in peanut to late leaf spot disease by plant growth-promoting rhizobacteria and chemical elicitors. Plant Dis. 85:879-884.

Zhu, F., D. Xi, S. Yuan, F. Xu, D. Zhang, and H. Lin. 2014. Salicylic acid and jasmonic acid are essential for systemic resistance against tobacco mosaic virus in nicotiana benthamiana. Mol. Plant-Microbe Interact. 27:567-577.

CHAPTER 6 - Synthetic elicitors of systemic acquired resistance administered to wheat (*Triticum aestivum* L) plants at the jointing stage induced phenolics levels in mature grains

Abstract

Wheat grain contains phenolic compounds that act as antioxidants when the bran is included in the human diet. Plants produce phenolics as a defense response to, among other factors, insect and fungal attack, presumably through systemic acquired resistance (SAR). Synthetic elicitors of SAR that act through the jasmonic and salicylic acid signaling pathway induced *de novo* phenolic synthesis in wheat foliage. This study investigated the possibility of using those synthetic elicitors to enhance the phenolic levels in the grains. Solutions of SAR elicitors were sprayed on plants of the hard red winter wheat cultivars Karl 92 and Ike at the tillering stage (Zadoks scale 29). Treatments included 50 mM thiamine (B1), 0.5 mM riboflavin (B2), 0.7 mM 2,6-dichloropyridine-4 carboxylic acid (DCPCA), 1 mM benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester (BTH), 44 mM methyl jasmonate (MeJa), and 50 mM sodium salicylate (SS). Bran extracts from these plants were analyzed for total phenolic content (TPC) with the Folin Ciocalteau assay. BTH and DCPCA significantly increased TPC in mature grains by 22 and 17% compared to control plants. Spraying these SAR elicitors is an effective and feasible strategy to increase the levels of phenolics in Karl 92 and Ike wheat crops.

Keywords: antioxidants, systemic acquired resistance, elicitors, phenolics, wheat bran

Introduction

De novo phenolic synthesis in wheat (*Triticum aestivum* L) plants is part of the defense responses to insects, pathogens, and abiotic stress. Previous investigations of the effect of stress factors on phenolic concentration in mature wheat grains demonstrated that feeding by the Bird-cherry oat aphid *(Rhopalosiphum padi* L*)* explained the most variation in concentration of phenolics per gram of bran, compared to leaf rust infection with an avirulent *Puccina triticina* strain, or heat stress (Ramos et al., 2015a; in review). Although this effect was not contingent on the phenological stage of wheat at the time of *R. padi* infestation, plants stressed at the 5-tiller stage had higher levels of grain phenolics compared to non-infested plants (Ramos et al., 2015b; in review). Since the phenolic induction was shown in organs distally located to the site of stress in terms of space and time, systemic acquired resistance (SAR) was hypothesized to be the defense response responsible for phenolic synthesis.

SAR is an inducible defense response that is activated by pathogens, insects, and synthetic elicitors (Durrant and Dong, 2004; Fu and Dong, 2013). Hard red winter wheat plants cv. Karl 92 and Ike responded to synthetic elicitors of SAR that act through SA- or JA- signaling by inducing phenolic synthesis in the leaves (Ramos et al, 2016, *in preparation*). This phenolic response peaked 36 h post application (hpa) and either disappeared or reached a plateau 12 h later. It has been shown that salicylic acid and methyl jasmonate move through storage organs as part of the SAR response (Luzzatto-Knaan et al, 2013), perhaps as part of the biochemical modification needed to pass on the SAR state from parents to offspring known as transgenerational resistance (Walters and Paterson, 2012), which indicates that elicitors that act through the SA- or JA-signaling pathway may have potential to increase phenolics levels in the grains. Here, we studied the effect of synthetic elicitors of SAR administered to hard red winter

cv. Karl 92 and Ike at the tillering stage on phenolic concentration in mature grains. The objectives were to 1) evaluate the phenolic response across growing seasons, 2) determine the cultivar effect, and 3) compare the phenolic response in mature grains with that observed previously in the leaves.

Materials and methods

Plant Materials. Plants of the hard red winter wheat cultivars Karl 92 and Ike were grown from seed in a greenhouse at 21/16º C day/night and a photoperiod of 14:10 (light:dark) h with supplemental light from high pressure sodium lamps. Fourteen day-old seedlings were kept in a vernalization chamber for 6 weeks, and subsequently transplanted into 15-cm diameter pots (3 plants/pot) and placed in a greenhouse room. Individual pots were used as experimental units. Karl 92 is a well-adapted semi-dwarf and early maturing Kansas cultivar (Narasimhamoorthy et al., 2006; Sears et al., 1997). Ike is the only double null partial waxy wheat cultivar released for cultivation in North America (Graybosch and Baenziger, 2004), and ranked second best among five cultivars from different wheat classes for its antioxidant properties (Carter et al., 2006).

Elicitor Treatments. Thiamine (B1) hydrochloride, riboflavin (B2), 2,6-dichloropyridine-4-carboxylic acid (DCPCA, analog to INA), sodium salicylate (SS), and methyl jasmonate (MeJa) were purchased from Sigma Aldrich. Benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH), commercial name Actigard 50WG®, was obtained from Syngenta. Solutions of elicitors were prepared at the following concentrations: 50 mM B1 hydrochloride, 0.5 mM B2, 0.7 mM DCPCA, 50 mM SS, 44 mM MeJa, and 1 mM BTH. These concentrations were sufficient to significantly express PR-1 (Ahn et al., 2005; Dong and Beer, 2000; Gorlach et

al., 1996; Wang and Wu, 2005; Ward et al., 1991; Xu et al., 1994). Fresh solutions of these elicitors were sprayed on Ike and Karl 92 plants with a household plastic spray bottle at the Zadoks scale=29. Two sets of Ike and Karl 92 plants were sprayed with either water, the solvent used for most elicitors, or ethanol, the solvent used for MeJa. Two sets of Ike and Karl 92 plants were kept intact without elicitor or solvent spray and their grains were used as non-sprayed controls. These sets of elicitor-treated, solvent-only, and non-sprayed Karl 92 and Ike plants were transferred back to the greenhouse after a 12-hour post-spray quarantine period. They were allowed to grow undisturbed until they reached physiological maturity. Insect influx to the greenhouse was monitored by scouting insects with sticky traps. Insect pests were controlled by releasing batches 4,500 adult lady bugs individuals (*Hippodamia convergens)* and 50000 adult predatory mites (*Neoseiulus cucumeris)* every two weeks (Hummert International, Earth city, MO).

Experimental Design. A randomized complete block design was used. Block was the growing season. Plants from both cultivars treated with SAR elicitors, solvent-only, and non-sprayed plants were randomly assigned to six rectangle-shaped compartments (dimensions: 2.3 x 1.2 x 1.2 m, length x width x height) made with insect-proof screen (81 x 81 mesh) with 0.15 x 0.15 mm opening size (Hummert International, Earth City, MO) inside a greenhouse with temperature and photoperiod stated above. There was at least one experimental unit (3 plants/pot) from each elicitor-treated, solvent only, or non-sprayed Karl 92 and Ike in each compartment. The experiment was replicated 3 times (blocks) during summer 2013, spring 2014, and summer 2014 for 21 weeks each time.

Grain sample preparation. Grain samples were cleaned and sorted by size using dockage test sieves with 5.16 mm round holes and a 1.6 x 9.5 mm slotted sieve. The sound, healthy grains (overs from the slotted sieve) were tempered to 15±0.5% moisture content before milling on a Quadrumat Jr. Mill (Brabender, South Hackensack, NJ). The bran was collected from the mill and sifted in a plansifter. Bran particles equal or larger than 400 microns (overs from US sieve no. 40) were kept for extraction and analysis.

Extraction Procedure. Phenolics were extracted from the bran with the procedure reported in Ramos et al (2015a, in review). Briefly, one gram of wheat bran was defatted, extracted with methanol:acetone:water solution (7:7:6 v/v/v, adjusted to pH 2 with concentrated hydrochloric acid), incubated for 2 hours at 23°C with shaking motion, and centrifuged at 5000 *g* for 10 minutes at 10°C. The supernatant was poured into a round-bottomed flask and concentrated under vacuum at 40°C. An aliquot of 5-10 ml of supernatant was mixed with 30 ml of ethyl ether/ethyl acetate solvent (1:1 v/v). The mixture was hand-shaken for 1 minute and poured into a separatory funnel. The upper and lower phases were collected separately. This procedure was repeated twice on the lower phase using fresh solvent each time. The three aliquots of solvent were pooled together. Magnesium sulfate was added to remove any remaining water. The mixture was concentrated under vacuum at 40°C. The concentrate was reconstituted with 10 ml methanol and saved for analysis.

Determination of Total Phenolic Content. The free phenolic extracts were analyzed for total phenolic content (TPC) with the Folin Ciocalteau assay. The procedure is briefly summarized here. An aliquot of 200 μL of the extracts was pipetted into a test tube and the following reagents were added: 1.5 ml of 0.2 N Folin-Ciocalteau reagent and 1.5 ml of 6%

sodium carbonate (w/v). This mixture was vortexed and allowed to incubate in the dark at 23°C for 90 minutes. Ferulic acid solutions with known concentrations and a methanol blank were also tested and incubated with the samples. After incubation, absorbance was measured at 725 nm with a UV-VIS Spectrophotometer (Shimadzu, model UV-1650, Columbia, MD). Absorbance vs. concentration was plotted for the ferulic acid standard solutions. The linear equation obtained was used to calculate concentration from the absorbance of the samples, and these values were reported as ferulic acid equivalents (FAE).

Statistical analysis. TPC data from the three blocks were analyzed with the PROC MIXED procedure in SAS 9.4 statistical software (SAS Institute, Cary, NC). Wheat cultivar and SAR elicitor treatments were fixed effects and block was treated as a random effect. The Least Squares Means test was used to calculate the means ± standard error (SE) for each wheat cultivar, elicitor, or cultivar*elicitor variable. Pairwise comparisons of means between elicitor treatments and solvent only or non-sprayed control were made with a Bonferroni adjustment ($P<0.004166$). Differences between elicitors for each cultivar were Bonferroni adjusted to $P<0.00208$.

Results

Covariance and ANOVA analysis. Covariance parameter estimates for block was larger than residuals (PROC MIXED procedure, σ^2_{block}=0.001596, $\sigma^2_{residual}$=0.001280, Appendix Table 9-54), which indicates that seasonal variability was larger than spatial effects or plant variability in this study. This indicates that RCBD with blocking by season was a good design choice. There was a significant cultivar effect (DF=1, 32; F=20.46; $P<0.0001$, Appendix Table

9-55) and elicitor treatment effect on TPC (DF=8, 32; F=5.44; P<0.0002, Appendix Table 9-55), but the cultivar*elicitor interaction was not significant.

Effect of elicitors on yield. Karl 92 grain yield decreased by 35% in response to DCPCA (P<0.1, Table 6-1). The rest of the elicitors showed more variable yields as reflected in the large standard deviations, especially in Ike wheat. Nevertheless the mean grain yields were not significantly different than control.

Effect of elicitor treatments. Application of B1, DCPCA, BTH, and SS, significantly increased TPC by 18%, 28%, 34%, and 23% respectively, relative to not sprayed control plants (P<0.004167, Figure 6-1). However, only BTH and DCPCA significantly increased TPC in mature grains by 22 and 17% compared to H2O control plants (P<0.004167, Figure 6-1).

Cultivar effects. Averaged TPC of all Ike wheat bran extracts was 10% higher than Karl 92 (P<0.0005, Appendix Table 9-56).

