Genotype and Environment Variation in the Phenolic Antioxidant Properties of Hard Spring Wheat

by

Archie Mpofu

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

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ABSTRACT

Phenolic compounds, synthesized naturally in wheat and other plants, are potent antioxidants that protect plants and their consumers against the damaging effects of oxidative stress. This thesis research project was carried out to investigate the effects of genotype (G), growing environment (E) and G × E interactions on the total phenolic content, phenolic acid composition and antioxidant activity of wheat genotypes grown in the Canadian Prairie region. Sets of samples comprised of six hard spring wheat genotypes: Neepawa, AC Elsa, AC Barrie, Superb, AC Vista and AC Snowbird were investigated. One set was comprised of sound wheat harvested from Regina, Swift Current, Melfort and Winnipeg during the 2003 crop year. The other was comprised of *Fusarium* damaged, frost damaged and sound wheat respectively harvested from Carman, Regina and Swift Current during the 2004 crop year. G effects were significant ($p<0.0001$) for total phenolic content (TPC), antioxidant activity (AOA) (% DPPH scavenging capacity) and concentrations of ferulic acid (FA), o-coumaric acid (OCA), p-coumaric acid (PCA), syringic acid (SA), caffeic acid (CA), and vanillic acid (VA) in 2003 wheat harvest. Significant G effects were also detected in the superoxide scavenging capacities of water (SSCW) and lipid (SSCL) soluble substances, but not for any of the peroxyl radical scavenging properties. For 2004 samples, genotype effects were significant ($p<0.05$) for TPC and concentrations of VA, SA, FA, PCA and OCA. Interaction effects were significant for TPC, SSCW and SSCL among the parameters measured in 2003 wheat. In the 2004 wheat, interaction effects were significant for concentrations of VA, SA and FA. E effects were significant ($p<0.01$) for all the parameters measured in the 2003 and 2004 samples. The relative contribution of G, E and

G × E interaction to total variation largely indicated domination of E. Frost-damaged wheat had a significantly higher TPC, AOA (% DPPH scavenging capacity) and higher concentrations of gallic acid (GA), p-hydroxybenzoic acid (PHBA), VA, CA, SA, PCA, FA and OCA than *Fusarium*-damaged and sound wheat. Significant genotype effects indicate the possibility of selecting for given phenolic antioxidant traits in genotype development programs. Such processes would, however, likely be complicated and /or delayed by significant influences of E and G × E interaction.

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LIST OF ABBREVIATIONS

AAE	Ascorbic acid equivalents
AAPH	2,2'-azobis (2-amino-propane) dihydrochloride
ABTS	2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt
AOA	Antioxidant activity
AUC	Area under the curve
CA	Caffeic acid
CH	Canada Western Hard White Spring
CHD	Coronary heart disease
CPS	Canada Prairie Spring White
CVD	Cadiovascular disease
CWRS	Canada Western Red Spring
DPPH	2,2-diphenyl-1picrylhydrazyl
FA	Ferulic acid
FAE	Ferulic acid equivalents
FC	Folin-Ciocalteu reagent
FL	Fluorescein
FRAP	Ferric reducing/antioxidant power
GA	Gallic acid
HAT	Hydrogen atom transfer
H-ORAC$_{FL}$	Hydrophilic oxygen radical absorbance capacity with fluorescein (FL) as probe
HPLC	High performance liquid chromatography
L-ORAC$_{FL}$	Lipophilic oxygen radical absorbance capacity with fluorescein (FL) as probe
OCA	*ortho*-coumaric acid
ORAC	Oxygen radical absorbance capacity
PCA	*para*-coumaric acid
PCL	Photochemiluminescence
TE	Trolox equivalents
PHBA	*para*-hydroxybenzoic acid
RMCD	Randomly methylated beta-cyclodextrin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SA	Syringic acid
SSCL	Superoxide scavenging capacity of lipid soluble substances
SSCW	Superoxide scavenging capacity of water soluble substances
T-ORAC$_{FL}$	Total oxygen radical absorbance capacity with fluorescein (FL) as probe
TPC	Total phenolic content
VA	Vanillic acid

ORIGINAL PUBLICATIONS

Chapter 2 of this thesis was originally published in the journal of Agricultural Food Chemistry: Mpofu, A., Sapirstein, H.D., and Beta, T. 2006. Genotype and environmental variation in phenolic content, phenolic acid composition and antioxidant Activity of hard spring wheat. J. Agric. Food Chem. 54:1265-1270.

This paper was re-formatted for presentation in this thesis. The author of this thesis was responsible for the planning and experimental part of the work, analyzed the data and prepared the manuscript. Drs. T. Beta and H. D. Sapirstein participated in the interpretation of the results and editing of the manuscript.

INTRODUCTION

Natural antioxidants are synthesized in biological systems as part of multifunctional defense systems against the detrimental effects of oxidation. Defense systems against oxidation control reactive oxidation species, oxidation catalysts and oxidation products through metal chelation, free radical scavenging and peroxide inactivation among other mechanisms (Decker 1988). Natural antioxidants are important intrinsic food components or additives in enhancing the quality, stability and safety of food products. When consumed in human diets, these antioxidants may also terminate free radical chain reactions and therefore reduce cell injury and death, slow down the aging process and reduce the incidence of such diseases as cancer, atherosclerosis and Parkinson's disease (Halliwell 1996).

Wheat is an important cereal commodity worldwide, owing largely to the fact that it is the only cereal grain suitable for the manufacture of leavened bread as it has a unique viscoelastic gluten protein complex. In recent years, there has been increasing evidence that wheat may act as a suitable functional food or nutraceutical ingredient source as it contains several antioxidants, most notably phenolic compounds that likely have the greatest potential of being beneficial to health. Wheat breeding efforts have over the years enabled the development of high yielding varieties adapted to local environmental conditions (Worland and Snape 2001). Genetic control of grain components has largely been focused on endosperm composition as the endosperm is the most profitable wheat fraction. Endosperm storage proteins have by far been the most studied wheat components as they contribute to the unique ability of wheat flour to form a viscoelastic gluten dough. Dough strength for breadmaking can now be partially predicted since much

of the germplasm in the world's wheat breeding programs has been characterized for its composition of high molecular weight glutenin subunits (Worland and Snape 2001).

High levels of phenolic compounds have advantages in human health, however, a large knowledge gap still exists on their genetic control. The genotype (G) of wheat, environment (E) in which wheat is grown and possibly genotype-environment (G × E) interactions can likely strongly influence the levels of grain antioxidants. Significant G and/or E effects have been detected on antioxidant properties of wheat (Onyeneho and Hettiarachchy 2003, Zhou and Yu 2004, Zhou et al 2004a,b, Beta et al 2005). Literature is, however, deficient on the relative contributions of G, E and G × E interaction effects to wheat phenolic composition and antioxidant activity. This kind of knowledge would create a good foundation for breeding and eventual marketing of wheat high in phenolic antioxidants and therefore beneficial to the plant and competitive in the nutraceuticals and functional foods industry.

In this study, the effects of genotype, growing environment and genotype × environment interactions on the total phenolic content, phenolic acid composition and antioxidant activity of six wheat genotypes grown in the Canadian Prairie region during the 2003 and 2004 growing seasons were investigated. The objectives of the study were:

- To determine the extent to which genotype, growing environment and their interaction affect the total phenolic content, phenolic acid composition and antioxidant activity of wheat.
- To establish the effects of genotype, growing environment and G × E interaction on the superoxide scavenging properties of wheat.
- To evaluate the effects of genotype, growing environment and G × E interaction on peroxyl radical scavenging properties of wheat.

- To evaluate effects of *Fusarium* damage and frost damage on the phenolic content, phenolic acid composition and antioxidant activity of wheat.

Chapter 1: Literature Review

1.1. Introduction

Antioxidants are substances that when present in foods at very low concentrations compared to potentially oxidizable substrates significantly inhibit or delay oxidation of the substrate (Halliwell 1990). Antioxidants as part of food or supplements are increasingly perceived by consumers to be an important component of diet whose role is to lessen the risk of chronic diseases such as cancer and atherosclerosis.

Natural antioxidants are synthesized in crops as part of multifunctional defense systems against the detrimental effects of oxidation. Defense systems against oxidation control reactive oxidation species, oxidation catalysts and oxidation products through metal chelation, free radical scavenging and peroxide inactivation among other mechanisms (Decker 1988). Natural antioxidants are important food components or additives to enhance the quality, stability and safety of food products. When consumed in human diets, these antioxidants may also terminate free radical chain reactions and therefore reduce cell injury and death, slow down the aging process and reduce the incidence of such diseases as cancer, atherosclerosis and Parkinson's disease (Halliwell 1996).

Wheat contains several classes of antioxidants among which, phenolic compounds likely have the greatest potential of being beneficial to human health (Baublis et al 2000). Phenolic compounds inhibit oxidation by scavenging free radicals such as hydroxyl radicals ($HO^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$) resulting in the formation of low energy

phenolic radicals which do not promote oxidation at biologically significant rates (Decker 1998).

Phenolic compounds are a diverse group of secondary metabolites that includes simple phenolics, phenylpropanoids, benzoic acid derivatives, flavonoids, tannins, hydroxycinnamate esters and the structural polymer lignin. Defined chemically, phenolic compounds are substances (and their functional derivatives) that have an aromatic ring bearing one or more hydroxyl groups (Shahidi and Naczk 2004). Ferulic, *p*-coumaric, and vanillic acids are the most common simple phenolics in wheat and are found together with other phenolics including caffeic, chlorogenic, gentisic, syringic, and *p*-hydroxybenzoic acids (Onyeneho and Hettiarachchy 1992). Apart from phenolic acids, other phenolic compounds present in wheat include alkylresorcinols, flavanoids, ferulates, tannins, suberin and lignin (Shahidi and Naczk 2004). The concentration and composition of phenolic compounds, and hence associated antioxidant activity of wheat likely depends on the genotype and growing environment. This literature review provides an insight into the phenolic antioxidants present in wheat (with major focus on the phenolic acids), how these are synthesized, their distribution in the wheat grain, how their biosynthesis is influenced by genotype and growing environments and their physiological effects when consumed as part of the human diet.

1.2. Phenolic compounds

1.2.1. Biosynthesis

Phenolic compounds are synthesized naturally in plants as part of the antioxidant systems that control levels of reactive oxygen species and protect cells under oxidative

stress. They share a common origin in the phenylpropanoid biosynthetic pathway. Details of this pathway have been reviewed by Shahidi and Naczk (2004) as well as by Dixon and Paiva (1995) among others. In this pathway, phenolic synthesis begins with either phenylalanine or tyrosine, which undergoes deamination by their respective amino lyases to yield *trans*-cinnamic or *p*-coumaric acids respectively (Fig. 1.1.). Phenylpropanoids, so termed because they possess a phenyl ring (C6) and a C3 side chain, including caffeic, ferulic and sinapic acids may be formed through hydroxylation and possibly methylation of *p*-coumaric acid in positions 3 and 5. Benzoic acid derivatives may subsequently be formed from C6–C3 compounds via the loss of a two-carbon moiety. These include protocatechuic acid, vanillic acid, syringic acid and gallic acid. Phenylpropanoids and benzoic acid derivatives are collectively termed phenolic acids. Phenolic acids can accumulate in free form, as stable sugar and/or organic acid esters, or act as precursors to more complex phenolics such as flavonoids, tannins and lignin (Dixon and Paiva 1995).

Lignin is a cell wall component of all vascular plants. Lignification is a normal cell wall thickening process; however it can also be induced by UV-radiation, herbicide application, wounding stress, nutrient stress and cold temperatures (Chalker-Scott and Fuchigami 1989).

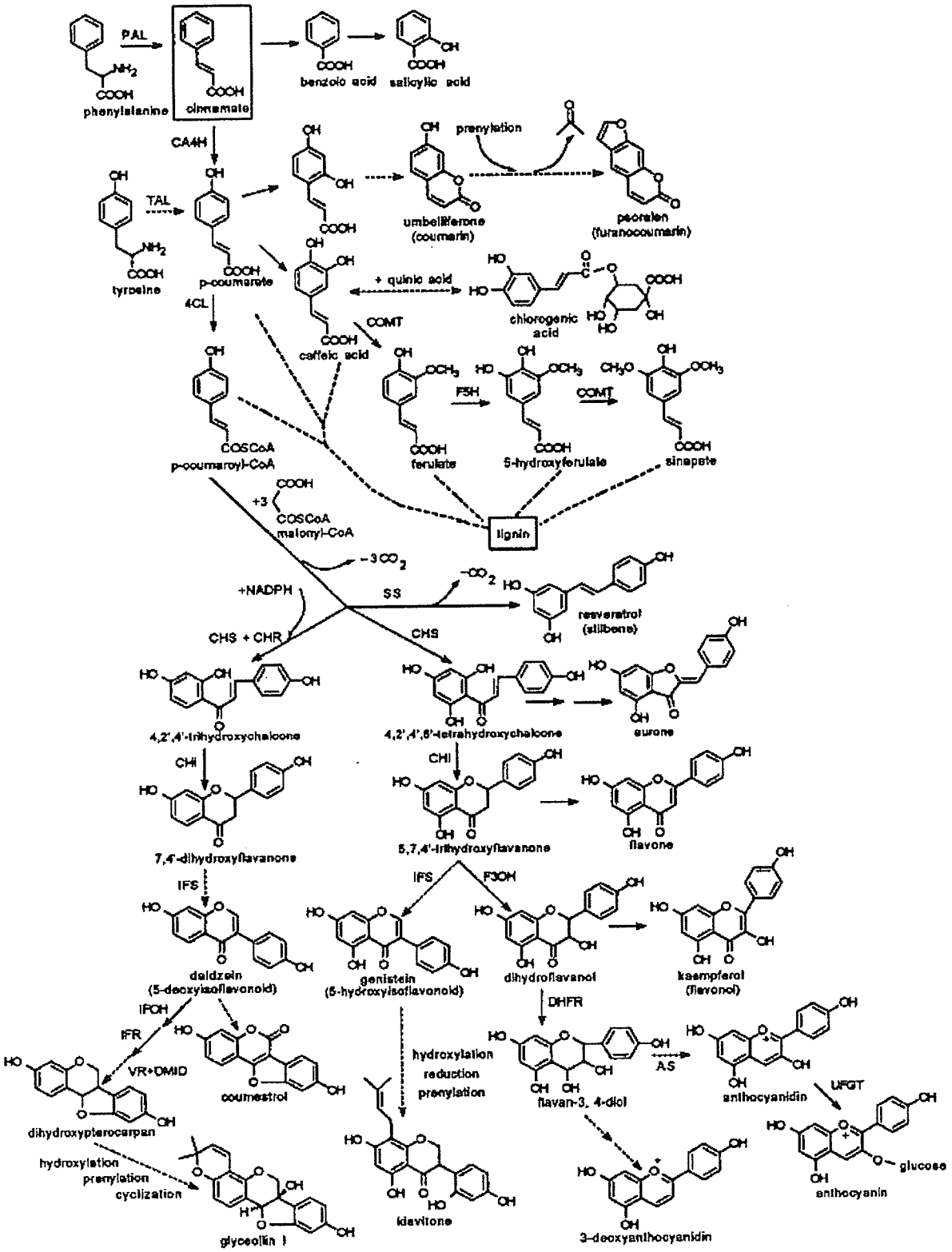

Figure 1.1. The phenylpropanoid biosynthetic pathway (Source: Dixon and Paiva 1995)

1.2.2. Antioxidant mechanisms

Structure-activity relationships have shown that the catechol group is the principal determinant of the hydrogen-donating (antioxidant) activity of phenolics (Rice-Evans et al 1996). Phenolics possess ideal structural chemistry for free radical scavenging activity, and they have been shown to be more effective antioxidants *in vitro* than tocopherols and ascorbate (Onyeneho and Hettiarachchy 1992). They are highly reactive as hydrogen or electron donors, and polyphenol-derived radicals are able to stabilize and delocalize the unpaired electron thereby breaking free radical chain reactions, and able to chelate transition metal ions and therefore terminate Fenton reaction which is implicated in generation of the highly reactive hydroxyl radicals ($HO^{\bullet}$) (Rice-Evans et al 1997). Takahama and Oniki (1997) showed that phenolic compounds are likely involved in the hydrogen peroxide scavenging cascade in plant cells. Flavonoids are additionally able to alter peroxidation kinetics by modification of the lipid packing order thereby decreasing fluidity of the membranes (Arora et al 2000). The changes induced could sterically hinder diffusion of free radicals and restrict peroxidative reactions.

The number of hydroxyl groups in phenolic acids and their esters is a key determinant of their antioxidant activity, which would be strengthened by steric hindrance (Dziedzic and Hudson 1983). Hydroxylated cinnamates are more effective antioxidants than benzoate counterparts because of the electron-withdrawing properties. The carboxylate group in benzoic acids has a negative influence on the H-donating abilities of the hydroxy benzoates (Dziedzic and Hudson 1983).

1.2.3. Health benefits

A number of human disease states, among them several forms of cancer, neurological disorders and stroke, inflammatory bowel disease, arthritis, toxic shock, and acute reperfusion injuries are characterized by cell death and tissue damage which are a result of oxidative stress (Castro et al 1994, Hausladen and Fridovich 1994, Szabó et al 1996). Oxidative stress occurs when there is an imbalance between the antioxidant defense system and formation of reactive oxygen species (ROS) in the human body (Halliwell et al 1992). The human body contains several endogenous antioxidant systems; however, dietary antioxidants such as vitamin C, vitamin E and phenolic compounds have an important role in maintaining the oxidative/antioxidative balance. The protective effects of phenolics against cardiovascular diseases and different forms of cancer are discussed in this section.