Discussion

In this study, B1, DCPCA, BTH, and SS elicited a phenolic response in mature grains of hard red winter wheat crops. In the previous chapter, we reported that DCPCA and MeJa acted through either the SA or JA-signaling pathway to increase the phenolic concentration in the leaves of the same cultivars studied here. A closer look at the individual block replications in Ike wheat showed that MeJa had either the lowest induction (N=1, 1.19%) or suppression (N=2, -3.67% and N=3, -14.56%) effect of grain phenolic concentration among all elicitors tested. This stands in stark contrast to the significant effect of MeJa on the leaves at 36 hpa. This leaf phenolic induction was gone at 48 hpa. Based on those findings and the effect on grain

phenolics observed here, we can conclude that MeJa has a transient induction effect on leaf phenolic content, but eventually any long-term SAR phenolic response is not observed. B1 acts as a supporting signal in the SAR response, but its effect on grain phenolics was significant (Figure 6-1). DCPCA, BTH, and SS are analogues of SA and as such were expected to activate the SA-dependent SAR. This was shown in the leaves for DCPCA, which acts upstream of SA accumulation, but not on BHT or SS perhaps due to its action downstream of SA accumulation and rapid metabolism in the plant.

A caveat of inducible responses is that not all cultivars react to cues or environmental stimuli to the same extent. Karl 92 showed potential for phenolic induction in response to four SAR synthetic elicitors that acted through SA or JA-dependent signaling pathway, while the phenolic induction for Ike wheat was shown in response to two synthetic elicitors, one on foliage and another one on grains. Natural variation in induced defense responses exists between genotypes in wild Solanum species (Smith et al 2014). This variation may be due to different isozymes that respond to either SA- or JA-signaling.

Most of the elicitors did not incur in a fitness cost in terms of grain yield on the plants, except for DCPCA on Karl 92 wheat. The percentage of induction in grain phenolics was offset by the same percentage of reduction in grain yield. This pattern is in agreement with the allocation cost theory, which is states that this is a type of fitness cost due to the need for the plant to allocate a limited resource to defense activation to the detriment to those required by the growth and reproduction (Zangerl and Bazzaz, 1992). While there is no grain yield data available for BTH in this study, other researchers have reported that there was a net effect of 0-18% gain on grain yield after BTH preventive application followed by pathogen infection in wheat plants (Stadnik and Buchenauer, 1999). This observation supports the notion that the

benefits of SAR are more evident under high pathogen pressure (Cipollini and Heil, 2010). Although, effective SAR elicitors do have a fitness cost to wheat as observed in this study, there is a potential to obtain a net positive effect under plant disease or herbivory. While this topic is beyond the scope of this study, it is worth pursuing in formal field trials with the SAR elicitors were effective at inducing phenolics in wheat.

It is not clear whether the revenues from high antioxidant wheat crops would off-set the loss due to reduced yield. Assume the regular revenues for an acre of regular wheat=$4/bushel*60bu/acre=$240. Supposing that there is 30% yield drag, and a 10 cent/bushel premium, the revenues from an acre of high antioxidant wheat would be= $4.10/bushel*(60 bu/acre- 60*0.3 for yield drag)=$172. In order to break even with a large yield drag of 30%, the wheat would need to be priced at $5.71, a $1.71 premium over $4/bu wheat. For a yield drag of 10% the breakeven price would be $4.44 or a premium of $0.41/bu. A substantial yield drag would require a substantial premium for the wheat farmer to profit from producing this enhanced antioxidant wheat. These estimates do not include the price of spray applications.

In conclusion, B1, DCPCA, BTH, and SS demonstrated effectiveness at inducing phenolics in mature wheat grains. There is a relationship between the total phenolic content in the leaves in response to DCPCA elicitor treatment and that in the grains. Although SS and MeJa act through SA and JA-signaling, their responses in the leaves and in the grain were not positively correlated.

Abbreviations used

SAR, systemic acquired resistance

PR, pathogenesis-related

BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester

DCPCA,2,6-dichloropyridine-4-carboxylic acid

SA, salicylic acid

B1, thiamine

B2, riboflavin

MeJa, methyl jasmonate

SS, sodium salicylate

RCBD, randomized complete block design

mm, milimeter

mM, milimolar

TPC, total phenolic content

N, normal

n, number of replications

FAE, ferulic acid equivalent

DF, degrees of freedom

Tables

Table 6-1. Mean±SD (g) grain yield per pot (3 plant/pot) in grams. P values shown for the difference between elicitors and control. Number in parenthesis is number of pots

Elicitor	Ike	Karl 92
B1	3.49±2.14 (8)	3.37±1.89 (16)
$\mu_{elicitor}$-$\mu_{control}$	P=0.710	P=0.658
B2	3.35±2.53 (4)	3.19±1.99 (12)
$\mu_{elicitor}$-$\mu_{control}$	P=0.862	P=0.880
DCPCA	2.83±1.42 (5)	2.01±1.38 (17)
$\mu_{elicitor}$-$\mu_{control}$	P=0.808	P=0.063
MeJa	3.9±2.44 (6)	3.44±1.74 (12)
$\mu_{elicitor}$-$\mu_{control}$	P=0.245	P=0.429
SS	5.58±2.75 (5)	4.12±2.37 (15)
$\mu_{elicitor}$-$\mu_{control}$	P=0.130	P=0.179
H2O	3.08±2.05 (7)	3.08±1.71 (15)
EtOH	2.46±1.50 (7)	4.07±1.97 (12)

Table 6-2 Mean ± standard error total phenolic content (µg ferulic acid equivalent/g bran) of wheat bran extract from both Karl 92 and Ike wheat non-sprayed (Null), solvent-only (H2O or EtOH), and plants treated with thiamine (B1), riboflavin (B2), 2,6-dichloropyridine-4 carboxylic acid (INA), sodium salicylate (SS), methyl jasmonate (MJ), and benzo(1,2,3)thiadiazole-7 carbothioic acid S-methyl ester (BTH).

Elicitor	Ike	Karl 92	Average
B1	457.9±30.96	408.9±30.96	433.4±27.3
B2	483.2±30.96	361.8±30.96	422.5±27.3
BTH	525.9±34.42	457.2±34.42	491.5±29.41
SS	467.8±30.96	434.1±30.96	451±27.3
DCPCA	471.3±30.96	465.2±30.96	468.2±27.3
MeJa	415.7±30.96	440.4±30.96	428.1±27.3
H2O	435.4±30.96	367.7±30.96	401.5±27.3
EtOH	439.7±30.96	415±30.96	427.4±27.3
Null	396.6±30.96	336±30.96	366.3±27.3

Figures

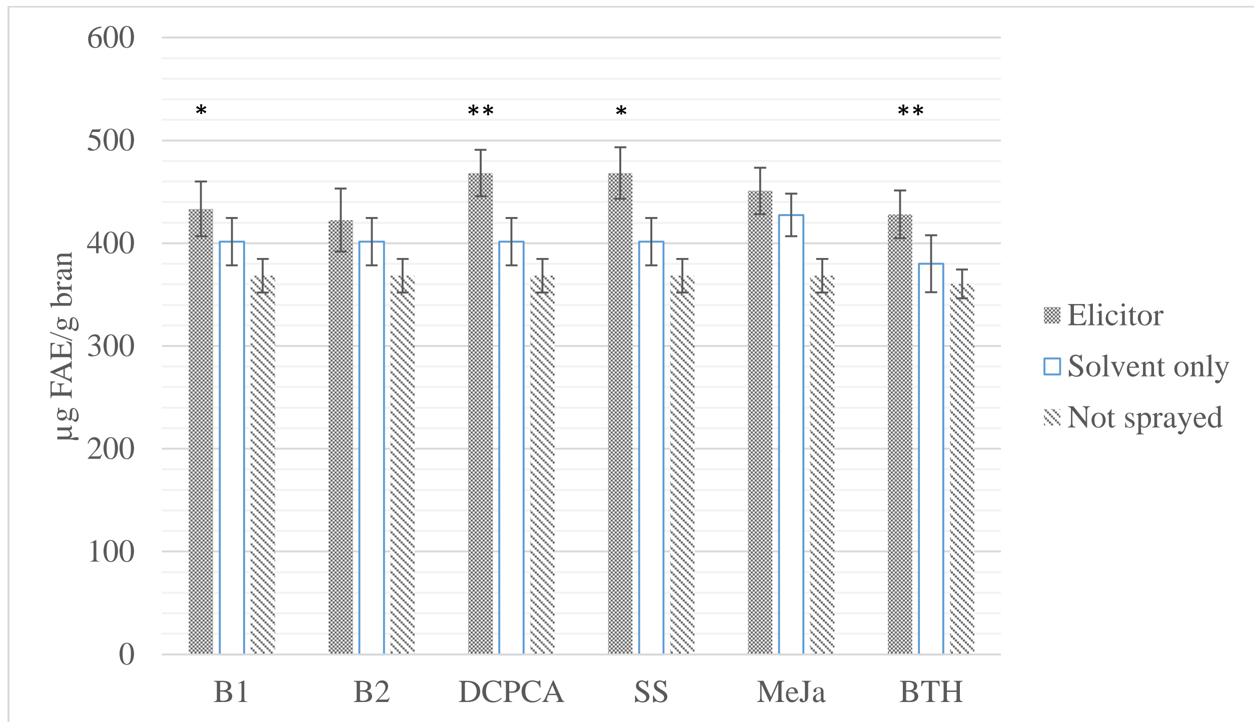

Figure 6-1. Total phenolic content of wheat bran extracts from not sprayed, solvent-only, and plants treated with thiamine (B1), riboflavin (B2), 2,6-dichloropyridine-4 carboxylic acid (INA), sodium salicylate (SS), methyl jasmonate (MJ), and benzo(1,2,3)thiadiazole-7 carbothioic acid S-methyl ester (BTH). Means and standard errors are shown (n=3). Bars with "*" indicates significant difference from not-sprayed control, and bars with "**" indicates significant difference from not-sprayed and solvent-only treated plants (Bonferroni adjusted $P<0.004167$).

References

Ahn, I.P., S. Kim, and Y.H. Lee. 2005. Vitamin B-1 functions as an activator of plant disease resistance. Plant Physiol. 138:1505-1515.

Carter, J.W., R. Madl, and F. Padula. 2006. Wheat antioxidants suppress intestinal tumor activity in min mice. Nutr. Res. 26:33-38.

Cipollini, D., and M. Heil. 2010. Costs and benefits of induced resistance to herbivores and pathogens in plants. Plant Sci.Rev 5:1-25.

Dong, H., and S.V. Beer. 2000. Riboflavin induces disease resistance in plants by activating a novel signal transduction pathway. Phytopathology 90:801-811.

Durrant, W., and X. Dong. 2004. Systemic acquired resistance. Annu. Rev. Phytopathol. 42:185-209.

Fu, Z.Q., and X. Dong. 2013. Systemic acquired resistance: Turning local infection into global defense. Annual Review of Plant Biology, Vol 64 64:839-863.

Gorlach, J., S. Volrath, G. KnaufBeiter, G. Hengy, U. Beckhove, K.H. Kogel, M. Oostendorp, T. Staub, E. Ward, H. Kessmann, and J. Ryals. 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8:629-643.

Graybosch, R.A., and P.S. Baenziger. 2004. Registration of three partial waxy winter wheats. Crop Sci. 44:2273-2274.

Luzzatto-Knaan, T., Z. Kerem, A. Lipsky, and I. Yedidia. 2013. A systemic response of geophytes is demonstrated by patterns of protein expression and the accumulation of signal molecules in zantedeschia aethiopica. Plant Physiology and Biochemistry 71:218-225.

Narasimhamoorthy, B., B. Gill, A. Fritz, J. Nelson, and G. Brown-Guedira. 2006. Advanced backcross QTL analysis of a hard winter wheat× synthetic wheat population. Theor. Appl. Genet. 112:787-796.

Ramos, O.; Smith, C.M.; Fritz, A.K.; Madl, R. 2015. Effect of insect feeding, pathogen infection, and heat stress on wheat bean antioxidants. Crop Science. In review.

Ramos, O.; Smith, C.M.; Fritz, A.K.; Madl, R. 2015. Bird-cherry oat aphid (*Rhopalosiphum padi*) feeding induces enhanced levels of phenolics in mature wheat grains. Crop Science. In review.

Ramos, O.; Smith, C.M.; Fritz, K.A.; Madl, R. 2015. Synthetic elicitors of systemic acquired resistance promote de novo synthesis of phenolics in foliage of hard red winter wheat (*Triticum aestivum* L). In preparation.

Stadnik, M.J., and H. Buchenauer. 1999. Effects of benzothiadiazole, kinetin and urea on the severity of powdery mildew and yield of winter wheat. Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection 106:476-489.

Sears, R.G., T.J. Martin, T.S. Cox, O.K. Chung, S.P. Curran, W.F. Heer, and M.D. Witt. 1997. Registration of 'karl 92' wheat. Crop Sci. 37:628-628.

Walters, D.R., and L. Paterson. 2012. Parents lend a helping hand to their offspring in plant defence. Biol. Lett. 8:871-873.

Wang, J.W., and J.Y. Wu. 2005. Nitric oxide is involved in methyl jasmonate-induced defense responses and secondary metabolism activities of taxus cells. Plant Cell Physiol. 46:923-930.

Ward, E.R., S.J. Uknes, S.C. Williams, S.S. Dincher, D.L. Wiederhold, D.C. Alexander, P. Ahl-Goy, J.P. Metraux, and J.A. Ryals. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3:1085-1094.

Xu, Y., P. Chang, D. Liu, M.L. Narasimhan, K.G. Raghothama, P.M. Hasegawa, and R.A. Bressan. 1994. Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell 6:1077-1085.

Zangerl, A. and F. Bazaaz. 1992. Theory and pattern in plant defense allocation. In: Plant resistance to herbivores and pathogens. Ecology, evolution, and genetics. The University of Chicago Press, Chicago, USA.

Chapter 7 - Summary and Conclusions

Several theories of general plant stress metabolism provided a framework to test and understand several of the factors influencing the variability in antioxidant levels in wheat grains. In Chapter 3, experiments tested the hypothesis that the variability in total phenolic content in wheat plants is part of an inducible defense response to abiotic and biotic stress factors. The ability of hard red winter wheat cv. Karl 92 to modulate this defense response in grain total phenolic content (TPC) was shown by the different responses to insect feeding, pathogen infection, and heat stress. Bird-cherry oat aphid (*Rhopalosiphum padi*) feeding and heat stress explained most of the variation in free TPC, but *R. padi* feeding induces it by 26% and heat stress suppresses it by 20%. Furthermore, heat stress suppressed the bound phenolics, which are said to be constitutive and not inducible, by 22%. Rust infection did not explain any of the variation in TPC in wheat bran extracts.

The theory of growth/differentiation balance was the basis for the design of experiments in Chapter 4. *R. padi* feeding stress was the choice of stress factor based on the findings in Chapter 3 and the fact that *R. padi* damage does not compromise the plant's photosynthetic capacity. The lady bug beetle, *Hippodamia convergens,* and the predatory mite, *Neoseiulus cucumeris*, were used to completely remove *R. padi* at the end of the stress period and keep undesired insects out. Finally, the lighting conditions were homogeneous to all stressed and non-stressed treatments. Contrary to the theory of growth/differentiation balance, wheat plant defense response to *R. padi* feeding stress had no ontogenetically-determined plant stage, but the relative allocation of carbon resources to growth and defense was dependent on the time at which *R. padi* stress started. More resources were allocated to defense when the stress occurred or

started early (5-tillers, 7 DPA or 5 tillers through 35 DPA), while less was allocated to defense when the stress occurred late (21 DPA). Plant stress response to *R. padi* feeding was concluded to be systemic acquired resistance because increased phenolic production was long lasting and systemic, i.e., activated in the leaves, but appearing in the grains.

This research started based on the principles of plant generalized stress responses, but based on experimental results in the previous chapters, phenolics were observed to be part of an active plant defense response, presumably through systemic acquired resistance (SAR). In order to test this hypothesis, six synthetic elicitors of SAR with different structures and modes of action were evaluated for their ability to induce a phenolic response in the leaves of Ike and Karl 92 wheat at 24-, 36-, and 48-h post application in Chapter 5. Phenolics were induced as part of the response of wheat foliage to two different SAR elicitors. DCPCA and Methyl jasmonate induced a transient but significant phenolic response in the leaves of Karl 92 and Ike of 49% and 177%, respectively.

As an additional test of the active defense theory, plant phenolic responses to the same six synthetic SAR elicitors applied to the foliage at the tillering stage was tested in mature grains in experiments described in Chapter 6. B1, DCPCA, BTH, and SS induced phenolics in mature grains. There was a positive correlation between phenolic induction in the leaves and in the grains in response to DCPCA. This study shows that phenolic synthesis in hard red winter wheat is part of an active inducible defense response known as SAR. The theories of inducible defense responses provided a framework to test the hypothesis that phenolics are produced in wheat as part of an active defense response to insect feeding stress via SAR.

Chapter 8 - Future Research

The research done in this dissertation can serve as a background to the following lines of future research:

1. Field trials to test the effectiveness of SAR elicitor applications (more cultivars and replications) and evaluate grain yield.
2. Test the use of jasmonic acid as an less costly hormone for phenolic synthesis because it only activates phenolic synthesis in the leaves for a short period of time, but its effect is long lasting because it is observed on mature grains too.
3. Combine applications of salicylic and jasmonic acid to evaluate whether there is a synergistic effect on phenolic synthesis.
4. Evaluate the effect of fertilization on wheat crops post elicitor application on phenolic synthesis. Test the theory of carbon/nutrient balance after inducible defense responses have been activated. Nitrogen fertilization can be made after elicitor applications and the phenolic responses and yield can be evaluated.
5. In the present study, wheat was grown across several seasons in the year to account for changes in sunlight. Additionally, supplemental light was provided. The results indicated that, even with the changing sunlight conditions, insect feeding stress had a significant effect on the phenolic response. However, the theory of photodamage states that phenolics are produced as a defense response to adverse light conditions following herbivory. Formal trials can be designed and conducted in which artificial adverse light (i.e. excessive photoperiod, or UV light) is supplied after insect feeding stress.

6. Create a QTL map of the genes responsible for inducible responses. The mapping population can be made from a cross of Karl 92, a very inducible cultivar, and Ike, a less sensitive cultivar. The F1 population can be selfed through double haploid technology. Markers of SAR can be used and applications of DCPCA and BTH can be used as screening tools for the phenotype of inducible responses.
7. A transgenic approach can be taken to increase the levels of β 1,3-glucanase in wheat lines. It was shown that such a modification increases the levels of bound phenolic compounds.
8. A quicker method to determine TPC needs to be developed if high antioxidant wheat becomes a specialty crop. This method needs to be quick and reliable to be used at grain receiving facilities to quickly determine the TPC content of bulk wheat. Perhaps a FTIR calibration could be developed.
9. Determine whether phenolics induced through the methods described in this study act as functional antioxidants in food systems. If so, evaluate the feasibility of replacing synthetic antioxidants with these natural ones.

Chapter 9 - Appendix list

Appendix A - Statistical analysis

Table 9-1. Two sample T-tests for unequal variances for the effect of insect feeding on grain yield per pot (grams)

	Insect-fed	Control
Mean	7.07	9.68
Variance	5.26	16.47
Observations	26	4
Hypothesized Mean Difference	0	
Degrees of freedom	3	
t Stat	-1.26	
P(T<=t) one-tail	0.1489	
t Critical one-tail	2.35	
P(T<=t) two-tail	0.2979	
t Critical two-tail	3.18	

Table 9-2. Two sample T-tests with unequal variances for the effect of insect feeding on number of spikes per pot

	Insect-fed	Control
Mean	25	26
Variance	35	23
Observations	26	4
Hypothesized Mean Difference	0	
Degrees of freedom	5	
t Stat	-0.60	
P(T<=t) one-tail	0.2873	
t Critical one-tail	2.02	
P(T<=t) two-tail	0.5747	
t Critical two-tail	2.57	

Table 9-3. Two sample T-tests with unequal variances for the effect of insect feeding on grain yield per spike (grams)

	Insect-fed	Control
Mean	0.307	0.384
Variance	0.018	0.035
Observations	26	4
Hypothesized Mean Difference	0	

Degrees of freedom	3
t Stat	-0.79
P(T<=t) one-tail	0.2444
t Critical one-tail	2.35
P(T<=t) two-tail	0.4888
t Critical two-tail	3.18

Table 9-4. Two sample T-test with unequal variances for the effect of heat stress on grain yield per pot (grams)

	Heat	Control
Mean	4.11	7.45
Variance	1.26	21.74
Observations	17	3
Hypothesized Mean Difference	0	
Degrees of freedom	2	
t Stat	-1.23	
P(T<=t) one-tail	0.1712	
t Critical one-tail	2.92	
P(T<=t) two-tail	0.3424	
t Critical two-tail	4.30	

Table 9-5. Two sample T-test with unequal variances for the effect of heat stress on number of spikes per pot

	Heat	Control
Mean	14	24
Variance	14	9
Observations	17	3
Hypothesized Mean Difference	0	
Degrees of freedom	3	
t Stat	-5.31	
P(T<=t) one-tail	0.0065	
t Critical one-tail	2.35	
P(T<=t) two-tail	0.0131	
t Critical two-tail	3.18	

Table 9-6. Two sample T-test with unequal variances for the effect of heat stress on grain yield per spike (grams)

	Heat	Control
Mean	0.325	0.307
Variance	0.007	0.027
Observations	17	3
Hypothesized Mean Difference	0	
Degrees of freedom	2	
t Stat	0.18	
P(T<=t) one-tail	0.4379	
t Critical one-tail	2.92	
P(T<=t) two-tail	0.8758	
t Critical two-tail	4.30	

Table 9-7. Two sample T-tests with unequal variances for the effect of rust infection on grain yield per pot (grams)

	Rust infection	Control
Mean	12.31	7.65
Variance	14.96	14.66
Observations	16	4
Hypothesized Mean Difference	0	
Degrees of freedom	5	
t Stat	2.17	
P(T<=t) one-tail	0.0410	
t Critical one-tail	2.02	
P(T<=t) two-tail	0.0821	
t Critical two-tail	2.57	

Table 9-8. Two sample T-test with unequal variances for the effect of rust infection on number of spikes per pot

	Rust infection	Control
Mean	23	24
Variance	19	9
Observations	16	3
Hypothesized Mean Difference	0	
Degrees of freedom	4	
t Stat	-0.56	
P(T<=t) one-tail	0.3023	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.6045	
t Critical two-tail	2.78	

Table 9-9. Two sample T-tests with unequal variances for the effect of rust infection on grain yield per spike (grams)

	Rust infection	Control
Mean	0.567	0.307
Variance	0.052	0.027
Observations	15	3
Hypothesized Mean Difference	0	
Degrees of freedom	4	
t Stat	2.31	
P(T<=t) one-tail	0.0410	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.0819	
t Critical two-tail	2.78	

Table 9-10. Proc Glimmix model information for the effect of insect feeding on free and bound total phenolic content (TPC) and % DPPH scavenged after 30 min (DPPH)

Model Information	
Data Set	WORK.INSECT
Response Variables	Free TPC, bound TPC, free DPPH, bound DPPH
Response Distribution	Gaussian
Link Function	Identity
Variance Function	Default
Variance Matrix	Diagonal
Estimation Technique	Restricted Maximum Likelihood
Degrees of Freedom Method	Residual