1.2.3.1. Protection against cardiovascular diseases (CVD)

Oxidation of low-density lipoproteins (LDL) initiates and promotes atherogenesis in several ways. Oxidized LDLs are atherogenic and are considered to be a crucial intermediate in the development of cardiovascular diseases (CVD) (Steinberg 1997). Plant phenolics show protective action against *in vitro* and most likely *in vivo* LDL oxidation (Nègre-Salvayre and Salvayre 1992, Wu et al 1996, Rice-Evans and Paganga 1996) indicating they can contribute to protection of consumers against CVD. Mechanisms proposed for the inhibition of LDL oxidation by phenolics, mainly flavanoids include the following:

- Scavenging of free radicals,

- Protection of α-tocopherol and carotenoids in the LDL particle from oxidation,
- Regeneration of vitamin E from oxidized α-tocopherol,
- Chelation of transition metal ions,
- Protection of cells against oxidative damage and as a result inhibition of cell mediated oxidation of LDL and
- Preservation of the activity of serum enzyme: paraoxonase (PON 1) and as a result hydrolysis of LDL associated lipid peroxides.

(Fuhrman and Aviram 2002)

The potentially protective role of phenolics in cardiovascular disease could also be due in part to their antithrombotic effects. Flavonoids have been shown to inhibit different aspects of blood platelet function *in vitro*, including platelet adhesion, aggregation and secretion (Beretz et al 1986).

Phenolic antioxidants, concentrated in the bran and germ components of whole grains are thought to be among the major components responsible for the protectiveness of whole grains against CHD (Slavin et al 1999). The protective role of phenolics against CVD is supported by epidemiological evidence. Several large prospective population studies have reported an inverse relationship between intake of whole grains and incidence of fatal and nonfatal coronary heart disease (CHD) (Jacobs et al 1998, Jacobs 1999, Liu et al 1999, Steffen at al 2003).

In four prospective cohort studies, flavonols from various food sources were found to have a protective effect against cardiovascular disease. These studies are the Zutphen Elderly Study, a cohort of 805 men in the Netherlands (Hertog et al 1993,

Hertog et al 1997), a cohort study of 5000 men and women in Finland (Knekt at al 1996), the Iowa women's health study, a cohort study of 34 500 women in the US (Yochum et al 1999) and the Erasmus Rotterdam Health for the Elderly (ERGO) study in the Netherlands, a cohort study of 8000 men and women (Geleijnse at al 1999).

1.2.3.2. Anticarcinogenic effects

The potential anticarcinogenic mechanism of phenolic compounds involves direct scavenging of the carcinogen and induction of detoxification systems, specifically the Phase II conjugation reactions (Wattenberg 1985). Caffeic acid and ferulic acid inhibit carcinogenesis by preventing the formation of carcinogens from precursor compounds and by blocking the reaction of carcinogens with critical cellular macromolecules (Wattenberg 1985).

Phenolics can also prevent cancer by stimulating one of the main types of programmed cell death; apoptosis, and blocking the replication of initiated cells that escape apoptosis (Chandra at al 2000). They likely have a role in inactivation or down-regulation of pro-oxidant enzymes and signal transduction enzymes, additional mechanisms that prevent tumor cell proliferation. Various phenolics have been found to be effective at inhibiting the pro-oxidant enzymes xanthine oxidase, cyclooxygenase (COX-2) and lipoxygenase (Chang et al 1993, Ding et al 1999, MacCarrone et al 1999, Michaluart et al 1999). Protein kinases are involved in the regulation of cell proliferation, differentiation and transformation. Inhibition of these kinases may reverse or suppress carcinogenic processes. Flavonoids have been reported to effectively inhibit protein tyrosine kinase (PTK) by binding to the ATP-binding site (Bames and Peterson 1995,

Huang et al 1999). The final stage of the cancer process involves growth of the tumor with destruction of surrounding tissues, increased formation of new blood vessels (angiogenesis) to supply nutrients to the dividing cancer cells, and spread to other tissues (metastasis). Plant phenolics may play a beneficial role in these later stages, as reported by Cao and Cao (1999) who found tea polyphenols to inhibit angiogenesis. Plant phenolics can additionally modulate steroid hormone concentrations in the body and may therefore play a role in the prevention of hormone-related cancers (Strom et al 1999).

Ferulic acid and p-coumaric acid were found to have the ability to protect against oxidative stress and genotoxicity in cultured mammalian cells (Ferguson et al 2005). Their activities were equal or superior to that of a known anticarcinogen, curcumin. Some epidemiological studies have reported the protective role of phenolics against lung cancer. A Finish, prospective cohort study carried out among 10 000 men and women found flavanoids to have a significant protective effect against lung cancer (Knekt et al 1997). A case control study (580 cases/580 controls) carried out in Hawaii found a tendency for a protective effect of flavanols against lung cancer, although the effect was not statistically significant (Le Marchand et al 2000).

1.3. Methods used for determining phenolic antioxidant properties

A vast array of *in vivo* and *in vitro* antioxidant determination methods exists. No simple universal method by which antioxidant capacity (AOC) can be measured accurately and quantitatively has yet been found (Prior et al 2005). *In vivo* methods generally involve measurement of the protection of DNA, lipid and protein from oxidative damage by reactive species (Prior and Cao 1999, King et al 1997). The most

commonly used *in vitro* methods for measuring AOC have been reviewed by Prior et al (2005). *In vitro* antioxidant determination methods can be broadly divided into three groups:

1. Methods that employ a Hydrogen Atom Transfer (HAT) mechanisms,
2. Single Electron Transfer (SET) based methods and
3. Methods that employ both HAT and SET mechanisms.

HAT based methods measure the ability of an antioxidant to quench free radicals by hydrogen donation whereas SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals (Wright et al 2001). SET and HAT mechanisms almost always occur together in all samples, with the balance determined by antioxidant structure and pH. HAT based assays include the oxygen radical absorbance capacity (ORAC) assay, total radical-trapping antioxidant parameter (TRAP), low-density lipoprotein (LDL) oxidation and the photochemiluminescence (PCL) assay. The ferric reducing antioxidant power (FRAP) assay and the copper reduction assay (CUPRAC, AOP-90) are examples of SET based antioxidant assays. Assays which utilize both HAT and SET mechanisms include the trolox equivalent antioxidant capacity (TEAC) assay and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Prior et al 2005).

Due to differences in antioxidant test systems, it is strongly recommended to use at least two methods in any given study for better elucidation of antioxidant properties (Schlesier et al 2002). DPPH radical scavenging capacity, ORAC and to a lesser extent the PCL assay have been used for determining antioxidant properties of grains including wheat in several studies. The Folin Ciocalteu method and high performance liquid

chromatography (HPLC) have been used extensively for the determination of the total phenolic content and phenolic acid composition, respectively. The Folin Ciocalteu (FC) method is primarily used as a measure of total phenolics in natural products, but its basic mechanism involves an oxidation/reduction reaction, as such, this method can be additionally considered as an AOC determination method (Prior et al 2005).

1.4. Wheat is an important cereal crop and source of phenolic antioxidants

Wheat is a major crop and component of the human diet across the world. It is the staple food in more than 40 countries for over 35% of the world's population (Bushuk 1998). Canada produces around 5% of the world's wheat, about 80% of this wheat is exported and accounts for about 20% of the wheat traded internationally (DePauw and Hunt 2001).The importance of wheat in the human diet stems from its ability to form a unique visco-elastic dough making it the only cereal suitable for breadmaking.

Significant antioxidant levels have been detected in wheat and wheat products (Onyeneho and Hettiarachchy 1992, Zielinski and Kozlowska 2000, Yu et al 2002a and b, Adom et al, 2003, Yu et al 2003, Yu et al 2004, Beta et al, 2005, Adom et al 2005). Knowledge of the relative contribution of genotype (G), the environment (E) in which wheat is grown and possibly genotype-environment (G × E) interactions to phenolic antioxidant properties is essential to explore the possibility of breeding wheat with elevated antioxidant levels. Breeding such wheat might likely improve the tolerance of the plant to environmental stress and expand the marketing opportunities of Canadian wheat to cover the lucrative and rapidly expanding nutraceuticals and functional food market.

1.4.1. Anatomy of the wheat kernel

The wheat caryopsis is comprised of three chemically and morphologically distinct fractions: the endosperm, germ and bran (Fig. 2). Arguably the most profitable fraction, the endosperm is the most abundant of the three accounting for 81 to 83% of the grain by weight. The endosperm is comprised of the starchy endosperm and the aleurone layer (Pomeranz, 1988). Bran constitutes 14.7 to 15.0% of the wheat grain and is comprised of at least six different tissue layers. The germ, comprised of the embryonic axis and scutellum is the smallest of the three fractions (2.4-3.6% of the caryopsis) (Pomeranz, 1988).

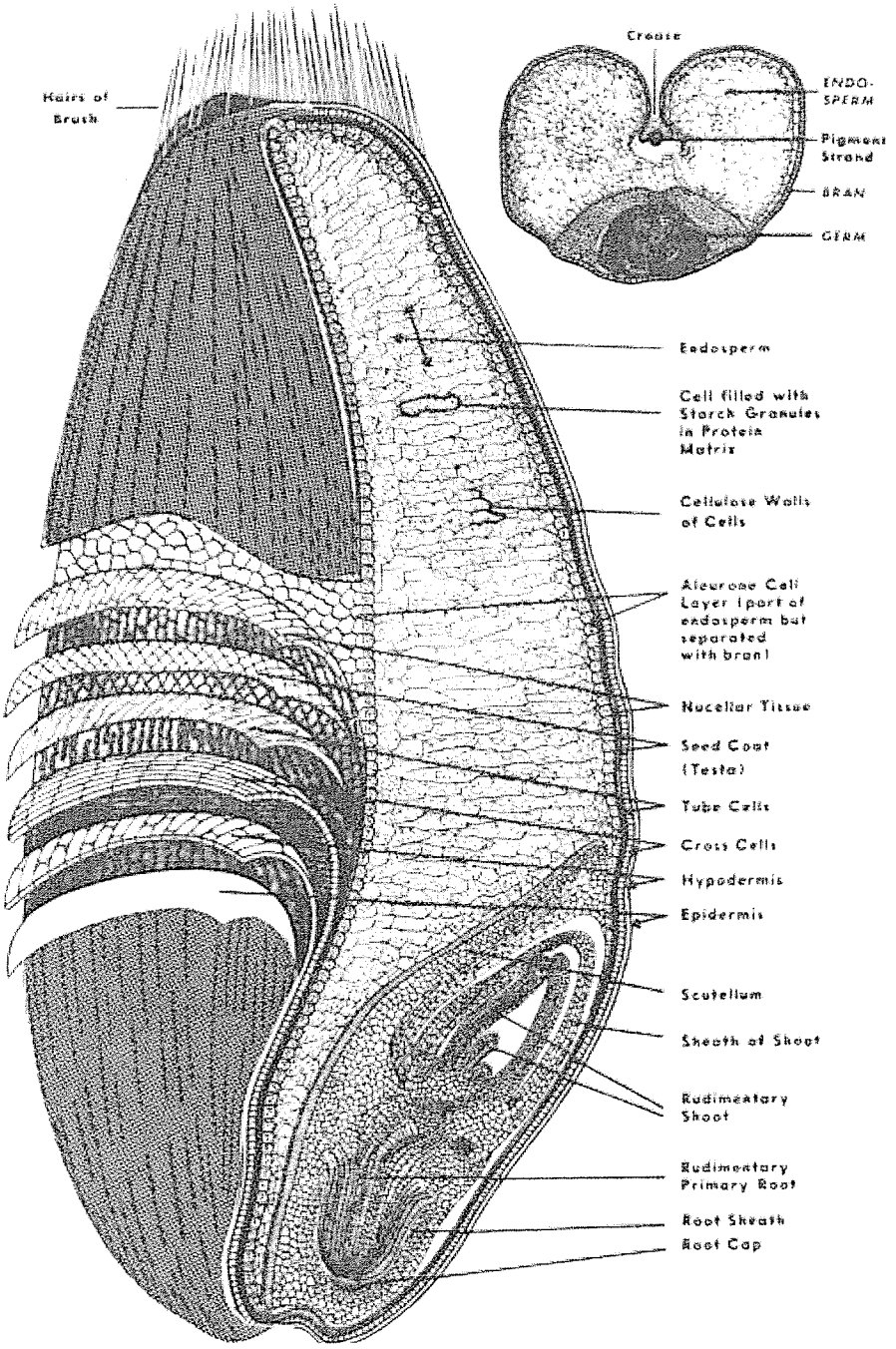

Figure 1.2. Longitudinal and cross sections of a wheat kernel (Source: Wheat Flour Institute, 1976, Washington, D.C.)

1.4.2. Nature and distribution of phenolic compounds in wheat

The phenolic compounds found in wheat include phenolic acids, alkylresorcinols, flavanoids, ferulates, tannins, suberin and lignin (Shahidi and Naczk 2004). Ferulic, *p*-coumaric, and vanillic acids are the most common phenolic acids in wheat, other phenolic acids present include caffeic, chlorogenic, gentisic, syringic, and *p*-hydroxybenzoic acids (Onyeneho and Hettiarachchy 1992). Among the phenolic acids present in wheat, ferulic acid is the most abundant (Onyeneho and Hettiarachchy 1992, Adom et al 2003, Zhou et al 2004a,b, Zhou and Yu 2004, Li et al 2005).

In one of the earliest studies on phenolic compounds, Fulcher et al (1972) using fluorescence microscopy and thin layer chromatography, observed that ferulic acid was most concentrated in the aleurone layer of wheat. Several studies have since reported that ferulic acid and other phenolic compounds are mainly concentrated in the aleurone cell walls and also in the seed coat and embryo of wheat, and are least concentrated in the starchy endosperm of the mature grain (Fulcher 1982, Pussayanawin and Wetzel 1987, Abdel-Aal et al 2001, Adom et al 2005, Beta et al 2005). During the production of flour, in the process of refining wheat, most of the bran and some of the germ are removed, resulting in loss of phenolic compounds together with dietary fiber, vitamins, minerals, lignans, phytoestrogens, and phytic acid (Slavin 1999). Compared to refined flour, whole wheat flour is therefore a much better source of phenolic compounds and other phytochemical components of wheat.

1.4.3. Genotype and environmental influences on the phenolic content and antioxidant activity of whole wheat and wheat fractions

Influences of genotype and the environment in which wheat is grown have been apparent in the study of wheat phenolic antioxidants (Abdel-Aal et al 2001, Onyeneho and Hettiarachchy 2003, Zhou and Yu 2004, Zhou et al 2004a,b, Beta et al 2005). The relative contribution of the G, E and G × E effects is, however largely undocumented. Results from studies which implied or suggested genotype and or growing environment influences on wheat antioxidant properties are summarized below.

Abdel-Aal et al (2001) reported ferulic acid concentration of between 535 and 783 μg/g in four wheat cultivars; Arin, Katepwa, AC Foremost and Roblin and three breeding lines, 94M-011, 94M-014, and 94M-025FA grown at four locations in Saskatchewan, Canada. The ferulic acid concentrations were similar for the four cultivars tested, but varied significantly among the locations demonstrating the likelihood of greater environment than genotype effects.

Antioxidant properties of Akron, a hard red winter wheat, and hard white winter wheat varieties Trego and Platte have been reported. These three wheat varieties, grown at a single dry land testing location in eastern Colorado differed in their capacities to quench or inhibit DPPH and $ABTS^{+\bullet}$ (Yu et al. 2002a). Akron showed the greatest DPPH radical quenching ability, Platte was second and Trego last. For the antioxidant capacity against $ABTS^{+\bullet}$, Trego had once again had the least activity, while Platte and Akron were in reverse order relative to the DPPH capacities. No significant inter-correlation was detected between TPC and radical scavenging capacities against DPPH and $ABTS^{+\bullet}$ (Yu et al. 2002a).

Investigation of the inhibitory effect of Akron, Trego and Platte on lipid peroxidation revealed Trego had the strongest inhibitory activity against lipid peroxidation in fish oils (Yu et al 2002b). Contrary to previous findings by the same researchers (Yu et al. 2002a), Akron and Trego had similar chelating activities while Platte had a lower activity (Yu et al 2002b) indicating likely mechanistic differences between lipid based antioxidant activity and the predominantly hydrophilic antioxidant capacities. In both studies (Yu et al 2002a,b) varietal differences were an evident indication of likely genotype influences on antioxidant properties.

Examination and comparison of the TPC and free radical scavenging properties of bran from Akron wheat grown at four non-irrigated and one irrigated testing locations in Colorado showed potential influences of growing conditions on antioxidant properties (Yu et al 2003). A significant negative correlation was detected between the chelating activities of the bran samples from the four non-irrigated locations and total solar or daily average solar radiation ($r = -0.999$ and $p = 0.001$) (Yu et al 2003). The bran from Trego wheat grown at the same testing locations had significantly different radical scavenging activities, chelating capacities, and total phenolic contents also suggesting that growing conditions may influence the antioxidant properties of wheat (Zhou and Yu 2004). Positive correlations were detected between the $DPPH^{\bullet}$ scavenging activity and either total solar radiation ($r = 0.97$, $p = 0.03$) or average daily solar radiation ($r = 0.97$, $p = 0.03$) (Zhou and Yu 2004). Unlike in the Akron wheat study (Yu et al 2003) chelating activities of the Trego bran samples were not significantly correlated with any recorded growing conditions including the hours exceeding 32°C, total solar radiation, or daily

average radiation showing possible genotype-based differential effects of environmental factors.

Gélinas et al (2006) reported that the total phenolic content of wheat samples from three varieties (AC Barrie, AC Brio, Celtic) harvested from several locations in Quebec, Canada varied more according to location than wheat variety. Mean values for varieties AC Barrie, AC Brio and Celtic were 631, 735 and 642 µg gallic acid equivalents/ g of whole meal respectively (Gélinas et al 2006), indicating an approximately 1.2 fold difference between AC Barrie and AC Brio. Averaged by growth location, the phenolic content data varied from 522 to 866 μg gallic acid equivalents per gram of whole meal (Gélinas et al 2006), a 1.6 fold difference and therefore a likely higher contribution of growing location than genotype to TPC variation.