Table 9-11. Type III of fixed effects for the effect of insect feeding stress, extraction, and milling on free TPC

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Stress	1	6	4.76	0.0719
Extraction	2	6	0.34	0.7223
Mill	1	6	2.47	0.1671
Stress*Mill	1	6	0.09	0.7781

Table 9-12. Type III of fixed effects for the effect of insect feeding, extraction, and milling on bound TPC

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Stress	1	6	2.48	0.1662
Extraction	2	6	0.16	0.8564
Mill	1	6	0.38	0.5581
Stress*Mill	1	6	0.01	0.9185

Table 9-13. Type III of fixed effects on the effect of insect feeding, extraction, and milling on DPPH scavenged by the free phenolics

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Stress	1	6	20.84	0.0038
Extraction	2	6	1.49	0.2976
Mill	1	6	1.00	0.3569
Stress*Mill	1	6	0.61	0.4661

Table 9-14. Type III of fixed effects on the effect of insect feeding, extraction, and milling on DPPH scavenged by the bound phenolics

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Stress	1	6	0.51	0.5004
Extraction	2	6	1.02	0.4167
Mill	1	6	0.79	0.4078
Stress*Mill	1	6	0.04	0.8483

Table 9-15. Proc Glimmix model information for the effect of heat stress on free and bound total phenolic content (TPC) and % DPPH scavenged after 30 min (DPPH)

Model Information	
Data Set	WORK.HEAT
Response Variable	Free TPC, bound TPC, free DPPH, bound DPPH
Response Distribution	Gaussian
Link Function	Identity
Variance Function	Default
Variance Matrix	Diagonal
Estimation Technique	Restricted Maximum Likelihood
Degrees of Freedom Method	Residual

Table 9-16. Type III test of fixed effects for the effect of heat stress, extraction, and milling on free TPC

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Stress	1	6	5.17	0.0633
Extraction	2	6	1.02	0.4168
Mill	1	6	3.86	0.0969
Stress*Mill	1	6	0.07	0.8048

Table 9-17. Type III of fixed effects for the effect of heat stress, extraction, and milling on bound TPC

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Stress	1	6	53.08	0.0003
Extraction	2	6	0.99	0.4237
Mill	1	6	10.74	0.0169
Stress*Mill	1	6	1.49	0.2674

Table 9-18. Type III of fixed effects on the effect of heat stress, extraction, and milling on DPPH scavenged by the free phenolics

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Stress	1	6	19.03	0.0048
Extraction	2	6	0.39	0.6928
Mill	1	6	0.38	0.5580
Stress*Mill	1	6	0.47	0.5186

Table 9-19. Type III test of fixed effects on the effect of heat stress, extraction, and milling on DPPH scavenged by the bound phenolics

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Stress	1	6	37.59	0.0009
Extraction	2	6	1.26	0.3492
Mill	1	6	0.00	0.9772
Stress*Mill	1	6	1.38	0.2846

Table 9-20. Proc Glimmix model information for the effect of rust infection on free and bound total phenolic content (TPC) and % DPPH scavenged after 30 min (DPPH)

Model Information	
Data Set	WORK.RUST
Response Variable	Free TPC, bound TPC, free DPPH, bound DPPH
Response Distribution	Gaussian
Link Function	Identity
Variance Function	Default
Variance Matrix	Diagonal
Estimation Technique	Restricted Maximum Likelihood
Degrees of Freedom Method	Residual

Table 9-21. Type III tests of fixed effects for the effect of rust infection, extraction, and milling on free TPC

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Stress	1	6	0.00	0.9724
Extraction	2	6	0.51	0.6260
Mill	1	6	2.48	0.1665
Stress*Mill	1	6	0.03	0.8755

Table 9-22. Type III tests of fixed effects for the effect of rust infection, extraction, and milling on the bound TPC

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Stress	1	6	0.09	0.7731
Extraction	2	6	0.22	0.8060
Mill	1	6	0.85	0.3911
Stress*Mill	1	6	0.00	0.9909

Table 9-23. Type III tests of fixed effects for the effect of rust infection, extraction, and milling on the DPPH scavenged by the free phenolics

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Stress	1	6	0.00	0.9560
Extraction	2	6	0.84	0.4779
Mill	1	6	1.55	0.2590
Stress*Mill	1	6	0.00	0.9678

Table 9-24. Type III test of fixed effects for the effect of rust infection, extraction, and milling on DPPH scavenged by the bound phenolics

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Stress	1	6	0.51	0.5030
Extraction	2	6	0.53	0.6143
Mill	1	6	2.76	0.1480
Stress*Mill	1	6	0.49	0.5104

Table 9-25. Proc Mixed model information for the effect of stress by bird-cherry oat aphid feeding at several growth stages on free total phenolic content (TPC) of wheat grains

Model Information	
Data Set	WORK.STAGE_RUN2
Dependent Variable	TPC_Free, TPC_Free and conjugated, TPC_Bound, DPPH_Free, DPPH_Free and conjugated, DPPH_Bound
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Satterthwaite

Table 9-26. Sources of variation and levels for each treatment used in ANOVA

Class Level Information		
Class	Levels	Values
R. padi feeding treatments	5	5-tillers -21 DPA, *R.padi* free, 7DPA, 21DPA, 5-tillers
Analytical order	13	1 2 3 4 5 6 7 8 9 10 11 12 13
Replicate	6	A B C1 C2 D E

Table 9-27. Type III test of fixed effects for the effect of *R. padi* feeding on free, free and conjugated, and bound TPC

Type 3 Tests of Fixed Effects				
TPC	Num DF	Den DF	F Value	Pr > F
Free	4	78.1	5.04	0.0012
Free and conjugated	4	70.4	3.23	0.0172
Bound	4	73	0.31	0.8706

Table 9-28. Mean and SE estimates of free TPC for each level of *R. padi* feeding treatments

Least Squares Means					
R. padi feeding	Estimate	Standard Error	DF	t Value	Pr > |t|
5-tillers	0.5667	0.01592	76	35.59	<.0001
7 DPA	0.5373	0.008173	29.6	65.74	<.0001
21 DPA	0.5241	0.02886	79.2	18.16	<.0001
5-tillers - 21 DPA	0.5469	0.009621	42.9	56.84	<.0001
R. padi free	0.5031	0.009488	53.1	53.02	<.0001

Table 9-29. Differences of LSM for free TPC among *R. padi* feeding treatments, Bonferroni adjusted (P<0.0125)

Differences of Least Squares Means							
Trt	Trt	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P
5-tillers – 21 DPA	*R. padi* free	0.04379	0.01228	76.1	3.57	0.0006	0.0062
R. padi free	7 DPA	-0.03422	0.01120	75.2	-3.06	0.0031	0.0308
R. padi free	21 DPA	-0.02103	0.02978	75.4	-0.71	0.4822	1.0000
R. padi free	5-tillers	-0.06364	0.01761	76.4	-3.61	0.0005	0.0053

Table 9-30. Mean and SE estimates of free and conjugated TPC for each level of *R. padi* feeding treatments

Least Squares Means					
R. padi feeding	Estimate	Standard Error	DF	t Value	Pr > |t|
5-tillers	1.1819	0.03128	44.4	37.79	<.0001
7 DPA	1.1630	0.02300	16.4	50.57	<.0001
21 DPA	1.1616	0.04829	78.3	24.05	<.0001
5-tillers - 21 DPA	1.1968	0.02436	20.2	49.12	<.0001
R. padi free	1.1308	0.02388	19.1	47.36	<.0001

Table 9-31. Differences of LSM for free and conjugated TPC among *R. padi* feeding treatments, Bonferroni adjusted (P<0.0125)

Differences of Least Squares Means							
Trt	Trt	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P
5-tillers – 21 DPA	*R. padi* free	0.06604	0.01890	69.9	3.49	0.0008	0.0083
R. padi free	7 DPA	-0.03217	0.01716	69.6	-1.87	0.0650	0.6496
R. padi free	21 DPA	-0.03085	0.04554	69.4	-0.68	0.5004	1.0000
R. padi free	5-tillers	-0.05110	0.02710	69.8	-1.89	0.0636	0.6352

Table 9-32. Mean and SE estimates of bound TPC for each level of *R. padi* feeding treatments

Least Squares Means					
R. padi feeding	Estimate	Standard Error	DF	t Value	Pr > |t|
5-tillers	4.1482	0.04063	65.9	102.10	<.0001
7 DPA	4.1322	0.02542	21.1	162.53	<.0001
21 DPA	4.1649	0.06856	79.6	60.74	<.0001
5-tillers - 21 DPA	4.1556	0.02809	29.1	147.92	<.0001
R. padi free	4.1575	0.02734	28.5	152.07	<.0001

Table 9-33. Differences of LSM for bound TPC among *R. padi* feeding treatments, Bonferroni adjusted (P<0.0125)

Differences of Least Squares Means							
Trt	Trt	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P
5-tillers – 21 DPA	*R. padi* free	-0.00189	0.02829	72	-0.07	0.9469	1.0000
R. padi free	7 DPA	0.02532	0.02572	71.4	0.98	0.3282	1.0000
R. padi free	21 DPA	-0.00738	0.06833	71.1	-0.11	0.9143	1.0000
R. padi free	5-tillers	0.009305	0.04059	71.9	0.23	0.8193	1.0000

Table 9-34. Test III tests of fixed effects for the effect of *R. padi* feeding on free, free and conjugated, and bound DPPH

Type 3 Tests of Fixed Effects				
DPPH	Num DF	Den DF	F Value	Pr > F
Free	4	58.7	5.11	0.0013
Free and conjugated	4	60	3.38	0.0148
Bound	4	21.8	1.45	0.2531

Table 9-35. Mean and SE estimates of free DPPH for each level of *R. padi* feeding treatment

Least Squares Means					
R. padi feeding	Estimate	Standard Error	DF	t Value	Pr > |t|
5-tillers	9.2108	0.8406	16.1	10.96	<.0001
7 DPA	9.1535	0.7639	11.1	11.98	<.0001
21 DPA	8.4525	1.0536	34.1	8.02	<.0001
5-tillers - 21 DPA	9.9322	0.7785	12	12.76	<.0001
R. padi free	8.3424	0.7735	11.7	10.78	<.0001

Table 9-36. Differences of LSM for free DPPH among *R. padi* feeding treatments, Bonferroni adjusted (P<0.0125)

Differences of Least Squares Means							
Trt	Trt	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P
5-tillers – 21 DPA	*R. padi* free	1.5898	0.3604	58.6	4.41	<.0001	0.0004
R. padi free	7 DPA	-0.8111	0.3298	58.5	-2.46	0.0169	0.1689
	21 DPA	-0.1101	0.7898	58.3	-0.14	0.8896	1.0000
	5-tillers	-0.8683	0.4754	58.4	-1.83	0.0729	0.7288

Table 9-37. Mean and SE estimates of free and conjugated DPPH for each level of *R. padi* feeding treatment

Least Squares Means					
R. padi feeding	Estimate	Standard Error	DF	t Value	Pr > |t|
5-tillers	13.1890	0.9231	30.2	14.29	<.0001
7 DPA	13.7233	0.7312	13.5	18.77	<.0001
21 DPA	12.5233	1.3766	62.8	9.10	<.0001
5-tillers - 21 DPA	14.0379	0.7694	16.2	18.24	<.0001
R. padi free	12.1740	0.7583	15.7	16.05	<.0001

Table 9-38. Differences of LSM for free and conjugated DPPH among *R. padi* feeding treatments, Bonferroni adjusted (P<0.0125)

Differences of Least Squares Means							
Trt	Trt	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P
5-tillers – 21 DPA	*R. padi* free	1.8639	0.5781	59.8	3.22	0.0020	0.0204
R. padi free	7 DPA	-1.5494	0.5293	59.6	-2.93	0.0048	0.0483
R. padi free	21 DPA	-0.3493	1.2699	59	-0.28	0.7842	1.0000
R. padi free	5-tillers	-1.0151	0.7641	59.1	-1.33	0.1892	1.0000

Table 9-39. Mean and SE estimates of bound DPPH for each level of *R. padi* feeding treatment

Least Squares Means					
R. padi feeding	Estimate	Standard Error	DF	t Value	Pr > |t|
5-tillers	25.0661	1.0624	15.2	23.59	<.0001
7 DPA	24.8026	0.9820	11.2	25.26	<.0001
21 DPA	25.9282	1.2838	28.2	20.20	<.0001
5-tillers - 21 DPA	25.8152	0.9964	11.9	25.91	<.0001
R. padi free	25.1752	0.9864	11.5	25.52	<.0001

Table 9-40. Differences of LSM for bound DPPH among *R. padi* feeding treatments, Bonferroni adjusted (P<0.0125)

Differences of Least Squares Means							
Trt	Trt	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P
5-tillers – 21 DPA	*R. padi* free	0.6399	0.4424	19.6	1.45	0.1639	1.0000
R. padi free	7 DPA	0.3727	0.4121	16	0.90	0.3792	1.0000
R. padi free	21 DPA	-0.7529	0.9168	34.3	-0.82	0.4172	1.0000
R. padi free	5-tillers	0.1092	0.5715	23	0.19	0.8502	1.0000

Table 9-41. Processing plan for extraction and analysis of wheat leaf samples

ORDER	*OBS*	*RUN*	*TIME*	*CULTIVAR*	*REP*	*ALIQUOT*	*POSITION*	*ELICITOR*	*TPC (mg FAE/g dry leaf)*
1	277	93	36	K92	B	3	1	24 hba	93.85
2	279	93	36	K92	B	3	2	EtOH	24.43
3	278	93	36	K92	B	3	3	MeJa	68.32
4	13	5	48	K92	A	2	1	DCPCA	134.37
5	15	5	48	K92	A	2	2	H2O	58.08
6	14	5	48	K92	A	2	3	*B2*	54.91
7	232	78	36	K92	A	1	1	*B1*	83.21
8	234	78	36	K92	A	1	2	H2O	72.36
9	233	78	36	K92	B	1	3	*B2*	58.88
10	41	14	48	K92	B	2	1	MeJa	32.62
11	42	14	48	K92	A	2	2	EtOH	20.85
12	40	14	48	K92	A	2	3	*BTH*	42.88
13	283	95	36	K92	B	3	1	*SS*	61.89
14	285	95	36	K92	B	3	2	Null	32.59
15	284	95	36	K92	B	3	3	*B2*	44.17
16	53	18	48	K92	B	3	1	24	44.80
17	54	18	48	K92	A	3	2	H2O	74.82
18	52	18	48	K92	B	3	3	*B1*	44.82
19	139	47	24	K92	A	3	1	*B1*	39.96
20	140	47	24	K92	A	3	2	Null	44.91
21	141	47	24	K92	A	3	3	H2O	34.04
22	334	112	36	IKE	B	3	1	Null	39.32
23	335	112	36	IKE	B	3	2	MeJa	129.76
24	336	112	36	IKE	B	3	3	EtOH	29.74
25	94	32	48	IKE	B	1	1	*B1*	32.71
26	96	32	48	IKE	B	1	2	Null	42.85
27	95	32	48	IKE	B	1	3	*BTH*	38.84
28	3	1	48	K92	A	1	1	EtOH	31.85
29	2	1	48	K92	B	1	2	MeJa	23.69
30	1	1	48	K92	A	1	3	*B2*	41.83
31	331	111	36	IKE	B	2	1	*BTH*	51.15
32	332	111	36	IKE	B	2	2	DCPCA	110.69
33	333	111	36	IKE	B	2	3	Null	71.57
34	311	104	36	IKE	A	3	1	Null	59.45
35	310	104	36	IKE	A	3	2	*B1*	82.51
36	312	104	36	IKE	A	3	3	H2O	75.14

37	130	44	24	K92	A	2	1	*SS*	30.71
38	131	44	24	K92	A	2	2	*B2*	33.59
39	132	44	24	K92	A	2	3	H2O	37.85
40	315	105	36	IKE	A	3	1	*BTH*	27.97
41	314	105	36	IKE	A	3	2	*B2*	21.47
42	313	105	36	IKE	A	3	3	*SS*	60.09
43	32	11	48	K92	A	1	1	*B2*	28.57
44	31	11	48	K92	B	1	2	DCPCA	52.79
45	33	11	48	K92	A	1	3	H2O	48.22
46	91	31	48	IKE	B	1	1	*B2*	36.29
47	92	31	48	IKE	B	1	2	DCPCA	37.95
48	93	31	48	IKE	B	1	3	H2O	69.29
49	35	12	48	K92	B	1	1	*B1*	33.44
50	36	12	48	K92	B	1	2	Null	39.19
51	34	12	48	K92	A	1	3	*SS*	28.49
52	197	66	24	IKE	A	3	1	*BTH*	28.21
53	196	66	24	IKE	A	3	2	*SS*	43.38
54	198	66	24	IKE	A	3	3	Null	
55	202	68	24	IKE	B	1	1	*SS*	54.00
56	203	68	24	IKE	B	1	2	DCPCA	46.52
57	204	68	24	IKE	B	1	3	H2O	44.99
58	17	6	48	K92	A	2	1	*SS*	
59	18	6	48	K92	A	2	2	Null	72.05
60	16	6	48	K92	A	2	3	*BTH*	90.81
61	110	37	48	IKE	B	3	1	*B1*	50.20
62	109	37	48	IKE	B	3	2	*SS*	68.04
63	111	37	48	IKE	B	3	3	H2O	154.60
64	226	76	24	IKE	B	3	1	DCPCA	27.63
65	228	76	24	IKE	B	3	2	Null	35.10
66	227	76	24	IKE		3	3	24	40.97
67	138	46	24	K92	A	3	1	EtOH	56.10
68	137	46	24	K92	A	3	2	MeJa	57.00
69	136	46	24	K92	A	3	3	*B2*	98.71
70	341	114	36	IKE	B	3	1	*B1*	39.23
71	342	114	36	IKE	B	3	2	*SS*	51.85
72	340	114	36	IKE	B	3	3	*B2*	56.15
73	183	61	24	IKE	A	2	1	EtOH	41.85
74	181	61	24	IKE	A	2	2	*SS*	53.40
75	182	61	24	IKE	A	2	3	MeJa	37.54
76	75	25	48	IKE	A	2	1	H2O	62.82
77	74	25	48	IKE	A	2	2	*B2*	35.35

78	73	25	48	IKE	A	2	3	*B1*	44.97
79	288	96	36	IKE	B	1	1	EtOH	27.53
80	286	96	36	IKE	B	1	2		
81	287	96	36	IKE	B	1	3	MeJa	139.44
82	211	71	24	IKE	B	2	1	*B2*	53.05
83	213	71	24	IKE	B	2	2	EtOH	46.68
84	212	71	24	IKE	B	2	3	MeJa	57.96
85	239	80	36	K92	A	2	1	MeJa	33.91
86	240	80	36	K92	A	2	2	EtOH	76.62
87	238	80	36	K92	A	2	3	*B1*	127.23
88	290	97	36	IKE	A	1	1	*BTH*	22.34
89	289	97	36	IKE	A	1	2	*B2*	32.37
90	291	97	36	IKE	A	1	3	H2O	24.00
91	250	84	36	K92	A	3	1	*B2*	22.41
92	251	84	36	K92	A	3	2	*SS*	71.52
93	252	84	36	K92	A	3	3	H2O	71.17
94	124	42	24	K92	A	1	1	*B1*	41.07
95	126	42	24	K92	A	2	2	*BTH*	26.26
96	125	42	24	K92	A	1	3	DCPCA	23.67
97	88	30	48	IKE	B	1	1	*SS*	47.05
98	89	30	48	IKE	B	1	2	MeJa	65.43
99	90	30	48	IKE	B	1	3	EtOH	19.51
100	129	43	24	K92	A	2	1	EtOH	35.57
101	128	43	24	K92	A	2	2	MeJa	29.80
102	127	43	24	K92	A	2	3		
103	165	55	24	K92	B	3	1	EtOH	45.89
104	163	55	24	K92	B	3	2	*BTH*	75.03
105	164	55	24	K92	A	3	3	MeJa	40.23
106	255	85	36	K92	A	3	1	Null	32.95
107	254	85	36	K92	A	3	2	*B1*	232.74
108	253	85	36	K92	A	3	3	DCPCA	56.89
109	66	22	48	IKE	A	1	1	*B2*	40.04
110	65	22	48	IKE	A	1	2	*B1*	37.37
111	64	22	48	IKE	A	1	3	DCPCA	126.30
112	296	99	36	IKE	A	2	1	*B2*	77.85
113	297	99	36	IKE	A	2	2	*BTH*	34.79
114	295	99	36	IKE	A	1	3	*SS*	67.76
115	274	92	36	K92	B	2	1	*B2*	35.52
116	275	92	36	K92	B	2	2	*SS*	43.92
117	276	92	36	K92	B	2	3	Null	58.46
118	262	88	36	K92	B	1	1	*B1*	52.72

119	263	88	36	K92	B	1	2	DCPCA	70.66
120	264	88	36	K92	B	1	3	*BTH*	115.38
121	192	64	24	IKE	A	3	1	EtOH	36.07
122	191	64	24	IKE	A	3	2	MeJa	47.70
123	190	64	24	IKE	A	3	3	DCPCA	44.22
124	261	87	36	K92	B	1	1	H2O	25.78
125	259	87	36	K92	B	1	2	*SS*	43.55
126	260	87	36	K92	B	1	3	Null	33.22
127	229	77	36	K92	A	1	1	Null	38.88
128	231	77	36	K92	B	1	2	EtOH	89.29
129	230	77	36	K92	A	1	3	MeJa	38.99
130	188	63	24	IKE	A	2	1	*B1*	64.48
131	187	63	24	IKE	A	2	2	*B2*	14.61
132	189	63	24	IKE	A	2	3	Null	43.75
133	134	45	24	K92	A	2	1	DCPCA	48.83
134	133	45	24	K92	A	2	2	*B1*	41.72
135	135	45	24	K92	A	2	3	Null	43.00
136	71	24	48	IKE	B	2	1	MeJa	32.34
137	72	24	48	IKE	A	2	2	EtOH	34.36
138	70	24	48	IKE	A	2	3		
139	208	70	24	IKE	B	1	1	*B1*	59.83
140	210	70	24	IKE	B	3	2	*B2*	50.90
141	209	70	24	IKE	B	3	3	*SS*	26.90
142	118	40	24	K92	A	1	1	*BTH*	29.84
143	119	40	24	K92	A	1	2	24	28.67
144	120	40	24	K92	A	1	3	H2O	37.51
145	151	51	24	K92	A	1	1	DCPCA	80.65
146	152	51	24	K92	B	1	2	*B2*	48.50
147	153	51	24	K92	B	1	3	Null	37.50
148	307	103	36	IKE	A	3	1	DCPCA	27.22
149	309	103	36	IKE	A	3	2	EtOH	33.12
150	308	103	36	IKE	A	3	3	MeJa	36.41
151	98	33	48	IKE	B	2	1	MeJa	86.09
152	99	33	48	IKE	B	2	2	EtOH	61.86
153	97	33	48	IKE	B	2	3	Null	144.82
154	43	15	48	K92	A	2	1	*SS*	113.05
155	45	15	48	K92	A	2	2	H2O	133.79
156	44	15	48	K92	B	2	3	Null	93.30
157	5	2	48	K92	A	1	1	*BTH*	174.22
158	4	2	48	K92	A	1	2	*B1*	87.97
159	6	2	48	K92	A	1	3	H2O	