Freeze dried aqueous extracts of bran reconstituted in absolute ethanol exhibited stronger antioxidant activity than similarly treated extracts of head shorts, tail shorts, low-quality flour, and low-grade flour (Onyeneho and Hettiarachchy 1992). These samples were obtained from durum spring wheat (*Triticum durum*) varieties, Lloyd, Monroe, Rugby, Stockholm, Reinville, and Vic purchased from a single farm (Onyeneho and Hettiarachchy 1992). The bran extracts had similar antioxidant activities in soy oil (PV 37.6-42.0 Mequiv/kg) indicating a likely lack of significant genotype effects. Protocatechuic, p-hydroxybenzoic, gentisic, caffeic, vanillic, chlorogenic, syringic, p-coumaric, and ferulic acid were present in the extract with ferulic, present in the highest amount (Onyeneho and Hettiarachchy 1992).

Zhou et al (2004b) examined bran samples of seven wheat varieties from four different countries and compared among other things their phenolic acid composition and

total phenolic content. They also measured antioxidant activities in terms of free radical scavenging properties against DPPH, ABTS, superoxide radical anion $O_2^{\bullet-}$, and oxygen radical and chelating capacities. Their results showed that the tested wheat bran samples differed in their phytochemical compositions and antioxidant properties. Ferulic acid, with a concentration range of 99-231 μg/g, was the predominant phenolic acid in all of the tested bran samples and accounted for about 46-67% of total phenolic acids on a weight basis. The ferulic acid concentration was, however, not correlated with any other identified phenolic acids or antioxidant properties in the wheat bran extracts. The TPC was correlated with the $O_2^{\bullet-}$ scavenging capacity of 50% acetone extracts ($r=0.83$, $p=0.02$) and the DPPH scavenging capacity of ethanol extracts ($r = 0.91$, $p = 0.01$). Although this work can not be considered a proper G × E study, given that the samples from each of the countries were different, the results seem to reflect possible genotype and growing location influences.

In pearled wheat (eight samples including six genotypes representing four commercial Canadian wheat classes with different intrinsic qualities), total phenolics were concentrated in fractions from the first and second pearlings (>4,000 mg ferulic acid equivalents (FAE) /kg) (Beta et al 2005). The phenolic content of wheat fractions from the third and fourth pearlings was also relatively high (>3,000 mg FAE /kg). Antioxidant activity measured in terms of DPPH radical scavenging capacity followed a similar trend, it ranged between 5 and 26% and was highly correlated with TPC ($r = 0.97$). Results from this study indicated a possibly strong growing location influence on TPC and AOA.

Adom et al (2003) determined the phytochemical profiles and total antioxidant activities of 11 wheat varieties and experimental lines. The results showed that total phenolic content (709.8-860.0 μmol of gallic acid equiv/100 g of wheat), total antioxidant activity (37.6-46.4 μmol of vitamin C/g), and total flavonoid content (105.8-141.8 μmol of catechin equiv/100 g of wheat) did not vary much among the 11 wheat lines. Significant differences were however more apparent in total ferulic acid content ($p < 0.05$ and total carotenoid content ($p < 0.05$) among the varieties.

The studies so far reviewed were mainly based on common red and white colored wheat. Significant antioxidant activities have also been reported in differently colored wheat (Abdel-Aal and Hucl 2003, Li et al 2005). Abdel-Aal and Hucl (2003) detected significant antioxidant levels in blue aleurone spring wheat line "Purendo 38" and commercial cultivars of purple (Konini) and red (Katepwa) wheat. The anthocyanin content of blue wheat was less influenced by growing environment than that of purple wheat. Li et al (2005) reported the free radical scavenging properties and phenolic content of extracts from Chinese black-grained wheat (BGW), Dongjian purple-grained wheat (DPGW), Wu blue-grained wheat (WBGW), and Dongjian white-grained wheat (DWGW). DPPH radical scavenging activities and phenolic contents differed significantly for bran as well as whole meal samples. DPPH radical scavenging activity and total phenolic content of bran were positively correlated (r=0.86). A higher degree of correlation was reported between DPPH radical scavenging activity and total phenolic content of whole meal (r=0.96). The following phenolic acids were detected in the wheat bran: gallic acid, *p*-hydroxybenzoic acid, caffeic acid, syringic acid, *p*-coumaric acid,

vanillic acid, gentisic acid, *o*-coumaric acid, and ferulic acid, with ferulic acid being the most predominant Li et al (2005).

1.4.4. Effects of processing on wheat phenolic antioxidant properties

Yu et al (2002c) reported on the free radical scavenging properties, chelating capacity, and total phenolic contents of Quaker Oat Bran™ ready-to-eat cold cereal, Quaker oatmeal, Mother's whole wheat hot natural cereal™, and Frosted mini- wheats™. The Mother's whole wheat hot natural cereal™ contained higher levels of total phenolic components than the Frosted mini-wheats™ cold cereal. Similarly, oatmeal had more TPC than the Quaker Oat Bran™ ready-to-eat cereal. TPC data suggested possible loss of phenolic components during food processing. DPPH scavenging data showed a similar tendency. This tendency was also observed in the $ABTS^{+\bullet}$ values for wheat cereals, but not for the oat based cereals. On the other hand, Fe^{2+} chelating capacity seemed to improve with processing for both wheat and oat based cereals (Yu et al 2002c).

Industrial processing of wheat products to elaborate tablets significantly decreased ($p < 0.05$) the phospholipid peroxidation inhibition percentages from those observed for the other wheat samples, to 51.8% for tablet of bran and 50.2% for tablet of bran with cellulose (Martinez-Tome 2004) . The decrease was attributed to a lower content of the basic ingredient (wheat bran) and some other additional ingredients that attenuated antioxidant activities. Tablets of bran are made with wheat bran powder, wheat germ, lactose, and calcium phosphate, the first two components having a very high antioxidant capacity, while a tablet of bran with cellulose contains wheat bran powder, malt flavoring, silicon dioxide, and cellulose (Martinez-Tome 2004).

Krings et al (2000) compared effects of roasting wheat germ at 140, 160, 180 and 200°C and found that increasing roasting temperatures improved the stability of stripped maize oil, indicating the possible involvement of Maillard-type antioxidants (Krings et al 2000). Krings and Berger (2001) reported similar results confirming the likely involvement of Maillard-type antioxidants.

Swiss red wheat grain, bran, aleurone, and micronized aleurone were examined and compared for their free radical scavenging properties against $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and peroxide radical anion $O_2^{\bullet-}$, oxygen radical absorbance capacity (ORAC), chelating capacity, total phenolic content (TPC), and phenolic acid composition (Zhou et al 2004a). Significant differences were found between the antioxidant properties, TPCs, and phenolic acid compositions of the Swiss wheat grain and its fractions. Micronized aleurone had the greatest antioxidant activities, TPC, and concentrations of all identified phenolic acids, suggesting the potential of post-harvest treatment on antioxidant activities and availability of TPC and phenolic acids.

Baking slightly increased the TPC in whole meal bread compared with refined flour bread regardless of baking time and whole meal bread was a much better source of phenolic compounds than white bread (Gélinas et al 2006).

1.4.5. Digestion and bioavailability of wheat antioxidants

Modification of phenolics in cereal grain products by the digestive process generally improves their bioavailability and antioxidant activity. Ferulic acid is released from wheat bran by microbial-dependent enzymic hydrolysis (Kroon et al 1997). More than 95% of the enzymic release of ferulic acid in humans occurs during fermentation in the colon. The proposed mechanism involves hydrolysis of arabinoxylans by xylanase to

produce feruloylated oligosaccharides that in turn are hydrolyzed by ferulic acid esterase to produce free ferulic acid. The antioxidant activities of these digestion products (feruloylated oligosaccharides and free ferulic acid) are higher than that of feruloylated polysaccharides. Plant lignans, another class of phenolic which possess antioxidant activity may also be influenced by digestion since they are converted to mammalian lignans such as enterolactone and enterodiol by bacterial fermentation in the colon. These mammalian lignans can then be absorbed into the blood (Thompson 1994). Bacterial fermentation in the colon also results in the hydrolysis of such phenolics as rutin and naringin from their sugar counterparts enabling their absorption into the blood (Manach et al 1995), further evidence that digestion improves bioavailability of phenolic antioxidants.

1.4.6. Comparison of wheat and wheat based foods to other foods as sources of dietary antioxidants

Miller et al (2000) compared the antioxidant activity of whole grain, ready-to-eat (RTE) breakfast cereals to that of fruits and vegetables. On average, the antioxidant activity of cereal products was equal to or exceeded that of most vegetables or fruits per serving. Whole grain breakfast cereals contained from 2200-3500 TE. In comparison, fruits generally ranged from 600-1700 TE, with a high of 2200 TE for red plums. Berries had exceptionally high AOA, 3700 TE on average, whereas vegetables averaged 450 TE with a high of 1400 TE for red cabbage (Miller et al 2000). Grain antioxidants are concentrated in the bran fraction; however, even the starchy fraction of grains was found to have relatively high antioxidant content (800 to 1000 TE) as compared to vegetables

and many fruits. Whole grain bread had an AOA of 2000 TE, compared to white bread at 1200 TE, indicating the contribution of bran and germ. Whole grain oat and wheat cereals were 2600 TE and 2900 TE, respectively, compared to 1300 TE for a cereal from white rice with the bran and germ removed. RTE corn cereal products, made from de-germed grain, were of intermediate value at 1900 TE (Miller et al 2000).

Products made with wheat bran (wheat bran alone, bran breakfast cereal, and wheat bran with malt flavor) exhibited a higher antioxidant activity than those made with oat bran (oat bran alone, oat breakfast cereal, and crunchy oat bran) (Martinez-Tome et al 2004). Roasted wheat germ and roasted press cake from wheat germ processing yielded the most efficient antioxidant extracts compared to other cereal products when added to stripped maize oil under accelerated-oxidation conditions (Krings and Berger 2001).

Zieliński and Kozlowska, (2000) compared the phenolic content of wheat (*Triticum aestivum L.*) cv. Almari and cv. Henika, barley (*Hordeum vulgare L.*) cv. Gregor and cv. Mobek, rye (*Secale cereale L.*) cv. Dańkowskie Zlote, oat (*Avena sativa L.*) cv. Slawko and buckwheat (*Fagopyrum esculentum* Moench) cv. Kora. They observed the following hierarchy of antioxidant activity (measured in terms of the capacity to scavenge the $ABTS^{\bullet+}$ in Trolox equivalents) for 80% methanolic extracts of the whole grain: buckwheat > barley > oat > wheat ≈ rye. This hierarchy was in agreement with total phenolic content (correlation coefficient r=0.96). Total phenolic content was reported in terms of μg of (±)-catechin/ mg, an uncommon measure of this parameter, which makes it impossible to compare the reported values to those from other studies. It is important to note that the observed hierarchy is likely limited to the cultivars

studied since significant cultivar and location effects on grain antioxidant properties have been reported in literature.

1.4.7. Response of wheat genotypes to oxidative stress through phenolic antioxidant biosynthesis

Oxidative injury at the cellular level as a result of water, temperature, pollution and other stresses is a major cause of crop damage (Allen 1995). Genotypes respond differentially to such stresses as a result of variations in their antioxidant systems (Pasitori and Trippi 1992, Turcsanyi et al 1994). Plant antioxidant systems include antioxidant enzymes, superoxide dismutase (SOD), catalase, peroxidases and glutathione peroxidase, detoxifying LP products (glutathione S-transferases, phospholipid-hydroperoxide glutathione peroxidase and ascorbate peroxidase), and a network of low molecular mass antioxidants (ascorbate, glutathione, phenolic compounds and tocopherols).

Studies on the response of wheat to frost related oxidative stress have largely focused on antioxidant enzymes (Scebba et al 1998, Kocsy et at 2001). Frost-resistant wheat genotypes, in comparison with less frost-resistant ones, maintain a better defense against ROS when exposed to low-temperatures (Scebba et al 1998). The effect of frost damage on the biosynthesis of phenolic compounds in wheat is largely deficient in literature although these compounds have been shown to be quite effective at combating other causes of oxidative stress.

Fusarium head blight (FHB) caused by *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) is a devastating fungal disease affecting

all classes of wheat and other small grains (McMullen et al. 1997). FHB reduces grain quality through destruction of starch and protein (Sutton 1982) and by accumulation of mycotoxins such as deoxynivalenol (DON), a vomitoxin, and the estrogenic zearalenone (Tuite et al 1990, Bai and Shaner 1994). McKeehen et al (1998) evaluated the phenolic acid profiles of six cultivars of wheat with known tolerance to *Fusarium* head blight during plant development from anthesis through maturity. The concentration of ferulic acid increased steadily during grain development prior to a 50% decrease during grain ripening. The accumulation of ferulic acid from anthesis until ~20 days after anthesis appeared to be related with cultivar resistance to *Fusarium*, however, at maturity concentrations of ferulic acid in the grain of resistant and susceptible cultivars were similar. The potency ferulic acid was confirmed through in-vitro testing where it demonstrated significant reductions ($p < 0.05$) of *Fusarium* species growth of up to 1.6 mg/mL (McKeehen et al 1998).

1.4.8. The possibility of breeding wheat to produce high phenolic antioxidant levels

Wheat has greatly diversified in terms of adaptation and end use since its emergence as a crop about 10 000 years ago (Worland and Snape 2000). Breeding programs in many wheat growing countries are primarily aimed at selecting new wheat cultivars for grain yield. End-use quality is an important secondary breeding objective in some countries, such as Australia and Canada, where a new cultivar must meet a prescribed minimum level of quality before it can be recommended for registration for commercial production (Bushuk 1998). The demand for phenolic antioxidant rich whole grains is likely to surge as the functional foods and nutraceuticals industry expands.

Wheat has been successfully bred for higher yield and bread-making end use quality. Some of the successes in wheat breeding are summarized here and the possibility of breeding wheat for high phenolic antioxidant levels explored.

Selection of genetically improved stocks capable of maximizing the benefits of agronomic practices by breeders has made possible worldwide increases in wheat yields. Adjustment of the life cycle to suit local seasonal climatic conditions has improved the adaptability of wheat (Worland and Snape 2000). Genes controlling ear emergence time have been identified and manipulated making it possible to select varieties with life cycles varying from seven weeks to a complete year. Several other genetic traits have been improved contributing to the increase in wheat yield. These include the control of plant stature, disease resistance, pest resistance, and stress tolerance (Worland and Snape 2000).

Wheat has been used for a variety of foods depending on cultural requirements since its early stages of development into a crop. Changes in end use were made possible through unconsciously changing the genetic characteristics of the grain constituents by exploiting natural variation of alleles at specific gene loci (Bushuk 1998). The same gene pool has been exploited to adapt the crop to compositional and processing and requirements (Bushuk 1998).

Wheat endosperm, by far the most abundant and most profitable gross anatomical component has received the most attention with respect to the genetic analysis of quality traits. Composition of the embryo and the grain coats, the pericarp and aleurone are complex and contain a range of constituents such as phenolic antioxidants whose

biochemistry is still partially unknown and genetic control is poorly understood (Worland and Snape 2000).

Eighty percent of the endosperm of wheat is comprised of starch and most of the remainder is protein. Protein quality and quantity are important factors in determining the end-use quality of wheat. Variation in grain storage proteins is thought to account for 50% of the variability between wheat varieties in breadmaking (Bushuk 1998). Other less studied grain constituents probably account for the remaining 50%. Genetic control of protein quality, protein quantity, sprouting resistance, starch synthesis, grain hardness and to lesser extent pentosans is now well understood (Bushuk 1998), making it possible to breed for these traits.

The genetic control of phenolic compounds is still unknown although high levels of these wheat components have been shown to be beneficial to human health. Selection and modification of particular genes could probably increase concentration of these compounds thereby improving the plant and human health beneficial effects of wheat.

Improvement of grain phenolic antioxidant properties in a wheat breeding program, just like selection for wheat color (Matus-Cadiz et al 2003) requires understanding the influences of genotype (G), environment (E), and their interaction (G × E). If both G and E effects are detected, knowledge of the magnitude of the G × E interaction becomes quite important. If G × E interactions exist, then genotypes selected as superior in one environment may not be superior in other environments. A significant G × E interaction may be either (i) a non-crossover G × E interaction where the ranking of genotypes remains constant across environments and the interaction is significant because of changes in the magnitude of the response, or (ii) a crossover G × E interaction where a

significant change in rank occurs from one environment to another. When selecting genotypes for wide adaptation, plant breeders look for a non-crossover G × E interaction or preferably the absence of a G × E interaction (Matus-Cadiz et al 2003).

Chapter 2: Genotype and Environmental Variation in Phenolic Content, Phenolic Acid Composition and Antioxidant Activity of Hard Spring Wheat

ABSTRACT

The health promoting effects of whole grain wheat likely derive from phenolic compounds and other antioxidants which also make wheat a potential source of functional food ingredients. The objective of this study was to determine the effects of genotype and growing environment on phenolic contents and antioxidant activities of alcohol-soluble extracts from commercial wheat cultivars. Total phenolic content (TPC), antioxidant activities (AOA) and concentrations of six phenolic acids were measured in six red and white-grained hard spring wheat genotypes grown at four diverse locations in Western Canada during the 2003 crop year. There were significant differences among genotypes and environments for TPC, AOA and concentrations of all the phenolic acids measured. The predominant indicators of antioxidant potential, i.e. TPC, AOA and ferulic acid (FA) concentration were highly inter-correlated ($r > 0.72$). For these indices, Canada Western (CW) Red Spring wheat cultivars Neepawa and AC Elsa had the highest levels, whereas an analogous CW hard white spring wheat cultivar, AC Snowbird, had the lowest levels. Grain color did not appear to be a factor in the expression of antioxidant-related parameters. For both TPC and AOA, as well as for vanillic acid, syringic acid, and ferulic acid, environment effects were considerably larger than genotype effects. Neither growing temperature nor rainfall from anthesis to maturity appeared to be related to the environmental variation that was observed. Genotype × environment interaction was small for all parameters compared with genotype and environment effects, and was

significant only for TPC. Genotype variation for antioxidant properties indicate that it would be possible to select for these quantitative traits in a breeding program. However, the significant environmental variation observed would delay and/or complicate this process.