160	103	35	48	IKE	B	2	1	*B2*	105.80
161	104	35	48	IKE	B	2	2	*SS*	195.42
162	105	35	48	IKE	B	2	3	DCPCA	107.59
163	321	107	36	IKE	B	1	1	H2O	361.74
164	319	107	36	IKE	B	1	2	DCPCA	133.22
165	320	107	36	IKE	B	1	3	*B2*	112.04
166	82	28	48	IKE	A	3	1	DCPCA	962.19
167	83	28	48	IKE	A	3	2	*SS*	196.02
168	84	28	48	IKE	A	3	3	H2O	165.42
169	19	7	48	K92	A	3	1	Null	106.59
170	20	7	48	K92	B	3	2	MeJa	87.81
171	21	7	48	K92	A	3	3	EtOH	84.90
172	55	19	48	K92	A	3	1	*BTH*	
173	56	19	48	K92	A	3	2	*SS*	236.68
174	57	19	48	K92	B	3	3	Null	96.62
175	28	10	48	K92	B	1	1		
176	30	10	48	K92	A	1	2	EtOH	119.58
177	29	10	48	K92	B	1	3	MeJa	84.61
178	235	79	36	K92	A	1	1	*SS*	186.08
179	236	79	36	K92	A	1	2	DCPCA	142.65
180	237	79	36	K92	A	1	3	*BTH*	132.15
181	215	72	24	IKE	B	2	1	Null	83.27
182	216	72	24	IKE	B	2	2	H2O	109.82
183	214	72	24	IKE	B	2	3	*BTH*	87.00
184	9	3	48	K92	A	1	1	Null	246.03
185	8	3	48	K92	A	1	2	DCPCA	439.48
186	7	3	48	K92	A	1	3	*SS*	123.19
187	113	38	48	IKE	B	3	1	*BTH*	50.93
188	114	38	48	IKE	A	3	2	Null	184.98
189	112	38	48	IKE	B	3	3	*B2*	84.72
190	194	65	24	IKE	A	3	1	*B2*	76.12
191	193	65	24	IKE	A	3	2	*B1*	88.66
192	195	65	24	IKE	A	3	3	H2O	84.85
193	280	94	36	K92	B	3	1	*BTH*	330.31
194	281	94	36	K92	B	3	2	DCPCA	242.91
195	282	94	36	K92	B	3	3	H2O	92.83
196	61	21	48	IKE	A	1	1	*BTH*	73.22
197	63	21	48	IKE	A	1	2	H2O	222.68
198	62	21	48	IKE	A	1	3	Null	197.48
199	303	101	36	IKE	A	2	1	H2O	156.68
200	302	101	36	IKE	A	2	2	DCPCA	95.41

201	301	101	36	IKE	A	2	3	*SS*	307.31
202	271	91	36	K92	B	2	1	*B1*	264.32
203	272	91	36	K92	B	2	2	*BTH*	1035.99
204	273	91	36	K92	B	2	3	H2O	166.97
205	222	74	24	IKE	B	3	1	EtOH	184.20
206	221	74	24	IKE	B	3	2	MeJa	113.59
207	220	74	24	IKE	B	3	3		
208	176	59	24	IKE	A	1	1	*SS*	64.32
209	175	59	24	IKE	A	1	2	*B2*	90.55
210	177	59	24	IKE	A	1	3	H2O	43.47
211	50	17	48	K92	B	3	1	MeJa	28.39
212	49	17	48	K92	B	3	2		
213	51	17	48	K92	A	3	3	EtOH	61.14
214	324	108	36	IKE	B	1	1	Null	58.48
215	323	108	36	IKE	B	1	2	*B1*	39.27
216	322	108	36	IKE	B	1	3	*BTH*	43.00
217	249	83	36	K92	A	3	1	EtOH	76.64
218	248	83	36	K92	A	3	2	MeJa	72.60
219	247	83	36	K92	A	3	3	*BTH*	50.60
220	122	41	24	K92	A	1	1	*SS*	109.77
221	121	41	24	K92	A	1	2	*B2*	107.46
222	123	41	24	K92	A	1	3	Null	110.72
223	37	13	48	K92	A	1	1	*BTH*	312.27
224	39	13	48	K92	B	3	2	DCPCA	153.50
225	38	13	48	K92	A	3	3	*B2*	53.95
226	69	23	48	IKE	A	3	1	*B1*	57.85
227	67	23	48	IKE	A	1	2	*SS*	124.12
228	68	23	48	IKE	A	2	3	DCPCA	319.42
229	24	8	48	K92	A	3	1	H2O	273.66
230	22	8	48	K92	A	3	2	*BTH*	157.19
231	23	8	48	K92	A	3	3	*SS*	50.93
232	306	102	36	IKE	A	2	1	Null	37.26
233	305	102	36	IKE	A	2	2	24	37.26
234	304	102	36	IKE	A	2	3	*B1*	45.49
235	26	9	48	K92	A	3	1	*B1*	48.28
236	25	9	48	K92	A	3	2	*B2*	120.06
237	27	9	48	K92	A	3	3	DCPCA	104.30
238	47	16	48	K92	B	2	1	*B2*	
239	46	16	48	K92	B	2	2	DCPCA	862.47
240	48	16	48	K92	B	2	3	*B1*	
241	108	36	48	IKE	B	3	1	EtOH	69.53

242	106	36	48	IKE	B	3	2	DCPCA	48.79
243	107	36	48	IKE	B	3	3	MeJa	59.91
244	77	26	48	IKE	A	2	1	*BTH*	84.40
245	76	26	48	IKE	A	2	2	*SS*	139.66
246	78	26	48	IKE	A	2	3	Null	133.61
247	87	29	48	IKE	A	3	1	Null	119.86
248	86	29	48	IKE	A	3	2	*B2*	36.83
249	85	29	48	IKE	A	3	3	*BTH*	36.35
250	150	50	24	K92	B	1	1	H2O	59.85
251	149	50	24	K92	B	1	2	*SS*	63.13
252	148	50	24	K92	B	1	3	*BTH*	71.91
253	174	58	24	IKE	A	1	1	EtOH	52.36
254	172	58	24	IKE	A	1	2	Null	71.08
255	173	58	24	IKE	A	1	3	MeJa	66.67
256	147	49	24	K92	B	1	1	EtOH	112.18
257	146	49	24	K92	B	1	2	MeJa	81.65
258	145	49	24	K92	B	1	3	*B1*	0.00
259	298	100	36	IKE	A	2	1		
260	299	100	36	IKE	A	2	2	MeJa	118.68
261	300	100	36	IKE	A	2	3	EtOH	37.39
262	329	110	36	IKE	B	2	1	*SS*	37.72
263	328	110	36	IKE	B	2	2	*B1*	29.63
264	330	110	36	IKE	B	2	3	H2O	202.68
265	294	98	36	IKE	A	1	1	Null	37.90
266	293	98	36	IKE	A	1	2	DCPCA	39.78
267	292	98	36	IKE	A	1	3	*B1*	87.11
268	116	39	24	K92	A	1	1	MeJa	67.00
269	117	39	24	K92	A	1	2	EtOH	13.49
270	115	39	24	K92	A	1	3		
271	200	67	24	IKE	B	1	1	MeJa	43.80
272	201	67	24	IKE	B	1	2	EtOH	53.05
273	199	67	24	IKE	B	1	3		
274	157	53	24	K92	A	2	1	DCPCA	135.28
275	158	53	24	K92	B	2	2	*B2*	55.51
276	159	53	24	K92	B	2	3	H2O	78.51
277	179	60	24	IKE	A	1	1	DCPCA	38.24
278	178	60	24	IKE	A	1	2	*BTH*	35.88
279	180	60	24	IKE	A	1	3	*B1*	68.85
280	266	89	36	K92	B	2	1	DCPCA	227.45
281	265	89	36	K92	A	1	2	*B2*	93.71
282	267	89	36	K92	B	3	3	*B1*	126.77

283	258	86	36	K92	B	1	1	EtOH	36.57
284	256	86	36	K92	B	1	2		
285	257	86	36	K92	B	1	3	MeJa	92.63
286	217	73	24	IKE	B	2	1	*SS*	42.01
287	218	73	24	IKE	B	2	2	DCPCA	84.55
288	219	73	24	IKE	B	2	3	*B1*	90.58
289	12	4	48	K92	A	2	1	EtOH	48.72
290	10	4	48	K92	A	2	2	*B1*	44.94
291	11	4	48	K92	B	2	3	MeJa	63.17
292	185	62	24	IKE	A	2	1	DCPCA	84.59
293	186	62	24	IKE	A	2	2	H2O	43.26
294	184	62	24	IKE	A	2	3	*BTH*	120.36
295	166	56	24	K92	B	3	1	*B1*	35.77
296	167	56	24	K92	A	3	2	DCPCA	332.24
297	168	56	24	K92	B	3	3	H2O	101.53
298	327	109	36	IKE	B	2	1	EtOH	12.31
299	326	109	36	IKE	B	2	2	MeJa	214.76
300	325	109	36	IKE	B	2	3	*B2*	99.19
301	154	52	24	K92	B	2	1	Null	72.95
302	155	52	24	K92	B	2	2	MeJa	133.67
303	156	52	24	K92	B	2	3	EtOH	162.01
304	224	75	24	IKE	B	3	1	*BTH*	128.83
305	223	75	24	IKE	B	3	2	*B1*	65.38
306	225	75	24	IKE	B	3	3	H2O	49.06
307	143	48	24	K92	A	3	1	*BTH*	18.19
308	142	48	24	K92	A	3	2	DCPCA	
309	144	48	24	K92	A	3	3	*SS*	82.08
310	339	113	36	IKE	B	3	1	H2O	596.73
311	337	113	36	IKE	B	3	2	*BTH*	69.70
312	338	113	36	IKE	B	3	3	DCPCA	130.08
313	242	81	36	K92	A	2	1	*SS*	59.25
314	243	81	36	K92	A	2	2	H2O	168.41
315	241	81	36	K92	A	2	3	DCPCA	31.30
316	246	82	36	K92	A	2	1	Null	89.89
317	245	82	36	K92	A	2	2	*BTH*	43.74
318	244	82	36	K92	A	2	3	*B2*	42.86
319	270	90	36	K92	B	2	1	EtOH	35.15
320	269	90	36	K92	B	2	2	MeJa	101.04
321	268	90	36	K92	B	2	3		
322	160	54	24	K92	B	2	1	*B1*	68.81
323	162	54	24	K92	B	2	2	*BTH*	75.77

324	161	54	24	K92	B	2	3	*SS*	157.24
325	169	57	24	K92	B	3	1	*B2*	158.42
326	171	57	24	K92	B	3	2	Null	134.10
327	170	57	24	K92	B	3	3	*SS*	184.86
328	205	69	24	IKE	B	1	1	*BTH*	46.99
329	206	69	24	IKE	B	1	2	*B2*	46.83
330	207	69	24	IKE	B	1	3	Null	59.46
331	81	27	48	IKE	A	3	1	EtOH	32.57
332	79	27	48	IKE	A	3	2	24	24.68
333	80	27	48	IKE	A	3	3	MeJa	54.11
334	100	34	48	IKE	B	2	1	*BTH*	
335	102	34	48	IKE	B	2	2	H2O	56.24
336	101	34	48	IKE	B	2	3	*B1*	34.84
337	59	20	48	IKE	A	1	1	MeJa	43.74
338	60	20	48	IKE	A	1	2	EtOH	56.65
339	58	20	48	IKE	A	1	3		
340	316	106	36	IKE	B	1	1	*SS*	43.84
341	317	106	36	IKE	B	1	2	MeJa	676.46
342	318	106	36	IKE	B	1	3	EtOH	54.13