2.1. Introduction

Regular consumption of whole grain foods has been found to be associated with reduced total mortality (Jacobs et al 1999, 2001), and reduced risk of CHD (Liu et al 1999), type 2 diabetes (Liu et al 2000a, Meyer et al 2000) and ischemic stroke (Liu et al 2000b). Health beneficial properties of whole wheat grains can be largely ascribed to the presence of phytochemicals in the diet that reduce oxidative stress thereby reducing the risk of chronic diseases. Wheat is a critically important commodity worldwide. It is grown on more land area than any other commercial crop and is the most important food grain source for humans. Quantification of health beneficial phytochemicals present in whole grain and its products is important for the breeding and marketing of wheat based on its potential to promote health in line with increasing consumer demands for healthier foods.

Wheat has significant levels of antioxidants (Onyeneho and Hettiarachchy 1992, Zielinski and Kozlowska 2000, Yu et al 2002a,b, Adom et al, 2003, Yu et al 2003, Yu et al 2004, Adom et al 2005, Beta et al 2005). Among the different antioxidants present in wheat, phenolic compounds seem to have the greatest potential of being beneficial to health (Baublis et al 2000). Phenolic compounds inhibit lipid peroxidation by scavenging free radicals such as hydroxyl radicals ($HO^{\bullet}$) and peroxyl radials ($ROO^{\bullet}$) resulting in the

formation of phenolic radicals whose energy is not sufficient to promote lipid oxidation at biologically significant rates (Decker 1998). Wheat phenolic compounds exist in free, bound and soluble conjugated forms (Onyeneho and Hettiarachchy 1992). Ferulic, p-coumaric, and vanillic acids are the most common free phenolics and are found together with other phenolics including caffeic, chlorogenic, gentisic, syringic, and p-hydroxybenzoic acids (Onyeneho and Hettiarachchy 1992).

The wheat genotype (G), the environment (E) in which wheat is grown and possibly genotype × environment (G × E) interactions can likely strongly influence the levels of grain antioxidants. The literature is, however, relatively deficient on this topic. Three hard winter wheat varieties (Akron, Trego and Platte) grown in a single field location differed significantly in their capacities to quench free radicals using scavengers such as 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-di [3-ethylbenzthiazoline-6-sulfonate] (ABTS) radicals (Yu et al 2002a). Growing location had a strong influence on the antioxidant activity of pearled wheat fractions of a leading Canadian bread wheat cultivar, Superb (Beta et al 2005). Yu et al (2003) reported significant effects of growing conditions including the number of hours exceeding 32°C on the antioxidant properties of Akron, a hard red winter wheat variety. Genotype and growing location had significant effects on the antioxidant activity and phenolic content of flours of Akron, Trego and Platte wheat grown at five locations in Colorado, the relative contributions of G and E to total variation were however not reported (Yu et al 2004). To our knowledge, there have been no reports on the relative contributions of genotype and the growing environment of wheat to its phenolic composition and antioxidant activity. This study was carried out to evaluate the effects of genotype, growing environment and genotype × environment

interactions on the total phenolic content, phenolic acid composition and antioxidant activity of six wheat genotypes grown in the Canadian Prairie region.

2.2. Materials and methods

2.2.1. Sample description

The wheat samples used in this study were all grown in the 2003 crop year and the harvested wheat was of sound milling condition. The samples comprised six western Canadian wheat genotypes representing three commercial classes with different technological qualities. Neepawa (Canada Western Red Spring (CWRS)), AC Elsa (CWRS), AC Barrie (CWRS), Superb (CWRS), AC Vista (Canada Prairie Spring White) and AC Snowbird (Canada Western Hard White Spring). The six genotypes were grown in triplicate according to a split-plot design, at each of four locations: Regina, Swift Current and Melfort (Saskatchewan) and Winnipeg (Manitoba). Accordingly, the total number of samples was 72. Wheat in each growing environment was planted in a randomized complete block design with replicates as blocks.

Table 2.1 summarizes the environmental conditions at the locations as well as some physical and chemical properties of the wheat (test weight and protein) that reflect typical differences in those growing locations. Test weight averaged across locations was lowest for wheat genotypes grown in Swift Current due to the low amounts of rainfall in this traditionally semi-arid growing location which attenuated normal grain filling, resulting in relatively high protein contents (Table 2.1). At the other extreme, protein content was lowest for genotypes grown in Winnipeg, mainly due to relatively high amounts of precipitation during the grain filling period. Samples were each ground to

pass through a 0.8-mm screen in a model 3100 falling number laboratory mill (Perten Instruments, Springfield, IL) prior to chemical analysis.

2.2.2. Determination of total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu method (Singleton and Rossi 1965) modified by Gao et al (2002) as described previously (Beta et al 2005). Briefly, ground wheat samples (200 mg) were extracted with acidified methanol (HCl/methanol/water, 1:80:10, v/v/v) (4 mL) at room temperature for 2 h. The extracts obtained were oxidized with Folin-Ciocalteu reagent, and the reaction was neutralized with sodium carbonate. The mixture was incubated at room temperature for 90 min and its absorbance was measured at 725 nm. Acidified methanol was used as the blank. Ferulic acid was used as the standard and results were expressed as ferulic acid equivalents per gram of wheat. All analyses were performed in duplicate.

Table 2.1. Environmental conditions at growing locations and outcomes of test weight and protein content for 2003 wheat samples

Location	Soil zone	Soil pH	*Temp (°C)	*Rainfall (mm)	Test weight (Kg/hL)	Protein (%)
Melfort	Transition black and grey	6.4	19.8	15.2	83.8	13.8
Swift Current	Brown	7.3	22.2	3.3	74.1	15.7
Regina	Transition black and brown	8.1	22.6	34.5	82.9	13.7
Winnipeg	Black	7.2	21.6	48.7	82.5	10.1

* Average per location from anthesis to maturity.

2.2.3. Determination of antioxidant activity

Antioxidant activity was measured using a modified version of the Brand-Williams et al (1995) DPPH radical scavenging method described by Beta et al (2005). Ground wheat samples were extracted in duplicate with 100% methanol. The extracts were mixed with DPPH solution (6×10^{-5} mol/ L of methanol). The absorbance (A) of the mixture at 515 nm was determined at 0 and 30 min. Methanol was used as a blank and antioxidant activity (AOA) calculated as percent discoloration

$$\text{percent discoloration} = (1 - [A \text{ of sample}_{t=30}/A \text{ of control}_{t=0}]) \times 100.$$

Each test was carried out in duplicate.

2.2.4. Determination of phenolic acid composition

2.2.4.1. Sample preparation

Wheat samples were hydrolyzed according to the method of Krygier et al (1982) with some modifications. Ground wheat samples (2 g) were hydrolyzed in duplicate using 4 M NaOH (60 mL) for 4 h under nitrogen. The pHs of the resulting mixtures were adjusted to between 1.5 and 2 using 6 M ice cold HCl and the pH-adjusted mixtures centrifuged at 13 000 rpm (Sorvall RC5C, Sorvall Instruments, DuPont, Wilmington, DE) for 15 min. Each supernatant was extracted 3 times with ethyl acetate (70 mL) and the organic phase was retained, dried by adding anhydrous Na_2SO_4 (1 g) and filtered through 0.45 μm membrane filters. The filtrates were evaporated to dryness at 35°C in a rotary vacuum evaporator (RE III Rotavapor, Büchi, Switzerland). The residues were re-dissolved in 50 % methanol (4 mL) and then filtered through a 0.45 μm nylon filter. The filtrates were stored in the dark at -20°C and subsequently analyzed by HPLC.

2.2.4.2. HPLC analysis

HPLC analysis was performed on a Waters model 2695 chromatograph instrument (Waters, Mississauga, ON, Canada) equipped with Waters 2996 photodiode array detector. Phenolic acids were separated on a reversed-phase Waters μBondapak C18 column (3.9 × 300 mm) with a gradient of solvent A (water containing 1% (v/v) HAc) and solvent B (100% methanol). The solvent gradient was programmed as follows: at 0 min 15% B, 10 min 20% B, 16 min 23% B, 24-28 min 27% B, 30-33 min 15% B in 33 min with a flow rate of 1.5 mL/min. Phenolic acids in the eluents were monitored at 280 nm. The following phenolic acids were quantified: ferulic acid (FA), o-coumaric acid (OCA), p-coumaric acid (PCA), syringic acid (SA), caffeic acid (CA), and vanillic acid (VA). Identification of the phenolic acids was accomplished by comparing the retention times of peaks in wheat samples to those of phenolic acid standards. The HPLC analyses were carried out in duplicate.

2.2.5. Statistical Analysis

The data were analyzed by analysis of variance (ANOVA). The ANOVA was performed with the general linear model (GLM) of the SAS software package (release 8.2) (SAS Institute, Cary, U.S.A.) using the split plot design and analyzing genotype and environment as fixed effects. Genotype (G), environment (E) and G × E effects were determined using replicates as blocks with measurements from duplicate analyses for each plot. Comparison of means was done at the 5% significance level using Duncan's multiple range test. Correlation analyses were performed with the PROC Corr procedure of the SAS software package using the Pearson correlation test.

2.3. Results

2.3.1. Genotype and environmental effects

There were highly significant differences ($p<0.0001$) among the six genotypes for total phenolic content (TPC), AOA and concentrations of all the phenolic acids assayed (Table 2.2). Differences among genotypes at each location were also highly significant for all these parameters. Averaged across growing environments, TPC was highest for AC Elsa and Neepawa (1990 and 1985 µg ferulic acid equivalents (FAE)/g, respectively), followed by AC Barrie, Superb and AC Vista. AC Snowbird had the lowest TPC of 1709 µg FAE /g. Differences in TPC were significant ($p<0.05$) for data combined across all locations and when analyzed on a by-location basis. AC Snowbird had the lowest TPC at every location except Winnipeg where although second lowest, it had a similar TPC to Superb, which had the lowest TPC. In Swift Current, AC Snowbird had the lowest TPC, its TPC was, however, not significantly different from that of AC Barrie, AC Vista and Superb. AC Elsa had the highest TPC in all locations but Regina where it was joint second highest with AC Barrie. Neepawa had a TPC similar to that of AC Elsa in Melfort and Swift Current. Neepawa had the highest TPC in Regina and was second highest with AC Barrie in Winnipeg.

Mean AOA among genotypes ranged between 13.21 and 14.22 % (Table 2.2). Similar to TPC results, AC Elsa had the highest AOA; its AOA was significantly different from those of all the other genotypes except Neepawa. The AOA of Neepawa was not significantly different from that of AC Barrie and AC Vista but significantly different from that of Superb and AC Snowbird which had the lowest AOA. AOA

differences were significant for data combined across all locations and when analyzed location by location.

There were significant differences in the concentration of each phenolic acid averaged across growing environments (Table 2.2), and at each location when results were evaluated by location. Moreover, the patterns of variation for phenolic acid concentrations were dissimilar among genotypes (Table 2.2). Ferulic acid was the predominant phenolic acid representing approximately 63% of the total content of individual phenolic acids averaged over genotypes and environments. The overall mean concentration of ferulic acid ranged across genotypes from 371 to 441 μg/g. Similar to results for TPC and AOA, the genotypes with the highest Ferulic acid concentrations were AC Elsa and Neepawa, with AC Snowbird and Superb having the lowest concentrations. In contrast, Superb had the highest concentration of o-coumaric acid (OCA) (229 μg/ g), which was the second most prominent phenolic acid that was measured. OCA comprised approximately 25% of the total content of individual phenolic acids averaged over genotypes and environments. The remaining phenolic acids VA, CA, SA and PCA comprised about 13% of the total phenolic acids that were quantified.

Table 2.2. Mean total phenolic content, antioxidant activity and phenolic acid concentrations of six wheat genotypes‡*

Genotype	Total phenolic content (µg/g)	AOA (% DPPH Discoloration)	Vanillic acid (µg/g)	Caffeic acid (µg/g)	Syringic acid (µg/g)	p-Coumaric acid (µg/g)	Ferulic acid (µg/g)	o-Coumaric acid (µg/g)
AC Barrie	1894b*±87	14.2b±0.8	9.7a±1.2	12.9a±2.8	13.3b±4.3	28.5d±3.6	417b±39	146c±37
AC Elsa	1990a±172	15.1a±1.3	9.7a±1.2	10.4b±1.9	16.1a±2.9	29.5d±3.5	441a±46	188b±50
Neepawa	1985a±82	14.6ab±0.9	9.4b±1.4	12.3a±2.4	13.2b±3.0	32.6c±3.8	437a±32	188b±31
AC Snowbird	1709d±170	13.2d±1.0	9.0b±1.1	7.6c±1.0	12.8bc±2.9	34.2b±3.1	371d±32	146c±32
Superb	1776c±87	13.5cd±0.7	8.2c±1.2	9.8b±2.3	11.6c±1.6	23.9e±2.0	379d±22	229a±35
AC Vista	1803c±109	14.0bc±1.0	7.8c±1.3	7.7c±0.7	12.8bc±2.5	37.2a±3.4	404c±26	166c±18

‡ Values expressed as mean ± standard deviation.

* Means in same column followed by different letters are significantly different ($p<0.05$).

There were significant differences among growing environments for TPC, AOA and concentrations of all the phenolic acids (Table 2.3). Winnipeg had the highest TPC, AOA, FA, PCA and SA concentration. Swift Current had the highest VA and CA concentrations, and second highest TPC, AOA, FA and SA concentrations. With one exception, there was no significant relationship between phenolic acid concentrations, TPC or AOA and either total rainfall or average temperature during the kernel development period (refer to Table 2.1). OCA concentration per location was negatively correlated ($r = - 0.970$, $p<0.05$) to average temperature during grain filling. However, given the limited number of site year results available to this study, this relationship must be interpreted with caution

Table 2.3. Mean total phenolic content, antioxidant activity and phenolic acid concentrations of wheat grown at four locations‡*

Growing location	Total phenolics (µg/g)	AOA (% DPPH Discoloration)	Vanillic acid (µg/g)	Caffeic acid (µg/g)	Syringic acid (µg/g)	p-Coumaric acid (µg/g)	Ferulic acid (µg/g)	o-Coumaric acid (µg/g)
Melfort	1794c±103	13.6c±0.9	7.8d±1.0	9.7b±2.1	10.2d±1.5	31.4b±5.4	384c±26	210a±40
Regina	1746d±157	13.6c±1.1	9.5b±1.1	9.0b±2.4	12.8c±1.7	29.9c±4.8	391c±32	156c±38
Swift Current	1889b±119	14.2b±1.0	10.3a±0.8	11.9a±3.4	14.0b±1.7	28.2d±3.8	409b±32	161c±39
Winnipeg	2009a±120	15.0a±1.0	8.3c±1.1	9.8b±2.1	16.1a±3.8	34.5a±4.9	450a±43	181b±44

‡ Values expressed as mean ± standard deviation.

* Means in same column followed by different letters are significantly different ($p<0.05$).

2.3.2. Inter-correlations among total phenolics, antioxidant activity and phenolic acids

There were highly significant ($p<0.01$) and strong correlations between TPC and AOA ($r = 0.73$), TPC and concentration of FA ($r = 0.84$), as well as between AOA and FA ($r = 0.72$) (Table 2.4). Other positive and significant correlations, but of a lesser magnitude were found between FA and SA ($r=0.61$), AOA and SA ($r=0.47$), TPC and CA ($r=0.45$), VA and SA ($r=0.41$), SA and VA ($r=0.41$), FA and CA ($r=0.40$), and between FA and PCA ($r=0.30$). There were significant, negative correlations ($p<0.05$) between VA and OCA ($r=-0.40$), CA and PCA ($r=-0.38$), VA and PCA ($r=-0.32$), and between OCA and PCA ($r=-0.27$).

Table 2.4. Correlation coefficients between total phenolic content, AOA and phenolic acid concentrations (n=72)

	Total phenolic content	AOA	Vanillic acid (VA)	Caffeic acid (CA)	Syringic acid (SA)	p-Coumaric acid (PCA)	Ferulic acid (FA)
AOA	0.729**						
Vanillic acid (VA)	0.213	0.143					
Caffeic acid (CA)	0.449**	0.205	0.510**				
Syringic acid (SA)	0.581*	0.474**	0.406**	0.151			
p-Coumaric acid (PCA)	0.101	0.166	-0.323*	-0.38*	0.152		
Ferulic acid (FA)	0.842**	0.718**	0.222	0.396*	0.609**	0.299*	
o-Coumaric acid (OCA)	0.107	0.064	-0.397*	0.013	-0.172	-0.265*	0.07

* Significant ($p<0.05$).

** Highly significant ($p<0.01$).

2.3.3. Relative influence of genotype, environment and G × E interactions

The magnitude of the variance components of genotype and environment and G × E interactions indicates their relative importance for specific antioxidant activities and related parameters. These results are summarized in Table 2.5 which compares the proportions of mean squares to the total mean square of each variance component. Similar to mean difference results for the measured parameters (Tables 2.2 and 2.3), variance due to genotype and environment was significant for TPC, AOA and all the phenolic acids. Depending on the measured parameters the combination of genotype and environment variance explained from 87% (AOA) to 96% (TPC) of the total variance. For both TPC and AOA, as well as for FA, VA and SA, environmental effects were considerably larger than genotype effects, by as much as 52% and 53% for TPC and FA, respectively, and by 37% for AOA. This result may be partly due to relatively close genetic relationships among some of the genotypes used in this study, which would limit genetic variability accordingly. Interestingly for two other phenolic acids, viz. CA and PCA, relative genotype variance (61 and 63%, respectively) was substantially greater than environmental variance (29 and 31%, respectively). Genetic and environment variance related to the phenolic acid OCA were comparable (47 and 41%, respectively).

Despite the large influence of both genotype and environment to essentially all response factors, variance attributable to G × E interactions was small (< 4% of total variance) and significant ($p < 0.0001$) only for TPC (Table 2.5). Genotypes by environment interactions become significant depending on the extent of crossover interactions (significant change of ranking of cultivars over environments) and/or non-

crossover interactions (significant change in magnitude of genotype variation across growing environments when rankings are unaffected).

The small level of G × E interaction is partly reflected in the similar patterns of genotype-by-location means and location-by-genotype means for TPC (Table 2.6.). The G × E interaction appears to partly stem from a significant change in ranking, relative to other cultivar TPC levels, of cultivar Neepawa (increased in Regina) and cultivar Elsa (increased in Winnipeg). Comparing all genotypes together, the greatest effect of G × E interactions for TPC was found jointly for AC Snowbird and Elsa which had coefficients of variation (average CV =10.1%) across growing locations more than double that of the average of the other four cultivars (CV = 4.4%). The TPC of cultivar Neepawa was least affected by environmental effects across growing locations (CV = 3.6%) indicating the relative stability of this genotype for TPC.

Table 2.5. Variance components (% of total mean squares) for genotype (G), growing environment (E), and G × E interaction effects for total phenolic content, antioxidant activity, and phenolic acid composition of six wheat genotypes grown at four locations

Variance component	G†	E‡	G × E†	G X Block/E	Block/E
Degrees of freedom	5	3	15	40	8
Parameter					
Total Phenolics (µg Ferulic acid equivalents /g)	38.12***	57.86***	3.34***	0.38	0.29
Antioxidant activity (% DPPH discoloration)	36.99***	50.61***	6.71	4.00	1.68
Vanillic Acid (µg/g)	24.42***	71.09***	0.79	1.43	2.28
Caffeic acid	60.89***	29.29***	1.93	2.03	5.87
Syringic acid (µg/g)	17.34***	70.91***	3.63	1.66	6.45
p-Coumaric acid (µg/g)	63.54***	30.61***	1.17	0.70	3.98
Ferulic acid (µg/g)	36.86***	56.57***	2.34	0.95	3.29
o-Coumaric acid (µg/g)	46.74***	41.44***	2.76	2.45	6.61

*** Significant ($p<0.0001$).

† Significance is based on genotype × block/E mean square.

‡ Significance is based on block/E mean square.

Table 2.6. Total phenolic content means and coefficients of variation (CV) of six wheat genotypes grown at each of four locations

Genotype	Location				CV
	Melfort	Regina	Swift Current	Winnipeg	(%)
AC Barrie	1862a	1837b	1844b	2030b	4.84
AC Elsa	1902a	1793b	2039a	2227a	9.42
Neepawa	1884a	1987a	2032a	2037b	3.57
AC Snowbird	1630c	1505d	1765b	1936cd	10.81
Superb	1736b	1677c	1821b	1873d	4.91
AC Vista	1747b	1680c	1830b	1956c	6.59

* Means in the same column followed by different letters are significantly different ($p<0.05$).

2.4. Discussion

The preeminence of wheat as a food crop is mainly due to the presence of a unique viscoelastic gluten protein complex that makes it the only cereal grain suitable for the manufacture of leavened bread. Significant antioxidant levels have been detected in wheat indicating its importance in a healthy diet to reduce the risk of many chronic diseases, as well as its potential as a source of functional food ingredients. Several authors have concluded that the antioxidant properties of wheat or wheat products are significantly influenced by genotype and/or the environment in which wheat is grown without quantifying the relative contributions of these factors (Onyeneho and Hettiarachchy 2003, Zhou and Yu 2004, Zhou et al 2004a,b, Beta et al 2005).

This study is the first to document the relative contribution of genotype and environmental conditions to the antioxidant properties of wheat genotypes grown in different locations. It is also the first to document predominant indicators of antioxidant activity of western Canadian wheat cultivars which are important in international wheat commerce.

There were highly significant genotype differences in TPC, AOA and concentration of all phenolic acids. The CWRS wheat cultivars AC Elsa and Neepawa had the highest TPC, AOA and FA concentrations compared to other genotypes. CWHWS wheat AC Snowbird had the lowest TPC, AOA and FA concentration. Its AOA and FA concentration was, however similar to that of Superb, a CWRS wheat. Canada Prairie Spring White wheat, AC Vista had mid-range AOA, FA concentration and TPC. Accordingly, no link was identified between wheat color (red vs. white) and antioxidant properties. A similar observation was made by Beta et al (2005).

Compared to genotype effects, the effects of the growing environment on TPC, AOA and all measured simple phenolic acids was even larger. However, the nature of the location effects was not apparent in this study. There was no clear link between levels of any of the predominant indicators of antioxidants (TPC, AOA and FA concentration) and environmental effects such as rainfall or temperature which otherwise had very significant effects on grain properties such as test weight and protein content. For example, the Swift Current and Winnipeg locations were the most diverse locations in this study based on weather patterns related to rainfall (Table 2.1.). The impact of a very dry grain development period in Swift Current and timeliness of ample precipitation in Winnipeg, resulted in crops with extreme differences in protein content for western Canadian hard spring wheat, and very large differences in bulk density or test weight; wheat of all genotypes harvested from the Winnipeg location were of high test weight (82.5 Kg/hL) and low protein content (10.1%) compared to genotypes grown in Swift Current (test weight 74.1 Kg/hL, protein content 15.7%). Moreover, the high protein wheat grown in Swift Current had technological properties for breadmaking that were very different from those obtained from the low protein wheat grown in Winnipeg (results not shown). Despite these very large differences in grain properties, the Swift Current and Winnipeg locations generated wheat with mean total phenolic contents, antioxidant activities, and phenolic acid concentrations that were more similar to each other than to wheat grown in either Melfort or Regina, Saskatchewan (Table 2.3.). It is known that plant phenolic compounds can be influenced by defense reactions related to pathogen attack (Nicholson and Hammerschmidt 1992). For example, FA which is the major phenolic acid in wheat has been found to be a significant variable correlated with

resistance to wheat midge (Abdel-Aal et al 2001) and *Fusarium* head blight (FHB) (McKeehen et al 1999). However, no significant effects of midge, FHB or damage by any other pest or pathogen were evident in the samples that were studied.

Yu et al (2003) reported significant effects of growing conditions including the number of hours exceeding 32°C on the antioxidant properties of Akron, a hard red winter wheat variety. Average temperature at growing locations was shown to have effects on the antioxidant properties of strawberries (Wang and Zhang 2001). Emmons and Peterson (2001) found significant cultivar effects for AOA and significant cultivar, location and cultivar × location interaction effects for the concentration of total free phenolic contents in oats. The cause of location effects was not identified in that study.

Phenolic compounds have potent antioxidant activity (Decker 1998). Total phenolic content ranged between 1709 and 1990 μg FA equivalents/g. The values were twice as high compared to levels reported by Li et al (2005) for four Chinese wheat samples, an indication of genotypic and/or environmental effects. Beta et al (2005) found TPC values of between 1300 and 5300 μg FA equivalents/ g in different mill fractions of wheat with the highest concentrations (>4000 μg FA equivalents/ g) in fractions from first and second pearlings. The larger TPC range reported by Beta and others (2005) reflects the increase in phenolic content of wheat from the inner to the outer parts of the grain. Whole wheat samples were used in the current study. Hence lower and less variable TPC values were observed.

Several phenolic compounds are present in wheat. These include phenolic acids, alkylresorcinols, flavanoids, and phenolic acid diacyl glycerols, phenolic aldehydes and ferulates (Shahidi and Naczk 2004). Phenolic acids present in wheat include ferulic, p-

coumaric, vanillic, caffeic, chlorogenic, gentisic, syringic, and *p*-hydroxybenzoic acids (Onyeneho and Hettiarachchy 1992). Ferulic acid was the predominant phenolic acid accounting for between 49.7 and 64.8 % of the total amount of measured phenolic acids in the wheat samples. This observation is in agreement with the findings of Onyeneho and Hettiarachchy (1992), Adom et al (2003), Zhou et al (2004a,b), Zhou and Yu (2004). Significant levels of syringic, vanillic, caffeic, p-coumaric and o-coumaric acids were also detected in line with observations by Li et al 2005. Levels of these phenolic acids were lower in our study, likely due to varietal differences.

Antioxidant activity (AOA) was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Brand Williams et al 1995). This assay is based on the measurement of the reducing ability of antioxidants toward DPPH. AOA was calculated as percentage discoloration and ranged between 13.21 and 14.22 %, with higher percentages indicating higher AOAs. Using a similar extraction protocol and AOA method, AOAs of between 2.5 and 26 % were obtained (Beta et al 2005). The differences in the magnitude of ranges are likely linked to the increase in concentration of phenolics in wheat from the inner to the outer parts of the grain. Wheat antioxidants are concentrated in the bran (Zhou et al 2004ab, Adom et al 2005, Beta et al 2005). Significant antioxidant activity has, however, also been detected in the endosperm (Martinez-Tome et al 2004) and in wheat germ (Krings et al. 2001).

2.5. Conclusion

Both the genotype and growing environment manifest significant differences in total phenolic content, antioxidant activity and phenolic acid composition of wheat.

Genotype effects were highly significant ($p<0.0001$). CWRS wheat genotypes AC Elsa and Neepawa had significantly higher antioxidant properties than the other genotypes. Environment effects were also highly significant ($p<0.0001$) and wheat grown in Winnipeg had the highest predominant indicators of antioxidant potential; TPC, AOA and FA concentration. Environment effects were greater for TPC, AOA and FA concentration than genotype effects. Interaction effects were much smaller than the genotype or environment effects, and were only significant for TPC. Genotype differences for TPC, AOA, VA, SA, FA CA, PCA and OCA indicate that it would be possible to select for these quantitative traits in a genotype development program. The significant effects of environment must, however, be considered. More research is needed to investigate the cause of environment effects, extend the study to several years and determine the heritability of the antioxidant properties.

Chapter 3: Superoxide Scavenging Capacities of Water and Lipid Soluble Substances as Measures of Antioxidant Activity in Wheat Genotypes Grown in Different Environments

ABSTRACT

Antioxidants present in wheat and other foods help quench reactive oxygen species such as the superoxide anion free radical and hence may be of great importance in the prevention of many chronic diseases. The present study was carried out to determine the influence of genotype and growing environment on the superoxide scavenging capacity of water soluble substances (SSCW) and lipid soluble substances (SSCL) of wheat. Methanolic extracts of six wheat genotypes, each grown in four locations in Western Canada, were assayed using a photochemiluminescence (PCL) assay. New knowledge has been generated on relative contributions of G, E and G × E interaction influences to the variation of superoxide scavenging capacities of water and lipid soluble substances in wheat. Wheat genotype (G) and growing environment (E) as well as G × E interactions had highly significant effects ($p < 0.0001$) on both the SSCW and SSCL. SSCW for the wheat genotypes ranged from 307 ± 38 μg ascorbic acid equivalents (AAE) /g in AC Snowbird to 497 ± 86 μg AAE/g in AC Barrie. SSCL per genotype ranged from 332 ± 49 μg trolox equivalents (TE) /g in AC Elsa to 412 ± 43 μg TE/g in Neepawa. Values per location for SSCW and SSCL ranged from 325 ± 71 to 438 ± 105 μg AAE/g and 330 ± 45 to 399 ± 38 μg TE/g, respectively. Wheat grown in Regina had the lowest SSCW and SSCL, while wheat grown in Winnipeg had the highest SSCW and SSCL. The pattern of variation of SSCW was different from that of SSCL indicating

significant differences in the response of SSCW and SSCL to genetic and environmental factors. The contribution of genotype variance to total variance in SSCW (53%) was greater than that of environment (36%). In contrast for SSCL, the contribution to total variance by genotype (25%) was less than that by growing environment (54%) likely indicating differences in the synthesis of the responsible compounds. The ability of wheat to scavenge the superoxide anion and therefore mitigate chronic disease conditions clearly varies with both genotype and growing environment. Improvement of the superoxide scavenging capacity of wheat in a breeding program will require identification of antioxidant compounds with high scavenging capacities and genotypes whose synthesis of these compounds is stable under diverse environmental conditions. In this study SSCW was more influenced by genotype than environment, indicating the likely relative ease of selecting for this parameter in a breeding program. G × E interaction was, however, highly significant and would hinder the selection program.

3.1. Introduction

An imbalance between the antioxidant defense system and formation of reactive oxygen species (ROS) in the human body can result in severe oxidative stress, which may damage life-important membrane lipids, proteins, carbohydrates and DNA (Halliwell et al 1992). Wheat has significant levels of antioxidants (Onyeneho and Hettiarachchy 1992, Yu et al 2002a,b,c, Adom et al 2003, Yu et al 2003, Yu et al 2004, Beta et al 2005, Li et al 2005, Mpofu et al 2006). Because wheat food products are so ubiquitous, it likely acts as a good dietary source of antioxidants and therefore helps reduce severe oxidative stress. Superoxide radical anion ($O_2^{\bullet-}$), a by-product of aerobic metabolism is a ROS that can cause broad spectrum damages to biological systems. Toxic effects of $O_2^{\bullet-}$ result from direct reactions with biological targets, such as lipids (Dix and Aikens 1993), catecholamines (Misra and Fridovich 1972, Heikkila and Cohen 1973, Rao and Hayon 1975, Macarthur et al 2000), and DNA (Dix et al 1996) as well as secondary formation of other oxygen radicals. $O_2^{\bullet-}$ promotes hydroxyl-radical formation and consequent DNA damage in cells of all types (Beauchamp and Fridovich 1970). $O_2^{\bullet-}$ also reacts with nitric oxide ($NO^{\bullet}$), one of the reactive nitrogen species (RNS) resulting in formation of the highly toxic peroxynitrite anion ($ONOO^{\bullet-}$) (Beckman et al 1990). The associated oxidative stress may cause cell death and tissue damage that characterize a number of human disease states, among them neurological disorders and stroke, inflammatory bowel disease, arthritis, toxic shock, and acute reperfusion injuries (Castro et al 1994, Hausladen and Fridovich 1994, Szabó et al 1996).

Wheat genotype (G), the environment (E) in which wheat is grown and possibly G × E interactions can likely strongly influence antioxidative properties. Three hard winter wheat varieties (Akron, Trego and Platte) grown in a single field location differed significantly in their capacities to quench DPPH and ABTS radicals (Yu et al 2002b). Growing location had a strong influence on the antioxidant activity of pearled wheat fractions of hard red spring wheat, Superb, (Beta et al 2005). Yu et al (2003) reported significant effects of growing conditions including the number of hours exceeding 32°C on the antioxidant properties of Akron, a hard red winter wheat variety. Genotype and growing location had significant effects on the antioxidant activity and phenolic content of flours of Akron, Trego and Platte wheat grown at five locations in Colorado (Yu et al 2004). We recently reported significant and greater environment than genotype effects on the total phenolic content, antioxidant activity as measured in terms of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacities and ferulic acid concentration of wheat (Mpofu et al 2006).

Lipophilic and hydrophilic antioxidants are utilized differently in the body. Lipophilic antioxidants can be absorbed through the lymphatic system and reach a higher level of bioavailability, whereas hydrophilic antioxidants can be absorbed in the small intestine and do not accumulate in the body (Burton and Ingold 1986). As there have been no reports on the effects of genotype, growing environment and their interactions on the superoxide anion scavenging capacity of water (SSCW) and lipid (SSCL) soluble substances in wheat, the main objective of this study was to obtain new information in this area. It also offered an opportunity to evaluate for the first time the superoxide scavenging capacities of wheat grown in the vast Canadian Prairie region.

3.2. Materials and methods

Samples were comprised of six Western Canadian wheat genotypes representing three commercial classes of diverse technological qualities. Neepawa (Canada Western Red Spring (CWRS)), AC Elsa (CWRS), AC Barrie (CWRS), Superb (CWRS), AC Vista (Canada Prairie Spring White) and AC Snowbird (Canada Western Hard White Spring). The genotypes were planted in triplicate according to a split-plot design, at each of four locations: Regina, Swift Current and Melfort in Saskatchewan and in Winnipeg, Manitoba. Table 2.1 summarized the environmental conditions at these locations. All wheat samples used in this study were grown in the 2003 crop year and harvested mature and in sound milling condition. The samples were all ground to pass through a 0.8 mm screen in a Falling number laboratory Mill model number 3100 (Perten Instruments, Springfield, IL) prior to analysis.

The superoxide scavenging capacity of the ground samples was assessed by photochemiluminescence (PCL) methods developed by Popov and Lewin (1994, 1996) and modified by Analytik Jena AG (Jena, Germany). The PCL assay was carried out in a photoluminometer Photochem®. SSCL was measured using the "ACL" kit and SSCW using the "ACW" kit provided, and the procedures followed as described by the manufacturer (AnalytikJena, The Woodlands, TX). ACL and ACW kits measure integral superoxide scavenging capacities of lipid and water soluble substances, respectively. The PCL assay involves the photochemical generation of $O_2^{\bullet -}$ free radicals combined with detection through chemiluminescence. The reaction is initiated by optical excitation ($h\nu$) of luminol, a photosensitizer (S), resulting in the generation of the superoxide radical anion (Popov and Lewin 1994).

$$S + h\nu + O_2 \rightarrow [S^* O_2] \rightarrow S^{\bullet+} + O_2^{\bullet-}$$

SSCW is assayed based on the length of the lag phase L in seconds:

$$L = L_0 - L_1$$

where L_0 and L_1 are respective parameters of blank and sample (Popov and Lewin 1994). The SSCW standard curve is a plot of L vs. ascorbic acid concentration. For any given sample, L_0 and L_1 are determined and L calculated then fitted into the standard curve to determine the equivalent ascorbic acid concentration. All these operations are automatically computed using PCL software in a computer attached to the Photochem. The shorter the lag phase of the sample (L_1), the higher the net lag phase L and the higher the SSCW.