Table 9-42. Proc Glimmix model information for the effect of synthetic elicitors of SAR on total phenolic content in wheat leaves

Model Information	
Data Set	WORK.DATA_121715
Response Variable	Log (Total phenolic content)
Response Distribution	Gaussian
Link Function	Identity
Variance Function	Default
Variance Matrix	Not blocked
Estimation Technique	Restricted Maximum Likelihood
Degrees of Freedom Method	Kenward-Roger

Table 9-43. Sources of variation and levels for each source used in ANOVA

Class Level Information		
Class	Levels	Values
Cultivar	2	IKE K92
Elicitor treatment	9	B1 B2 BTH DCPCA EtOH H2O MeJa Null SS
Post application time (hours)	3	24 36 48
Replicate	2	A B
Subsample	3	1 2 3

Table 9-44. Test III test of fixed effects for the effect of cultivar, elicitor treatment, and post application time on total phenolic content in wheat leaves

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Cultivar	1	42.34	1.56	0.2190
Elicitor treatment	8	42.34	2.40	0.0313
Cultivar*Elicitor treatment	8	42.34	0.91	0.5172
Post application time	2	42.43	2.08	0.1376
Cultivar*Post application time	2	42.43	0.06	0.9426
Elicitor treatment*Post application time	16	42.53	1.40	0.1901
Cultivar*Elicitor treatment*Post application time	16	42.53	1.10	0.3859

Table 9-45. Least Squares Means and SE estimates of total phenolic content for each cultivar

Cultivar	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*	*Alpha*	*Lower*	*Upper*
IKE	63.26	1.07	46.79	62.71	<.0001	0.05	55.37	72.25
K92	71.39	1.07	38.92	60.28	<.0001	0.05	61.86	82.38

Table 9-46. Least Square Means and SE estimates of total phenolic content for elicitor treatments

Elicitor	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*	*Alpha*	*Lower*	*Upper*
B1	58.80	1.15	49.15	28.7	<.0001	0.05	44.21	78.21
B2	53.83	1.16	41.07	27.24	<.0001	0.05	40.06	72.34
BTH	68.68	1.16	42.31	28.67	<.0001	0.05	51.00	92.49
DCPCA	98.77	1.16	41.07	31.39	<.0001	0.05	73.50	132.73
EtOH	46.91	1.16	39.88	26.38	<.0001	0.05	34.94	63.00
H2O	83.53	1.16	41.07	30.24	<.0001	0.05	62.16	112.24
MeJa	65.48	1.16	39.9	28.6	<.0001	0.05	48.73	88.00
Null	66.49	1.15	47.62	29.76	<.0001	0.05	50.07	88.30
SS	76.24	1.16	41.07	29.62	<.0001	0.05	56.74	102.44

Table 9-47. Elicitor treatment effect sliced by cultivar*collection time

Tests of Effect Slices for CULTIVAR*ELICITOR*TIME Sliced By CULTIVAR* POST APPLICATION TIME					
CULTIVAR	TIME	Num DF	Den DF	F Value	Pr > F
IKE	24	8	47.01	0.17	0.9941
IKE	36	8	46.12	2.20	0.0448
IKE	48	8	47	2.05	0.0608
K92	24	8	44.07	0.59	0.7792
K92	36	8	46.12	1.68	0.1295
K92	48	8	32.67	1.66	0.1460

Table 9-48. Least Square Means and SE estimates of total phenolic content for cultivar*elicitor treatment combinations

Cultivar	*Elicitor*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr> |t|*	*Alpha*	*Lower*	*Upper*
IKE	B1	53.18	1.22	46.13	20.15	<.0001	0.05	35.76	79.08
IKE	B2	51.83	1.22	46.13	20.02	<.0001	0.05	34.85	77.08
IKE	BTH	50.57	1.22	49.15	19.54	<.0001	0.05	33.78	75.70
IKE	DCPCA	81.26	1.22	46.13	22.3	<.0001	0.05	54.64	120.83
IKE	EtOH	41.72	1.22	46.11	18.83	<.0001	0.05	28.00	62.17
IKE	H2O	92.86	1.22	46.13	22.98	<.0001	0.05	62.44	138.10
IKE	MeJa	74.63	1.22	46.09	21.66	<.0001	0.05	49.98	111.41
IKE	Null	68.46	1.22	49.1	20.95	<.0001	0.05	45.65	102.67
IKE	SS	72.33	1.22	46.13	21.71	<.0001	0.05	48.63	107.57
K92	B1	65.02	1.23	52.22	20.44	<.0001	0.05	43.16	97.95
K92	B2	55.92	1.24	37.44	18.61	<.0001	0.05	36.09	86.64
K92	BTH	93.26	1.24	37.44	20.98	<.0001	0.05	60.19	144.50
K92	DCPCA	120.06	1.24	37.44	22.14	<.0001	0.05	77.49	186.05
K92	EtOH	52.76	1.24	35.45	18.52	<.0001	0.05	34.16	81.48
K92	H2O	75.13	1.24	37.44	19.98	<.0001	0.05	48.49	116.41
K92	MeJa	57.46	1.24	35.45	18.92	<.0001	0.05	37.21	88.74
K92	Null	64.58	1.22	46.13	21.14	<.0001	0.05	43.42	96.04
K92	SS	80.37	1.24	37.44	20.29	<.0001	0.05	51.86	124.52

Table 9-49. Least Square Means and SE estimates for the total phenolic content of elicitor treatment * post application time combinations

*Elicitor treatment*Post application time (HPA) Least Square Means*									
Elicitor	HPA	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
B1	24	56.66	1.28	50.68	16.28	<.0001	0.05	34.44	93.22
B1	36	79.62	1.27	46.13	18.13	<.0001	0.05	48.97	129.46
B1	48	45.06	1.28	50.68	15.35	<.0001	0.05	27.39	74.15
B2	24	59.41	1.27	46.13	16.91	<.0001	0.05	36.54	96.60
B2	36	50.59	1.27	46.13	16.25	<.0001	0.05	31.11	82.24
B2	48	51.91	1.32	34.71	14.32	<.0001	0.05	29.66	90.88
BTH	24	51.81	1.27	46.13	16.35	<.0001	0.05	31.86	84.23
BTH	36	76.23	1.27	46.13	17.95	<.0001	0.05	46.88	123.94
BTH	48	82.02	1.33	37.45	15.66	<.0001	0.05	46.39	145.05
DCPCA	24	68.01	1.32	34.71	15.3	<.0001	0.05	38.85	119.06
DCPCA	36	87.32	1.27	46.13	18.51	<.0001	0.05	53.70	141.97
DCPCA	48	162.26	1.27	46.13	21.07	<.0001	0.05	99.79	263.80
EtOH	24	55.25	1.27	46.13	16.61	<.0001	0.05	33.98	89.84
EtOH	36	40.04	1.28	46.07	15.06	<.0001	0.05	24.45	65.58
EtOH	48	46.67	1.31	32.08	14.21	<.0001	0.05	26.90	80.97
H2O	24	55.70	1.27	46.13	16.65	<.0001	0.05	34.26	90.56
H2O	36	104.50	1.27	46.13	19.25	<.0001	0.05	64.27	169.90
H2O	48	100.12	1.32	34.71	16.71	<.0001	0.05	57.19	175.27
MeJa	24	60.78	1.28	46.1	16.88	<.0001	0.05	37.25	99.18
MeJa	36	92.36	1.28	46.1	18.6	<.0001	0.05	56.60	150.70
MeJa	48	50.02	1.31	32.19	14.38	<.0001	0.05	28.74	87.05
Null	24	60.41	1.28	50.68	16.54	<.0001	0.05	36.72	99.40
Null	36	46.44	1.27	46.13	15.89	<.0001	0.05	28.57	75.51
Null	48	104.76	1.28	46.1	19.12	<.0001	0.05	64.21	170.95
SS	24	63.88	1.27	46.13	17.21	<.0001	0.05	39.29	103.86
SS	36	70.13	1.27	46.13	17.6	<.0001	0.05	43.13	114.03
SS	48	98.91	1.32	34.71	16.66	<.0001	0.05	56.50	173.16

Table 9-50. Least Square Mean and SE estimates for the total phenolic content of elicitor treatment * post application time combinations in Ike wheat

*Elicitor treatment * Post application time (HPA) Least Square Means*									
Elicitor	HPA	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
B1	24	72.01	1.41	46.13	12.52	<.0001	0.05	36.21	143.19
B1	36	49.62	1.41	46.13	11.43	<.0001	0.05	24.95	98.66
B1	48	42.09	1.41	46.13	10.95	<.0001	0.05	21.17	83.71
B2	24	48.32	1.41	46.13	11.35	<.0001	0.05	24.30	96.09
B2	36	56.85	1.41	46.13	11.83	<.0001	0.05	28.59	113.06
B2	48	50.69	1.41	46.13	11.49	<.0001	0.05	25.49	100.80
BTH	24	63.28	1.41	46.13	12.14	<.0001	0.05	31.82	125.83
BTH	36	38.65	1.41	46.13	10.7	<.0001	0.05	19.44	76.85
BTH	48	52.89	1.43	55.33	11.03	<.0001	0.05	25.73	108.76
DCPCA	24	49.96	1.41	46.13	11.45	<.0001	0.05	25.12	99.35
DCPCA	36	76.36	1.41	46.13	12.69	<.0001	0.05	38.40	151.84
DCPCA	48	140.63	1.41	46.13	14.48	<.0001	0.05	70.72	279.64
EtOH	24	57.47	1.41	46.13	11.86	<.0001	0.05	28.90	114.29
EtOH	36	30.24	1.41	46.07	9.84	<.0001	0.05	15.05	60.74
EtOH	48	41.78	1.41	46.13	10.93	<.0001	0.05	21.01	83.09
H2O	24	58.15	1.41	46.13	11.9	<.0001	0.05	29.24	115.64
H2O	36	130.28	1.41	46.13	14.26	<.0001	0.05	65.52	259.07
H2O	48	105.70	1.41	46.13	13.65	<.0001	0.05	53.15	210.19
MeJa	24	57.04	1.41	46.13	11.84	<.0001	0.05	28.68	113.42
MeJa	36	135.82	1.41	46.07	14.17	<.0001	0.05	67.61	272.82
MeJa	48	53.65	1.41	46.07	11.49	<.0001	0.05	26.71	107.76
Null	24	55.78	1.43	55.33	11.18	<.0001	0.05	27.13	114.70
Null	36	48.99	1.41	46.13	11.39	<.0001	0.05	24.64	97.42
Null	48	117.41	1.41	46.07	13.75	<.0001	0.05	58.45	235.85
SS	24	45.69	1.41	46.13	11.19	<.0001	0.05	22.97	90.85
SS	36	73.03	1.41	46.13	12.56	<.0001	0.05	36.73	145.24
SS	48	113.40	1.41	46.13	13.85	<.0001	0.05	57.02	225.50

Table 9-51. Least Square Means and SE estimates for total phenolic content of elicitor treatment * Post application time combinations in Karl 92 wheat