The evaluation parameter for the SSCL is the degree of PCL inhibition I, which is calculated as follows:

$$I = 1 - S/S_0$$

where S_0 and S are the integrals of the area under the blank and sample curves respectively. The SSCL standard curve is a plot of I vs. trolox concentration. For any given sample, S_0 and S are determined and I calculated then fitted into the standard curve to determine the equivalent trolox concentration. These mathematical operations are computed automatically using PCL software in a computer attached to the Photochem. The higher the integral of area under the sample curve (S), the higher the inhibition I and the higher the resultant SSCL.

3.2.1. Sample preparation and analysis

Samples (500 mg) were extracted under nitrogen with 70% methanol (5 mL) for 1 h on a wrist action shaker (Burrel, Pittsburg, PA). The suspension was centrifuged at 3000 rpm for 10 min on a Sorvall table centrifuge (GLC-1, Newton, CT). The supernatants (10 μL) were used for the determinations. Reagent mixtures for calibration and measurement of superoxide scavenging capacities of lipid and water soluble substances were prepared according to the pipetting schemes described by Analytik Jena AG (2004). Briefly, for determination of SSCW, the extract (10 μL) was added to components of the ACW kit: 1.5 mL reagent 1 (buffer solution, pH 10.5), 1 mL reagent 2 (water) and 25 μL reagent 3 (photosensitizer). For determination of SSCL, the extract (10 μL) was added to components of the ACL kit: 2.3 mL reagent 1 (methanol), 200 μL reagent 2 (buffer solution) and 25 μL reagent 3 (photosensitizer). The mixtures were run in the Photochem and results obtained from a workstation employing PCL software. SSCW was assayed by means of the lag phase and expressed as μg ascorbic acid equivalent (AAE) /g of wheat based on an ascorbic acid standard curve. SSCL was assayed by means of the area under the curve and expressed as μg trolox equivalent (TE) /g of wheat based on a trolox standard curve. All extractions and tests were run in duplicate.

The samples used in this study were analyzed for total phenolic content, phenolic acid composition and DPPH scavenging capacity in a related study (Mpofu et al 2006). SSCW and SSCL data were correlated to data generated from the reported study (Mpofu et al 2006) for purposes of comparison.

3.2.2. Statistical Analysis

The data were analyzed by analysis of variance (ANOVA). The ANOVA was performed with the general linear model (GLM) of the SAS software package (release 8.2) (SAS Institute, Cary, U.S.A.) using the split plot design and analyzing genotype and growing environment as fixed effects. Genotype, growing environment and genotype × growing environment effects were determined using blocks as replicates with measurements from duplicate extractions for each plot. Comparison of means was done at the 5 % significance level using Duncan's multiple range test. Correlation analyses were performed with the PROC Corr procedure of the SAS software package using the Pearson correlation test.

3.3. Results and discussion

3.3.1. Effects of genotype and growing environment on superoxide scavenging capacities

Wheat genotype had highly significant effects ($p < 0.0001$) on the superoxide scavenging capacities of water (SSCW) and lipid (SSCL) soluble substances. Averaged across locations, SSCW was highest for AC Barrie (497 ± 86 μg AAE/g) and lowest for AC Snowbird (307 ± 38 μg AAE/g) (Fig. 3.1.). SSCL followed a different trend among the genotypes, i.e. there was only a weak relationship between SSCW and SSCL values ($r = 0.38$). SSCL ranged from 332 ± 49 μg TE/g for AC Elsa to 412 ± 43 μg TE/g for Neepawa (Fig. 3.2.).

Growing environment likewise had highly significant effects ($p < 0.0001$) on SSCW and SSCL. Wheat grown in Winnipeg had the highest SSCW (Fig. 3.3) and SSCL

(Fig. 3.4) levels of 438 ± 105 μg AAE/g and 399 ± 38 μg TE/g, respectively. Wheat grown in Regina had the lowest levels of SSCW (325 ± 71 μg AAE/g) (Fig. 3) and SSCL (330 ± 45 μg TE/g) (Fig. 3.4).

Despite the significant environment effects, no correlation was detected between SSCW, or SSCL and the background data summarized in table 2.1: soil pH, mean seasonal temperature, average rainfall for the period from anthesis to maturity, protein content or average test weight. Environmental influences on the antioxidant properties and phenolic content of wheat and other crops have been reported in literature (Emmons and Peterson 2001, Wang and Zheng 2001 Yu et al 2003, Mpofu et al 2006). Our laboratory recently reported significant and greater environment than genotype effects on total phenolic content, antioxidant activity as measured by DPPH scavenging capacities and ferulic acid concentration (Mpofu et al 2006). Effects of the interaction between genotype and growing environment in that study were only significant for TPC and much smaller than the genotype or environment effects (Mpofu et al 2006). Yu et al (2003) reported significant effects of the number of hours exceeding 32°C on the antioxidant properties of Akron wheat. Effects of growing temperature on phenolic acid, flavonol, and anthocyanin contents and antioxidant activities of two strawberry cultivars were evaluated and strawberries grown at higher temperature conditions exhibited greater antioxidant activities and higher phenolic contents (Wang and Zheng 2001). Emmons and Peterson (2001) detected significant growing environment effects for the concentration of caffeic and vanillic acid (which both have significant antioxidant activity) in oats and suggested that the effects were due to temperature differences at the locations. They however, did not identify the specific cause(s) of the environmental effects.

It is of interest to note the relative differences in the contribution of genotype and environment to the total variance of superoxide scavenging capacities of water (SSCW) and lipid (SSCL) soluble substances of wheat. For SSCW, genotype effects were much larger than environment effects (Table 3.3.); the variance due to genotype and growing environment contributed 53% and 36% to the total variance of SSCW. In contrast for SSCL, the contribution to total variance by genotype (~25%) was less than that by growing environment (~54%). The differences in variation patterns observed can likely be attributed to differences in chemical properties of water and lipid soluble substances in wheat genotypes and their responses to different environmental conditions. Phenolic acids and flavonoids are typically water soluble compounds, but lipid soluble derivatives are common to grains and include caffeic and ferulic acid esters of C_{20-28} chain mono- and dialcohols (Collins 1986). Tocopherols and tocotrienols are also lipid soluble compounds that are common to vegetables and grains.

3.3.2. Effects of the interaction between genotype and growing environment superoxide scavenging capacities

G × E interaction effects on both SSCW and SSCL were highly significant ($p < 0.0001$). The significant interaction effects on SSCW and SSCL are indicated in Tables 3.2. and 3.3. which show genotypes having non-uniform rankings in the different locations. As suggested by the coefficients of variation (CVs) of the genotypes across different locations, interaction effects were of greater influence on the SSCW of AC Elsa than other genotypes. With comparable CVs (11.31 and 11.51% respectively), the SSCW values of AC Snowbird and AC Vista were the most stable to interaction. AC Barrie had

a CV of 4.33% for SSCL concentration making it the most stable genotype for this measure of antioxidant activity. Unlike for SSCW where it was most stable, AC Barrie (CV=15.69%) was one of the less stable genotypes to G × E interaction on SSCL. Having a CV of 15.47% for SSCL across the locations, AC Elsa was of comparable instability with AC Barrie for SSCL.

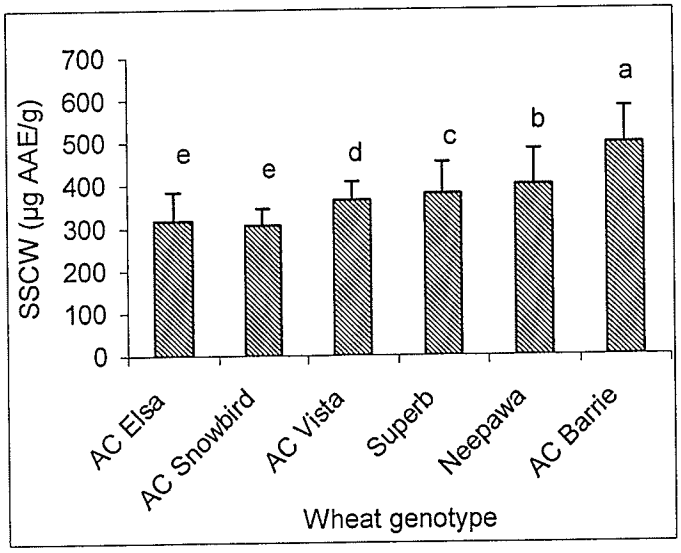

Figure 3.1. Water soluble superoxide scavenging capacities (SSCW in µg ascorbic acid equivalents (AAE) per g) of six wheat genotypes (averages for all locations). Averages of genotypes marked by the same letter are not significantly different ($p < 0.05$).

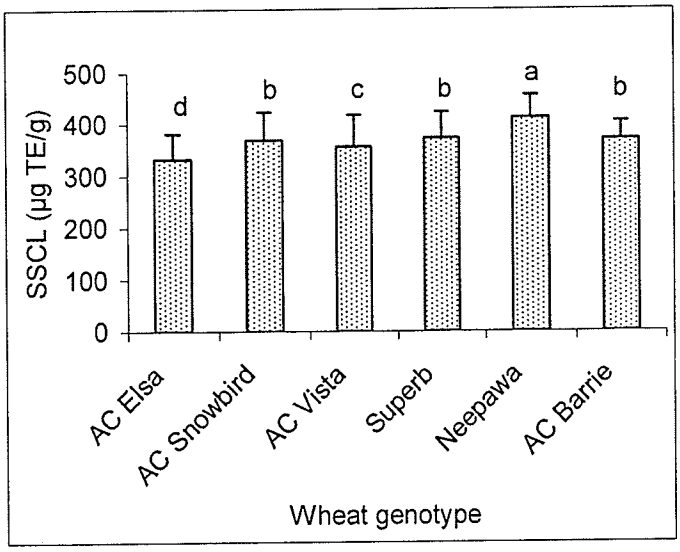

Figure 3.2. Lipid soluble superoxide scavenging capacities (SSCL in µg Trolox equivalents (TE) per g) of six wheat genotypes (averages for all locations). Averages of genotypes marked by the same letter are not significantly different ($p < 0.05$).

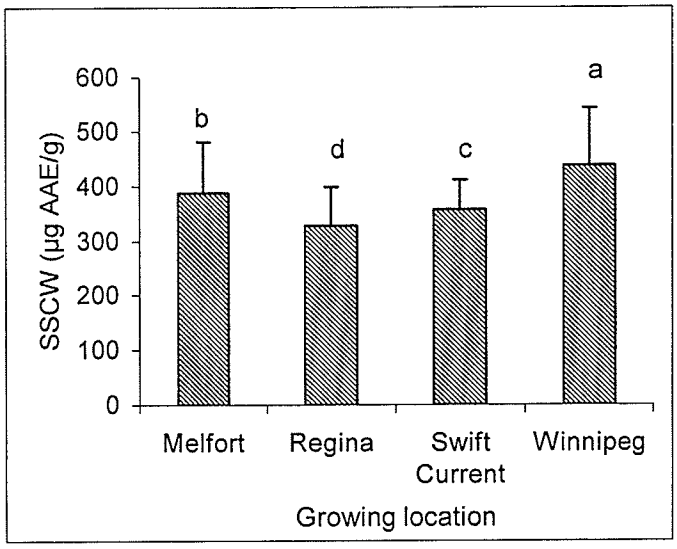

Figure 3.3. Water soluble superoxide scavenging capacities (SSCW in µg ascorbic acid equivalents (AAE) per g) of wheat grown at four locations. Averages of locations marked by the same letter are not significantly different ($p < 0.05$).

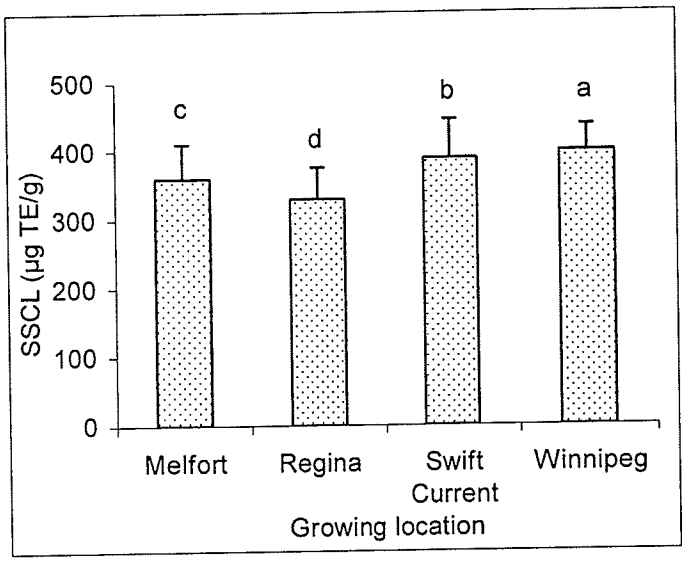

Figure 3.4. Lipid soluble superoxide scavenging capacities (SSCL in µg trolox equivalents (TE) per g) of wheat grown at four locations. Averages of locations marked by the same letter are not significantly different ($p < 0.05$).

Table 3.1. Variance components (% of total mean squares) for genotype (G), growing environment (E) and G × E interaction effects for SSCL and SSCW[a]

Variance component	G[b]	E[c]	G × E[b]	G × block/E	block/E
degrees of freedom:	5	3	15	40	8
Parameter					
SSCW (µg AAE/g)	52.50*	36.42*	9.12*	0.76	1.20
SSCL (µg TE/g)	25.31*	53.68*	14.30*	2.05	4.67

[a] SSCW = Superoxide scavenging capacity for water soluble substances.
SSCL = Superoxide scavenging capacity for lipid soluble substances.
* = Highly significant ($p < 0.0001$). [b] Significance is based on genotype × block/E mean square.
[c]Significance is based on block/E mean square.

Table 3.2. Water soluble superoxide scavenging capacities (μg AAE/g) of six wheat genotypes grown at each of four locations[*a,b]

Genotype	Location				CV (%)
	Melfort	Regina	Swift Current	Winnipeg	
AC Barrie	558a±26	425a±80	428a±20	578a±44	16.51
AC Elsa	269f ±9	268c±17	305d±12	424c±28	23.29
Neepawa	360d±21	332b±52	386c±13	525b±48	21.39
AC Snowbird	320e±24	343b±28	302d±29	261e±9	11.31
Superb	418b±15	254c±14	406b±21	438c±13	22.26
AC Vista	401c±26	345b±30	315d±13	398d±19	11.51

[*] Values expressed as mean ± standard deviation.
[a]Values within the same column with different letters are significantly different at $p < 0.05$.

Table 3.3. Lipid soluble superoxide scavenging capacities (μg TE/g) of six wheat genotypes grown at each of four locations[*a,b]

Genotype	Location				CV (%)
	Melfort	Regina	Swift Current	Winnipeg	
AC Barrie	392b±31	375a±44	357c±34	360d±18	4.33
AC Elsa	316d±12	284d±13	325d±32	405c±19	15.47
Neepawa	413a±19	351ab±25	451a±21	433a±19	10.56
AC Snowbird	333c±16	323bc±30	407b±55	418b±16	13.28
Superb	409a±10	309cd±11	429ab±20	347dC±17	14.83
AC Vista	295e±13	339bc±62	364c±36	429ab± 21	15.69

[*] Values expressed as mean ± standard deviation.
[a]Values within the same column with different letters are significantly different at $p < 0.05$.

3.3.3. Correlation between superoxide scavenging capacities and total phenolic content, ferulic acid concentration and DPPH scavenging capacities

Significant ($p<0.05$), but weak correlations were detected between SSCW and total phenolic content, ferulic acid concentration and DPPH scavenging capacities (Table 3.4). Correlations between SSCL and these parameters were also significant ($p<0.05$), but weak (table 3.3). Phenolic compounds have potent antioxidant activity (Decker 1988). The phenolic compounds present in wheat include phenolic acids, alkylresorcinols, flavanoids, and phenolic acid diacyl glycerols, phenolic aldehydes and ferulates (Shahidi and Naczk 2004). Wheat phenolic acids include ferulic, p-coumaric, vanillic, caffeic, chlorogenic, gentisic, syringic, and *p*-hydroxybenzoic acids (Onyeneho, and Hettiarachchy 1992). Ferulic acid is the most abundant phenolic acid in wheat (Onyeneho and Hettiarachchy 1992, Adom et al 2003, Zhou and Yu 2004, Zhou et al 2004a,b, Li et al 2005, Mpofu et al 2006).Weak correlations between superoxide scavenging capacities and total phenolic content as well as ferulic acid content can likely be attributed to the presence of other compounds in the extracts that can quench the superoxide radicals more effectively. There is need for more compositional analysis involving a wider spectrum of antioxidant compounds to enable identification of the most potent superoxide anion free radical scavengers. As well differences in the solvents and extraction conditions used likely contributed to the low correlations. Ideally similar extraction conditions and solvents should be used for determination of all antioxidant properties, this is however not possible since some solvents (for example 50% acetone when used in the DPPH assay (Zhou et al 2004b)) interfere with assay reagents. The weak correlations between superoxide scavenging capacities and DPPH scavenging capacities were not surprising

because although these parameters are both measures of antioxidant activity, they employ completely different free radical sources, luminol in the case of superoxide scavenging capacities and DPPH on the case of the DPPH scavenging capacities.

Table 3.4. Coefficients of correlations between SSCL and SSCW with total phenolic content*, ferulic acid content* and DPPH scavenging capacity* ‡

	Total phenolics	Ferulic acid content	DPPH scavenging capacity
SSCW	0.315	0.366	0.243
	0.007	*0.002*	*0.040*
SSCL	0.392	0.317	0.325
	0.001	*0.007*	*0.005*

* Previously reported data (Mpofu et al 2006)
‡Cell contents: Pearson correlation coefficient
P-value

3.3.4. Physiological implications of results

In the absence of adequate physiological or dietary antioxidants, the superoxide anion radical contributes to cell death and tissue damage that characterize human disease states, among them neurological disorders and stroke, inflammatory bowel disease, arthritis, toxic shock, and acute reperfusion injuries (Castro et al 1994, Hausladen and Fridovich, 1994, Szabó et al 1996a). This study revealed that wheat is likely a good source of dietary antioxidants that can quench the superoxide anion (and possibly other free radicals), thereby likely preventing the incidence of related diseases. Future studies should involve use of *in-vivo* assays to directly determine the physiological effects of the antioxidants.

3.4. Conclusions

The relative contribution of genotype and growing environment on the SSCW and SSCL of wheat has been reported. Genotype had a greater effect on SSCW than growing environment; the opposite is likely true for SSCL, but effects were smaller. Genotype ×

growing environment interaction effects on both SSCW and SSCL were highly significant ($p < 0.0001$). It therefore seems important to be mindful of both genotype and growing environment effects in studying antioxidative properties and in processing of wheat. As there were weak correlations between superoxide scavenging capacities and total phenolic content as well as ferulic acid concentration, there is need for greater characterization of antioxidant compounds to enable identification of potent superoxide scavenging compounds. Extensive testing of genotypes over years and locations would be desirable in breeding and selection of wheat with superior protective effects against the toxic effects of the superoxide radical anion and other ROS.

Chapter 4: Variation of Hydrophilic, Lipophilic and Total Oxygen Radical Scavenging Capacity (ORAC) Values of Canadian Wheat Genotypes Grown in Different Environments

ABSTRACT

The peroxyl radical is implicated in several pathophysiological conditions including atherosclerosis, diabetes, cancer, chronic inflammatory diseases and neurodegenerative diseases. The oxygen radical scavenging capacity (ORAC) assay is likely a suitable *in-vitro* method for determining the capacity of dietary antioxidants to protect against this radical. Wheat, a popular component of the human diet increasingly being shown to possess antioxidant properties is likely an ideal ingredient for functional food and nutraceutical use. Use of wheat for functional food and nutraceutical production would benefit from an ability to scavenge peroxyl radicals among other reactive species and thus confer health properties to the consumer. In this study the hydrophilic (H-$ORAC_{FL}$), lipophilic (L-$ORAC_{FL}$) and total (T-$ORAC_{FL}$) ORAC values of six Canadian wheat genotypes grown in four locations were determined using fluorescein (FL) as the fluorescent probe. The influence of genotype, growing environment and genotype by growing environment effects was evaluated to determine the feasibility of breeding wheat with superior antioxidant properties. For all the three parameters measured (H-$ORAC_{FL}$, L-$ORAC_{FL}$ and T-$ORAC_{FL}$), only growing environment effects were significant ($p<0.05$). Averaged by location, H-$ORAC_{FL}$ and T-$ORAC_{FL}$ values showed similar trends. Wheat grown in Winnipeg had the highest H-$ORAC_{FL}$ and T-$ORAC_{FL}$ values of

35.6 and 40.0 μg Trolox equivalents (TE)/g respectively. H-$ORAC_{FL}$ and T-$ORAC_{FL}$ values of Winnipeg wheat were similar to those of Melfort and Regina wheat but significantly different from lowest ranked Swift Current wheat. Averaged by location, L-$ORAC_{FL}$ values ranged between 4.31 and 5.68 μg TE/ g, with Swift Current wheat ranking significantly higher than wheat from the other three locations whose L-$ORAC_{FL}$ values were all similar. Growing environment contributed about four times as much as genotype to total variation for H-$ORAC_{FL}$ and L-$ORAC_{FL}$. For T-$ORAC_{FL}$, the contribution of growing environment to total variation was approximately three times as much as that of genotype. The cause of the large growing environment influence was not identified although a high, significantly negative correlation ($r = -0.999$) was detected between L-ORAC and test weight. Such large growing environment compared to genotype influences would likely complicate the process of breeding wheat with superior antioxidant properties and associated health benefits.

4.1. Introduction

Peroxyl radicals (R-COO$^{\bullet}$) are biologically relevant reactive oxygen species (ROS) with high capacity to damage cellular constituents. R-COO$^{\bullet}$ radicals are formed, during reactions between free radicals such as $^{\bullet}OH$, $^{1}O_2$ or $ONOO^{-}$ and carbon containing compounds, for example, polyunsaturated fatty acids within cell membrane (Asano et al 1991, Sevanian and McLeod 1997). The highly devastating effects of R-COO$^{\bullet}$ radicals are due to their high reactivity and the fact that they re-initiate (propagate) the process of the lipids oxidation (Cheesman 1993). The products of lipid oxidation accumulate in the cell membrane and may cause leakage of the plasmolemma and dysfunction of membrane bound receptors among other detrimental effects on cellular function. These products include unsaturated aldehydes which have cytotoxic and mutagenic properties (Herbst et al 1999). Diseases caused by lipid peroxidation include: atherosclerosis, diabetes, cancer, chronic inflammatory diseases and neurodegenerative diseases (Gutteridge 1993, Halliwell and Gutteridge, 1990, Halliwell et al 1992, Delanty and Dichter 1998, Sastre et al 2000).

The antioxidant properties of wheat, a very important cereal crop which is a staple food in more than 40 countries for over 35% of the world's population (Bushuk 1998) and a potential functional food ingredient or nutraceutical source are well documented (Onyeneho and Hettiarachchy 1992, Yu et al 2002a,b,c, Adom et al 2003, Yu et al 2003, Yu et al 2004, Zhou et al 2004a,b,2005, Beta et al 2005, Li et al 2005, Mpofu et al 2006). Several assays have been used in determination of antioxidant properties and the ORAC assay based upon the early work of Glazer (1988) and Ghiselli et al. (1994), as developed further by Cao et al (1993) and subsequently modified by Ou et al (2001) is one of the

most commonly used. ORAC measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects radical chain breaking antioxidant activity by H atom transfer, the predominant antioxidant mechanism for the autoxidation initiated by oxygen radicals (Ou et al 2001).

Most of the ORAC values reported for wheat are based on the traditional ORAC method which determines mainly hydrophilic antioxidant capacity (Zhou et al 2004a,b,2005, Moore et al 2005). Hydrophilic and lipophilic antioxidant values of wheat have been reported based on the recently developed peroxyl radical scavenging capacity (PSC) assay (Adom and Liu 2005). Based on the PSC assay, Adom and others (2005) reported significant differences in hydrophilic as well as in lipophilic antioxidant activities of bran/germ extracts. To our knowledge, hydrophilic and lipophilic antioxidant activities of wheat based on the ORAC assay have not been reported in literature. Moreover, the genotype by environment variation of these and other antioxidant parameters of wheat is still largely under-documented.

This study was carried out to determine the genotype by environmental variation in lipophilic and hydrophilic ORAC values of six Canadian grown wheat genotypes, establish how these values are related, how they relate to other measures of antioxidant properties and how they were influenced by measured environmental phenomena. Information generated from this work is additionally aimed at complementing previously reported work (Mpofu et al 2006) and unpublished work on superoxide radical scavenging capacities of the same wheat samples. These studies were carried out to gain understanding of the possibility of breeding and marketing wheat based on its antioxidant properties.

4.2. Materials and methods

4.2.1. Sample description

Wheat samples, all grown in the 2003 crop year, harvested mature and of sound milling condition were used in this study. The samples comprised six western Canadian wheat genotypes representing three commercial classes with different technological qualities. Neepawa (Canada Western Red Spring (CWRS)), AC Elsa (CWRS), AC Barrie (CWRS), Superb (CWRS), AC Vista (Canada Prairie Spring White) and AC Snowbird (Canada Western Hard White Spring). The six genotypes were grown in triplicate according to a split-plot design at each of four locations, Regina, Swift Current and Melfort (Saskatchewan) and Winnipeg (Manitoba), bringing the total number of samples to 72. In each of the growing environments, wheat was planted in a randomized complete block design with replicates as blocks.

Typical differences in the growing locations are reflected in Table 2.1., which is a summary of the environmental conditions at the locations as well as some physical and chemical properties of the wheat from each location. Samples were each ground to pass through a 0.8-mm screen in a model 3100 Falling number laboratory mill (Perten Instruments, Springfield, IL) prior to extraction and chemical analysis.

4.2.2. Determination of hydrophilic, lipophilic and total ORAC values

The hydrophilic (H-$ORAC_{FL}$) and lipophilic (L-$ORAC_{FL}$) $ORAC_{FL}$ values of the wheat samples were determined separately. Total $ORAC_{FL}$ (T-$ORAC_{FL}$) was calculated by adding together the L-$ORAC_{FL}$ and H-$ORAC_{FL}$. The procedures for L-$ORAC_{FL}$ and

H-ORAC$_{FL}$ determination were carried out according to the method of Wu and others (2004) with several modifications. Description of the modified assay procedures follow.

4.2.2.1. Sample preparation

4.2.2.1.1. L-ORAC$_{FL}$

For the determination of L-ORAC$_{FL}$, ground wheat samples (250 mg) were extracted in duplicate using hexane/dichloromethane (1:1, Hex/Dc) (10 mL). The sample-solvent mixture was shaken for 1 h at room temperature on a rotary shaker (Fermentation Design Inc., Allentown, PA) at 250 rpm for 1.5 h. Following this extraction step, the mixture was centrifuged at 5900 rpm on a Sorvall model RC5C centrifuge (Sorvall Instruments, DuPont, Wilmington, DE) for 15 min. The supernatant was collected and dried at 30°C on a rotary vacuum evaporator (RE III Rotavapor, Büchi, Switzerland) and the resulting residue reconstituted with 10 mL of acetone. The samples were labeled appropriately and stored in the dark at -20°C for up to 7 days before analysis.

4.2.2.1.2. H-ORAC$_{FL}$

Sample preparation for determination of H-ORAC$_{FL}$ involved duplicate extraction of ground wheat samples (250 mg each) in acetone/water/acetic acid (AWA; 70:29.5:0.5 v/v/v) (10 mL) using a rotary shaker (Fermentation Design Inc., Allentown, PA) at 250rpm for 1 ½ h. Extract mixtures were centrifuged at 5900 rpm on a Sorvall model RC5C centrifuge (Sorvall Instruments, DuPont, Wilmington, DE) for 15 min. The supernatants were collected, labeled appropriately and stored in the dark at -20°C for up to 7 days before analysis.

4.2.2.2. Preparation of ORAC assay plates

Sample extracts were thawed and appropriately diluted just before analysis. Hydrophilic extracts were diluted with phosphate buffer (pH 7.4) whilst lipophilic ones were diluted with 7% randomly methylated β-cyclodextrin (RMCD) in acetone/water (1:1, v/v). Sample dilution was in both cases carried out in such a way that the concentration of diluted samples would fit within a trolox standard curve.

Duplicates of the diluted samples (300 μL) were manually pipetted in duplicate into black-walled 96-well plates (Fisher Scientific, Hanover Park, IL) for each run. Wheat samples, control samples, Trolox standards and chemical control samples (Rutin) were transferred to separate 96-well assay plates for testing on a FLx800 fluorescence plate reader (Bio-Tek Instruments, Inc., Winooski, VT) using a robotic pipettor (Biotek Precision 2000 well automated microplate pipetting system, Bio-Tek Instruments, Inc., Winooski, VT).

Using the robotic pipettor, reactants were added in the following order according to a pre-set plate layout: 120 μL of Fluorescein (8.16×10^{-2} μM); either 20 μL of blank, or 20 μL each of appropriately diluted samples and control samples; Trolox standards (6.25, 12.5, 25 and 50 μM); 20 μL Rutin (20 μM); and 60 μL of 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) (153 mM). The blank solution for determination of H-$ORAC_{FL}$ was phosphate buffer (75 mM) and for determination of L-$ORAC_{FL}$ it was 7% RMCD in acetone/water (1:1, v/v). Before addition of AAPH, the microplate was sealed and incubated at 37°C for 20 min with a 3 min shaking using the microplate reader. Immediately after addition of AAPH, plates were placed in the FLx800 fluorescence plate reader and read for 50 min. Final fluorescence measurements

were expressed relative to the initial reading. Results were calculated based on differences in areas under the fluorescein decay curve between the blank and a sample,

$$\text{net AUC} = \text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}.$$

The final ORAC_{FL} values were calculated using a linear regression equation:

$$Y = a + bX$$

between trolox concentration (Y) (μM) in the range of 6.25-50 μM trolox and the net area under the FL decay curve (X). Results were expressed as μmol of trolox equivalents (TE)/g of wheat.

4.2.3. Statistical Analysis

Analysis of variance (ANOVA) was carried out on data combined for all locations. The ANOVA was performed using SAS software package (release 8.2) (SAS Institute, Cary, U.S.A.) in the general linear model (GLM) according to a split plot design, analyzing genotype and environment as fixed effects. Genotype (G), environment (E) and G × E effects were determined using replicates as blocks with measurements from duplicate analyses for each plot. Data from each location were additionally analyzed individually by ANOVA as randomized complete block designs. Comparison of means was done at the 5% significance level using Duncan's multiple range test. Correlation analyses were performed with the PROC Corr procedure of the SAS software package using the Pearson correlation test declaring as significant correlations those with p values less that 0.05.

4.3. Results

4.3.1. Genotype and environmental effects

H-ORAC$_{FL}$, L-ORAC$_{FL}$ and T-ORAC$_{FL}$ were all significantly influenced by growing environment ($p<0.05$), but not genotype or genotype × environment effects (Table 4.1). A high, significantly negative correlation was detected between L-ORAC and test weight ($r = -0.999$). No other significant correlation was observed between the measures of ORAC and any of the environmental, chemical or physical parameters presented in Table 2.1.

Table 4.1. Variance components (% of total mean squares) for genotype (G), growing environment (E), and G × E interaction effects for H-ORAC$_{FL}$, L-ORAC$_{FL}$ and T-ORAC$_{FL}$[a]

Variance component: degrees of freedom:	G[b] (5)	E[c] (3)	G × E[b] (15)	G × Block/E (40)	Block/E (8)
Parameter					
H-ORAC$_{FL}$	13.16	52.33*	8.36	14.35	11.80
L-ORAC$_{FL}$	11.94	45.99*	14.08	13.17	14.82
T-ORAC$_{FL}$	14.76	48.93*	8.30	16.16	11.84

[a] * = significant ($p < 0.05$). [b] Significance is based on genotype × block/E mean square. [c] Significance is based on block/E mean square.

Table 4.2. presents the average H-ORAC$_{FL}$, L-ORAC$_{FL}$ and T-ORAC$_{FL}$ values per growing location. Wheat grown in Winnipeg had the highest H-ORAC$_{FL}$ value which differed significantly with only that of lowest ranked Swift Current grown wheat. The trend for T-ORAC$_{FL}$ values was similar to that for H-ORAC$_{FL}$. L-ORAC$_{FL}$ values followed a different trend with Swift Current wheat having the highest value which was

significantly different from that of wheat grown at the other three sites; Melfort, Regina and Winnipeg which had a similar ranking.

Table 4.2. Mean H-ORAC$_{FL}$, L-ORAC$_{FL}$ and T-ORAC$_{FL}$ values (μmol TE/g) of wheat per growing location‡*

	L-ORAC$_{FL}$	H-ORAC$_{FL}$	T-ORAC$_{FL}$
Swift Current	5.7a±1.9	23.5b±10.6	29.2b±10.6
Melfort	4.3b±1.2	31.2ab±9.7	35.5ab±9.2
Regina	4.4b±1.5	31.1ab±10.9	35.6ab±10.1
Winnipeg	4.4b±1.3	35.6a±10.7	40.0a±10.4

‡ Values expressed as mean ± standard deviation (n=18).
* Means in same column followed by different letters are significantly different ($p<0.05$).

The mean L-ORAC$_{FL}$, H-ORAC$_{FL}$ and T-ORAC$_{FL}$ values for each of the six genotypes investigated are presented in Table 4.3. No significant differences were detected between the genotypes averaged over all locations for any of the parameters tested. The mean values averaged per genotype were also not significantly different at any of the growing locations for all the three parameters (data not shown). Accordingly, no significant interaction effects were detected for L-ORAC$_{FL}$, H-ORAC$_{FL}$ and T-ORAC$_{FL}$.

Table 4.3. Mean L-ORAC$_{FL}$, H-ORAC$_{FL}$ and T-ORAC$_{FL}$ values (μmol TE/g) of wheat per genotype‡*

	L-ORAC$_{FL}$	H-ORAC$_{FL}$	T-ORAC$_{FL}$
AC Elsa	4.9±1.0	28.1±9.4	33.0±8.9
Neepawa	4.9±1.7	28.8±9.0	33.7±8.0
AC Snowbird	4.9±1.5	30.0±13.3	35.0±12.3
Superb	5.1±2.1	29.6±9.4	34.7±10.0
AC Vista	4.0±1.6	29.1±14.4	33.1±13.3
AC Barrie	4.5±1.2	36.5±10.6	41.0±10.3

‡ Values expressed as mean ± standard deviation (n=12)
* Means in same column followed by different letters are significantly different ($p<0.05$).

4.3.2. Correlations between ORAC values and other measures of antioxidant properties

H-$ORAC_{FL}$ and T-$ORAC_{FL}$ were significantly correlated (r= 0.991) likely owing to the high contribution (at least 80% for a given growing location) of H-$ORAC_{FL}$ to the T-$ORAC_{FL}$ values. H-$ORAC_{FL}$ and L-$ORAC_{FL}$ were significantly negatively correlated (r = - 0.420). Neither H-$ORAC_{FL}$ nor L-$ORAC_{FL}$ or T-$ORAC_{FL}$ was significantly correlated with any of the previously measured phenolic antioxidant properties: DPPH scavenging capacities, total phenolic content (TPC), superoxide radical scavenging capacities or concentration of any of the phenolic acids.