*Elicitor treatment * Post application time (HPA) Least Square Means*									
Elicitor	HPA	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
B1	24	44.58	1.43	55.33	10.56	<.0001	0.05	21.68	91.66
B1	36	127.78	1.41	46.13	14.2	<.0001	0.05	64.26	254.09
B1	48	48.25	1.43	55.33	10.78	<.0001	0.05	23.47	99.20
B2	24	73.05	1.41	46.13	12.56	<.0001	0.05	36.74	145.27
B2	36	45.01	1.41	46.13	11.15	<.0001	0.05	22.64	89.51
B2	48	53.17	1.54	29.54	9.18	<.0001	0.05	21.95	128.82
BTH	24	42.42	1.41	46.13	10.97	<.0001	0.05	21.33	84.35
BTH	36	150.34	1.41	46.13	14.68	<.0001	0.05	75.60	298.96
BTH	48	127.21	1.54	29.54	11.19	<.0001	0.05	52.50	308.15
DCPCA	24	92.59	1.54	29.54	10.46	<.0001	0.05	38.22	224.30
DCPCA	36	99.86	1.41	46.13	13.48	<.0001	0.05	50.21	198.58
DCPCA	48	187.20	1.41	46.13	15.32	<.0001	0.05	94.14	372.26
EtOH	24	53.12	1.41	46.13	11.63	<.0001	0.05	26.71	105.63
EtOH	36	53.03	1.41	46.07	11.46	<.0001	0.05	26.40	106.53
EtOH	48	52.13	1.52	25.92	9.42	<.0001	0.05	22.01	123.51
H2O	24	53.35	1.41	46.13	11.64	<.0001	0.05	26.83	106.09
H2O	36	83.81	1.41	46.13	12.97	<.0001	0.05	42.15	166.67
H2O	48	94.84	1.54	29.54	10.51	<.0001	0.05	39.15	229.75
MeJa	24	64.77	1.41	46.07	12.04	<.0001	0.05	32.25	130.11
MeJa	36	62.80	1.41	46.13	12.12	<.0001	0.05	31.58	124.89
MeJa	48	46.64	1.52	25.92	9.16	<.0001	0.05	19.69	110.50
Null	24	65.43	1.41	46.13	12.24	<.0001	0.05	32.90	130.11
Null	36	44.03	1.41	46.13	11.08	<.0001	0.05	22.14	87.56
Null	48	93.48	1.41	46.13	13.29	<.0001	0.05	47.01	185.90
SS	24	89.32	1.41	46.13	13.15	<.0001	0.05	44.92	177.61
SS	36	67.35	1.41	46.13	12.33	<.0001	0.05	33.87	133.93
SS	48	86.28	1.54	29.54	10.3	<.0001	0.05	35.62	209.03

Table 9-52. Proc MIXED model information for the effect of synthetic SAR elicitor on the total phenolic content of mature wheat grains

Model Information	
Data Set	WORK.ELICITOR
Dependent Variable	Phenolics
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Satterthwaite

Table 9-53. Sources of variation, covariance parameters, and levels of each source used in ANOVA

Class Level Information		
Class	Levels	Values
Block (Season)	3	Summer 2013, Spring 2014, Summer 2014
Cultivar	2	IKE K92
Elicitor	9	B1 B2 BTH H2O EtOH DCPCA MeJa Null SS

Table 9-54. Covariance parameters estimates on the effect of block and residuals on the fixed effects

Covariance Parameter Estimates							
Covariance Parameter	Estimate	Standard Error	Z Value	Pr > Z	Alpha	Lower	Upper
Block	0.001596	0.001676	0.95	0.1704	0.05	0.000415	0.08721
Residual	0.001280	0.000320	4.00	<.0001	0.05	0.000828	0.002239

Table 9-55. Type III tests of fixed effects for the effect of synthetic elicitors of SAR on total phenolic content in mature wheat grains

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Cultivar	1	32	20.46	<.0001
Elicitor	8	32	5.44	0.0002
Cultivar*Elicitor	8	32	2.04	0.0735

Table 9-56. Least Square Means and SE estimates for the total phenolic content in mature grains of wheat cultivars

Cultivar	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*	*Alpha*	*Lower*	*Upper*
IKE	0.4548	0.02413	2.17	18.85	0.0019	0.05	0.3585	0.5511
K92	0.4096	0.02413	2.17	16.97	0.0024	0.05	0.3133	0.5059

Table 9-57. Least Square Means and SE estimates for the total phenolic content in mature grains of elicitor-treated plants

Elicitor	*Estimate*	*Standard error*	*DF*	*t Value*	*Pr > |t|*	*Alpha*	*Lower*	*Upper*
B1	0.4334	0.0273	3.54	15.87	0.0002	0.05	0.3535	0.5133
B2	0.4225	0.0273	3.54	15.47	0.0002	0.05	0.3426	0.5024
BTH	0.4915	0.02941	4.69	16.71	<.0001	0.05	0.4144	0.5686
H2O	0.4015	0.0273	3.54	14.71	0.0003	0.05	0.3216	0.4814
EtOH	0.4274	0.0273	3.54	15.65	0.0002	0.05	0.3475	0.5072
DCPCA	0.4682	0.0273	3.54	17.15	0.0002	0.05	0.3884	0.5481
MeJa	0.4281	0.0273	3.54	15.68	0.0002	0.05	0.3482	0.508
Null	0.3663	0.0273	3.54	13.42	0.0004	0.05	0.2864	0.4462
SS	0.451	0.0273	3.54	16.52	0.0002	0.05	0.3711	0.5308

Table 9-58. Least Square Means and SE estimates for the total phenolic content in mature grains of cultivar*elicitor treatment combinations

Cultivar	*Elicitor*	*Estimate*	*Standard error*	*DF*	*t Value*	*Pr > |t|*	*Alpha*	*Lower*	*Upper*
IKE	B1	0.4579	0.03096	5.71	14.79	<.0001	0.05	0.3812	0.5346
IKE	B2	0.4832	0.03096	5.71	15.61	<.0001	0.05	0.4065	0.5599
IKE	BTH	0.5259	0.03442	8.33	15.28	<.0001	0.05	0.447	0.6047
IKE	H2O	0.4354	0.03096	5.71	14.06	<.0001	0.05	0.3587	0.5121
IKE	EtOH	0.4397	0.03096	5.71	14.2	<.0001	0.05	0.363	0.5164
IKE	DCPCA	0.4713	0.03096	5.71	15.22	<.0001	0.05	0.3946	0.548
IKE	MeJa	0.4157	0.03096	5.71	13.43	<.0001	0.05	0.339	0.4924
IKE	Null	0.3966	0.03096	5.71	12.81	<.0001	0.05	0.3198	0.4733
IKE	SS	0.4678	0.03096	5.71	15.11	<.0001	0.05	0.3911	0.5445
K92	B1	0.4089	0.03096	5.71	13.21	<.0001	0.05	0.3322	0.4856
K92	B2	0.3618	0.03096	5.71	11.68	<.0001	0.05	0.2851	0.4385
K92	BTH	0.4572	0.03442	8.33	13.28	<.0001	0.05	0.3783	0.536
K92	H2O	0.3677	0.03096	5.71	11.87	<.0001	0.05	0.291	0.4444
K92	EtOH	0.415	0.03096	5.71	13.4	<.0001	0.05	0.3383	0.4917
K92	DCPCA	0.4652	0.03096	5.71	15.02	<.0001	0.05	0.3885	0.5419
K92	MeJa	0.4404	0.03096	5.71	14.22	<.0001	0.05	0.3637	0.5172
K92	Null	0.336	0.03096	5.71	10.85	<.0001	0.05	0.2593	0.4127
K92	SS	0.4341	0.03096	5.71	14.02	<.0001	0.05	0.3574	0.5108

Table 9-59. Estimates of the differences between total phenolic content in mature grains of elicitor-treated plants and controls. Significant differences were determined at P<0.004167, Bonferroni adjusted

Elicitor	*Controls*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*	*Alpha*	*Lower*	*Upper*
B1	H2O	0.03187	0.02065	32	1.54	0.1327	0.05	-0.0102	0.07394
B1	Null	0.06712	0.02065	32	3.25	0.0027	0.05	0.02505	0.1092
B2	H2O	0.02099	0.02065	32	1.02	0.3173	0.05	-0.02109	0.06306
B2	Null	0.05623	0.02065	32	2.72	0.0104	0.05	0.01416	0.09831
BTH	H2O	0.08999	0.02337	32.1	3.85	0.0005	0.05	0.0424	0.1376
BTH	Null	0.1252	0.02337	32.1	5.36	<.0001	0.05	0.07765	0.1728
DCPCA	H2O	0.06673	0.02065	32	3.23	0.0029	0.05	0.02466	0.1088
DCPCA	Null	0.102	0.02065	32	4.94	<.0001	0.05	0.05991	0.1441
SS	H2O	0.04944	0.02065	32	2.39	0.0227	0.05	0.00737	0.09151
SS	Null	0.08469	0.02065	32	4.1	0.0003	0.05	0.04262	0.1268
MeJa	EtOH	0.00072	0.02065	32	0.04	0.9723	0.05	0.04135	0.0428
MeJa	Null	0.06182	0.02065	32	2.99	0.0053	0.05	0.01975	0.1039

Table 9-60. Estimates of the differences between total phenolic content in mature grains of elicitor treated and control Ike plants. Significant differences were determined at P<0.00208, Bonferroni adjustment

Elicitor	*Controls*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*	*Alpha*	*Lower*	*Upper*
B1	H2O	0.02252	0.02921	32	0.77	0.4464	0.05	-0.03698	0.08202
B1	Null	0.06133	0.02921	32	2.1	0.0437	0.05	0.001831	0.1208
B2	H2O	0.04785	0.02921	32	1.64	0.1112	0.05	-0.01165	0.1073
B2	Null	0.08666	0.02921	32	2.97	0.0057	0.05	0.02716	0.1462
BTH	H2O	0.09049	0.03285	32	2.75	0.0096	0.05	0.02358	0.1574
BTH	Null	0.1293	0.03285	32	3.94	0.0004	0.05	0.06239	0.1962
DCPCA	H2O	0.03593	0.02921	32	1.23	0.2276	0.05	0.02357	0.09543
DCPCA	Null	0.07475	0.02921	32	2.56	0.0154	0.05	0.01525	0.1342
SS	H2O	0.03246	0.02921	32	1.11	0.2747	0.05	0.02704	0.09196
SS	Null	0.07128	0.02921	32	2.44	0.0204	0.05	0.01178	0.1308
MeJa	EtOH	-0.0239	0.02921	32	0.82	0.4177	0.05	-0.08348	0.03552
MeJa	Null	0.01917	0.02921	32	0.66	0.5164	0.05	-0.07867	0.04033

Table 9-61. Estimates of the differences between total phenolic content in mature grains of elicitor-treated and control Karl 92 plants. Significant differences were determined at P<0.00208, Bonferroni adjustment

Elicitor	*Controls*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*	*Alpha*	*Lower*	*Upper*
B1	H2O	0.04123	0.02921	32	1.41	0.1678	0.05	-0.01827	0.1007
B1	Null	0.07291	0.02921	32	2.5	0.0179	0.05	0.01341	0.1324
B2	H2O	-0.00588	0.02921	32	-0.2	0.8418	0.05	-0.06538	0.05362
B2	Null	0.02581	0.02921	32	0.88	0.3836	0.05	-0.03369	0.08531
BTH	H2O	0.0895	0.03285	32	2.72	0.0104	0.05	0.02258	0.1564
BTH	Null	0.1212	0.03285	32	3.69	0.0008	0.05	0.05427	0.1881
DCPCA	H2O	0.09753	0.02921	32	3.34	0.0021	0.05	-0.03803	0.157
DCPCA	Null	0.1292	0.02921	32	4.42	0.0001	0.05	0.06972	0.1887
SS	H2O	0.06642	0.02921	32	2.27	0.0298	0.05	0.00692	0.1259
SS	Null	0.0981	0.02921	32	3.36	0.002	0.05	0.0386	0.1576
MeJa	EtOH	0.02543	0.02921	32	0.87	0.3905	0.05	-0.03407	0.08493
MeJa	Null	0.1045	0.02921	32	3.58	0.0011	0.05	0.04497	0.164