4.4. Discussion

Previous reports on the ORAC properties of wheat have been based on the traditional ORAC method which mainly determines hydrophilic antioxidants. Given the potential impact of lipophilic antioxidants, on human health, determining both lipophilic and hydrophilic antioxidant properties seems imperative. This study is one of the few if not the first to report both hydrophilic and lipophilic ORAC values of wheat genotypes grown in different locations.

The influence of growing environment on the H-$ORAC_{FL}$, L-$ORAC_{FL}$ and L-$ORAC_{FL}$ values was far greater than that of genotype. For H-$ORAC_{FL}$ and L-$ORAC_{FL}$, growing environment influenced total variation by approximately four times more than genotype. We previously reported that growing environment contributed up to about 1.5 times more than genotype to the variation in key antioxidant parameters: TPC, DPPH scavenging capacity and ferulic acid concentration (Mpofu et al 2006). Given these

observations, it seems sensible to conclude greater contributions of growing environment than genotype to variation in antioxidant parameters, however in the previous chapter (Chapter 3 of this thesis report) greater environment influences were reported for SSCL while SSCW was influenced more by genotype. None of the measured ORAC parameters was significantly correlated to background growing environment data except for L-$ORAC_{FL}$, which was negatively correlated to test weight. In line with the detected negative correlation, Swift Current wheat, having the lowest test weight had the highest L-$ORAC_{FL}$ value. No significant heat stress, drought, frost damage, or any other condition that reduces test weight was, however reported for this wheat. Examination of G × E effects using a larger sample size from a larger selection of growing environments will be necessary before such a conclusion can be reached.

Similar to observations made for TPC, AOA (% DPPH scavenging capacity) and FA concentration, wheat grown in Winnipeg exhibited superior H-$ORAC_{FL}$ and T-$ORAC_{FL}$ properties than wheat from the other locations. No particular ranking pattern was however observed for the other locations. It is apparent therefore that Winnipeg likely provided a conducive environment for synthesis of antioxidants in wheat during the 2003 growing season. The factors that promoted antioxidant synthesis were however not identified necessitating further studies in this direction. Such factors could possibly include high UV light, low temperature and low nitrogen, phosphate or iron (Dixon and Paiva 1995).

Lack of correlation between ORAC values (based on the tradition largely hydrophilic assay) and other antioxidant properties have been reported in literature (Moore et al 2005, Zhou et al 2004a,b, 2005). The ORAC values of eight soft wheat

varieties and experimental lines was correlated with $ABTS^{\bullet+}$ scavenging activity (r = 0.908) but not total phenolic content, concentration of tocopherols, carotenoids, and phenolic acids or antioxidant properties, including Fe^{2+} chelating capacity and free radical scavenging activities against $DPPH^{\bullet}$ (Moore et al 2005). The ORAC values of extracts from red Swiss wheat grain and its fractions was correlated with $O_2^{\bullet-}$ scavenging activity, $ABTS^{\bullet+}$ scavenging capacity, $DPPH^{\bullet}$ scavenging capacity, and chelating activity (r = 0.94), but not with TPC (Zhou et al 2004a). Zhou et al (2004b) detected no correlation between ORAC value of wheat bran and any of the antioxidant activity they measured including DPPH radical scavenging capacity, ferulic acid concentration and TPC. ORAC values were not correlated to radical scavenging properties against the hydroxyl radical ($HO^{\bullet}$), 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$), and superoxide radical anion ($O_2^{\bullet-}$) or chelating capacities against Cu^{2+} and Fe^{2+} (Zhou et al 2005). No significant correlation was detected as well between ORAC and total phenolic content, tocopherol concentrations, concentrations of carotenoids or the concentration of phenolic acids including ferulic acid in the same wheat bran samples (Zhou et al 2005).

Although the studies reviewed did not find any significant correlation between ORAC values and tocopherol concentration, tocopherols are known to be potent scavengers of peroxyl radicals, for example; each molecule of α-Tocopherol (vitamin E) is known to scavenge two $R\text{-}COO^{\bullet}$ (Burton et al 1983, Pieri et al 1995). In studies on antioxidant properties, known antioxidant compounds are often of specific interest, but studies of the antioxidant activity of some extracts have shown that known compounds account for only a fraction of total activity suggesting that a large share of biologically important compounds may still be unknown (Rice-Evans et al 1997, Cao et al 1998).

Determination of G, E and G × E variation in H-ORAC$_{FL}$, L-ORAC$_{FL}$ and T-ORAC$_{FL}$ adds to the knowledge on the antioxidant properties of wheat since the assays measure the scavenging capacity against the highly devastating peroxyl radical. There is currently no single assay in use which can be considered a total antioxidant capacity assay even though it can be performed both in an aqueous solution and in a lipophilic environment (Prior et al 2005). In order to fully elucidate a profile of antioxidant capacity, scavenging capacities against various ROS/RNS, such as $O_2^{\bullet-}$, $HO^{\bullet}$, and $NO^{\bullet}$, have to be determined using different methods specific for each ROS/RNS (Prior et al 2005).

H-ORAC$_{FL}$ values ranged between 28.1 and 36.5 μmol TE/g of whole meal wheat. This range is comparable to the 32.9 to 47.7 μmol TE/g observed for Maryland soft wheat varieties (Moore et al 2005). Lower ranges, 3.41-6.25 and 4.46-5.60 μmol of TE/g of bran were detected in ethanol extracts of Trego and Akron wheat bran, respectively (Yu et al 2005). The observed differences can likely be explained by the effect of extraction solvent on antioxidant activity estimation. Zhou and Yu (2005) determined ORAC values using ethanol extracts, whereas in the present study acetone/waters /acetic acid (AWA) (70:29.5:0.5, v/v/v) was used. Bran has a higher antioxidant concentration than that of whole wheat meal. It is therefore not surprising to note that the H-ORAC$_{FL}$ values reported here are lower than the predominantly hydrophilic ORAC values reported on bran samples (45.02-124.29 μmol of TE/g of bran (Zhou et al 2004a,b) and 50.59 to 65.94 μmol of TE/g of bran (Zhou et al 2005)).

4.5. Conclusions

Growing environment accounted for the greatest and only significant influences on the variation in H-ORAC$_{FL}$, L-ORAC$_{FL}$ and T-ORAC$_{FL}$. The cause of the large growing environment influence was not identified although a high, significantly negative correlation ($r = -0.999$) was detected between L-ORAC$_{FL}$ and test weight. The observed H-ORAC$_{FL}$, L-ORAC$_{FL}$ and T-ORAC$_{FL}$ were not correlated to any of the previously reported antioxidant properties highlighting the need for further studies to determine a wider array of antioxidant compounds. In future studies, *in-vivo* antioxidant measurement will be carried out to determine the physiological effects of the antioxidants based on their G and E origins. Heritability of the antioxidant properties will also be a subject of interest in future research. The observed lack of genotype effects indicates the potential difficulty one can face in selecting wheat for these parameters in a breeding program.

Chapter 5: Genotype and Environment Variation in Phenolic Antioxidant Properties of Frost Damaged, *Fusarium* Damaged and Normal Wheat

ABSTRACT

Phenolic compounds are synthesized in plants as part of multifunctional defense systems against the detrimental effects of oxidation. When consumed in food, these compounds likely contribute to the health and well being of consumers. *Fusarium* head blight (FHB) and frost damage contribute to yield loss and reduce the edibility and processing quality of wheat. Phenolic compounds have been found to inhibit *Fusarium* species which cause FHB and although their involvement in resistance to frost damage is implied in literature, it has not been investigated in wheat grain. In this study, the influence of genotype, growing environment, and genotype × growing environment interaction on the total phenolic content (TPC), antioxidant activity (AOA) and phenolic composition of sound, frost-damaged and *Fusarium*-damaged wheat was determined and compared. The samples were comprised of four red and two white-grained hard spring wheat genotypes grown at three sites in the 2004 crop year. Wheat from Carman (Ca-04) had undergone *Fusarium* damage to different degrees, wheat from Swift Current (SC-04) was of sound milling quality, and wheat from the Regina site (Re-04) was predominantly frost damaged. Significant differences were detected among genotypes for TPC and concentrations of the following phenolic acids: vanillic acid (VA), syringic acid (SA), p-coumaric acid (PCA), ferulic acid (FA) and o-coumaric acid (OCA). Environmental effects were significant for all the parameters measured and their contribution to total variation was between 18% (in VA concentration) and 85% (in AOA) greater than the

contribution of genotype considering all the variables determined for sound, frost- and *Fusarium*-damaged wheat. The frost-damaged (Re-04) wheat had higher levels of TPC and AOA, and higher concentrations of all the phenolic acids measured. *Fusarium*-damaged (Ca-04) and sound (SC-04) wheat had similar levels of TPC and AOA as well as concentrations of all phenolic acids but VA and SA, both of which were lower in the *Fusarium*-damaged wheat. Average concentrations of gallic acid, p-hydroxybenzoic acid and ferulic acid were negatively correlated to average test weight per location ($r=-0.997$, $r=-0.998$ and $r=-0.999$ respectively) indicating that concentrations of these phenolic acids were on average higher in damaged (low test weight) wheat. In this case, Re-04 wheat had the lowest test weight, with Ca-04 and SC-04 wheat having comparable, lower test weights. No net accumulation of any of the phenolic acids was indicated in *Fusarium*-damaged wheat. Frost damage resistant wheat appeared to have higher concentrations than susceptible wheat. Protection of wheat from frost and other sources of oxidative stress in plants can likely be achieved through selecting for wheat high in phenolic compounds, key among them being ferulic acid. Such wheat would not only have agronomical advantages but also added human health benefits. Presence of a significant genetic component in the variation of TPC and concentrations of VA, SA, PCA, FA and OCA indicates that these traits can potentially be selected for in a genotype development program. These traits were, however, found to be more likely influenced by environmental factors and in the case of concentrations of VA, SA and FA also significantly influenced by G × E interaction. Successful breeding of wheat with high phenolic antioxidant properties will most likely depend on identification of a genotype

that is more stable to influences of the environment and interactions between genotype and environmental factors.

5.1. Introduction

Phenolic compounds are widely distributed secondary plant metabolites synthesized via the phenylpropanoid pathway (Dixon and Paiva 1995). These compounds are part of multifunctional defense systems against the detrimental effects of oxidation (Decker 1988). Phenolic compounds protect plants from a variety of stress factors, including high light, low temperatures, pathogen infection and nutrient deficiency (Harborne 1994, Dixon and Paiva 1995). The ability of plant cells to synthesize phenolic compounds indirectly reflects their resistance to stress (Zagoskina et al 2003). Phenolic compounds do not only have a bioactive role in plants, they are also increasingly being found to prevent a myriad of diseases in humans (Beretz et al 1986, Nègre-Salvayre and Salvayre 1992, Wattenberg 1992, Chang et al 1993, Hertog et al 1993, Wu et al 1996, Hertog at al 1997, Rice-Evans and Paganga 1996, Knekt et al 1997, Jacobs et al 1998, Jacobs 1999, Liu et al 1999, Slavin et al 1999, Yochum et al 1999, Geleijnse at al 1999, Ding et al 1999, MacCarrone et al 1999, Michaluart et al 1999, Fuhrman and Aviram 2002, Steffen at al 2003).

Wheat is a predominant component of the human diet; it is grown on more land area than any other commercial crop and is consumed by over 35% of the world's population (Bushuk 1998). Several studies have reported significant antioxidant levels in wheat and wheat products mainly attributable to phenolic compounds (Onyeneho and Hettiarachchy 1992, Yu et al 2002a,b,c, Adom et al 2003, Yu et al 2003, Yu et al 2004, Zhou et al 2004a,b,2005, Beta et al 2005, Li et al 2005, Mpofu et al 2006). The protective role of phenolics against different forms of plant stress has also been reported (Harborne 1994, Dixon and Paiva 1995, Sgherri et al 2003). Although the agronomical and human

health benefits of phenolic compounds are well known, wheat breeding programs have largely focused on improving other properties mainly related to yield and end use quality for breadmaking. Breeding wheat with elevated levels of phenolics seems highly desirable for improving resistance to several forms of plant stress and increasing human health benefits. Wheat with these characteristics would increase the income earned by farmers by improving yield and increasing the marketability of wheat in the highly lucrative functional foods and nutraceuticals industry.

Phenolics fulfill diverse functions related to photosynthesis, respiration, and plant defense against various stressors (pathogens, UV-B radiation, heavy metals, and others) (Harborne 1994, Dixon and Paiva 1995, Sgherri et al 2003). Studies on the response of wheat to frost-related oxidative stress have largely focused on antioxidant enzymes such as catalase, ascorbate peroxidase, guaiacol peroxidase (GPx), glutathione-S-transferase and glutathione reductase (GR) (Scebba et al 1998, Kocsy et al 2001, Janda et al 2003). Phenolics are, however, undoubtedly involved in this process, particularly due to their high antioxidant activity, which is of importance for cell survival under stress conditions (Harborne 1987). Soluble phenolic compounds accumulate in the leaves of cold hardened wheat (Zagoskina et al 2005) and growth of soybeans at lowered temperature results in the accumulation of phenolic compounds in their roots (Janas 2002). Effects of frost damage on the phenolic content or composition of wheat grain have not been reported in literature. Frost-resistant wheat genotypes, in comparison with less frost-resistant ones likely maintain a better defense against reactive oxygen species (ROS) when exposed to low-temperatures (Scebba et al 1998).

Fusarium head blight (FHB) caused by *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) is a devastating disease affecting all classes of wheat and other small grains (McMullen et al 1997). Wheat has successfully been bred for resistance to FHB and the mechanisms involved in resistance likely include the antioxidant activity of phenolics. McKeehen et al (1998) evaluated the phenolic acid profiles of six cultivars of wheat with known tolerance to *Fusarium* head blight and reported that accumulation of ferulic acid from anthesis until ~20 days after anthesis appeared to be related with cultivar resistance to *Fusarium*. In mature wheat, concentrations of ferulic acid in the grain of resistant and susceptible cultivars were, however, similar. The potency ferulic acid was confirmed through in-vitro testing where it demonstrated significant reductions ($p < 0.05$) of *Fusarium* species growth at concentrations of up to 1.6 mg/mL (McKeehen et al 1998).

Apart from inducing resistance against *Fusarium* species, phenolic compounds, specifically ferulic and p-coumaric acid also likely induce resistance against wheat midge (*Sitodiplosis mosellana*) which attacks developing wheat kernels causing severe reduction in their quantity and quality (Ding et al 2000, Abdel-Aal et al 2001).

Knowledge of the relative influences of genotype (G), environment (E), and their interaction (G × E) on a desired trait is essential to successfully breed a plant for that trait. We previously reported (based on sound wheat of good milling quality) that key antioxidant parameters in wheat (AOA (DPPH scavenging capacity), TPC and FA concentration) were influenced more by growing environment than genotype or genotype × growing environment interaction (Mpofu et al 2006). The present study was carried out to determine the magnitude of genotype, growing environment and genotype × growing

environment interaction effects on the phenolic antioxidant properties of Canadian wheat harvested in the 2004 crop year. The wheat investigated was either harvested mature and in sound milling condition, *Fusarium*- or frost-damaged offering the opportunity to compare for the first time the phenolic antioxidant properties of wheat affected by two forms of environmental stress (*Fusarium* and frost damage) and investigate the influence of G, E and G × E effects on their antioxidant properties.

5.2. Materials and methods

5.2.1. Sample description

Wheat samples from the 2004 harvest of the on-going University of Manitoba G × E study on the quality and chemical composition of wheat grown in the Canadian Prairie region were used for this study. The samples comprised six western Canadian wheat genotypes from three commercial classes: Canada Western Red Spring (CWRS), Canada Prairie Spring White (CPS) and Canada Western Hard White Spring (CWHW). Four of the genotypes used: Neepawa, AC Elsa, AC Barrie and Superb belong to the CWRS class, while the other two, AC Vista and AC Snowbird are classified as CPS and CWHW respectively. The six genotypes were grown in triplicate according to a split-plot design, at each of three locations: Regina, Swift Current and Carman bring the total number of samples to 54. The growing locations under investigation are from here on referred to as the following site-years: Regina (Re-04), Swift Current (SC-04 and Carman (Ca-04) in order to distinguish wheat grown at some of the sites from that grown during the 2003 crop year under different environmental conditions. At each site, wheat was planted in a randomized complete block design with replicates as blocks.

Table 5.1. summarizes some physical and chemical properties of the wheat (test weight and protein) that reflect typical differences in those sites. Each sample was ground to pass through a 0.8 mm screen in a model 3100 Falling number laboratory mill (Perten Instruments, Springfield, IL) prior to chemical analysis.

Table 5.1. Environmental conditions at the growing locations and outcomes for test weight, protein content and degree of *Fusarium* damage

Location	Soil zone	Soil pH	*Temp (°C)	*Rainfall (mm)	Test weight (Kg/hL)	Protein (%)	Degree of *Fusarium* damage (%)	Major characteristic of harvested wheat
Ca-04	Sandy loam	5.8	15.5	1.52	81.25±1.42	14.34±0.83	0.822±0.48	*Fusarium* damaged
Re-04	Transition Black and brown	7.7	13.9	3.11	72.05±2.43	14.89±0.93	0.000±0.39	Frost damaged
SC-04	Brown	7.3	13.3	2.24	81.25±1.34	15.44±0.78	0.004±0.54	Normal

* Average per location from anthesis to maturity

5.2.2. Determination of total phenolic content

The level of total phenolic content was determined using the Folin-Ciocalteu method (Singleton and Rossi 1965) modified by Gao et al (2002) as described previously (Mpofu et al 2006). Samples of ground wheat (200 mg) were extracted with acidified methanol (HCl/methanol/water, 1:80:10, v/v) (4 mL) then oxidized with the freshly diluted Folin-Ciocalteu reagent, and the reaction subsequently neutralized with sodium carbonate. The mixture was incubated at room temperature and its absorbance measured at 725 nm using acidified methanol as the blank. All analyses were performed in duplicate. The total phenolic content was expressed as ferulic acid equivalents (FAE) in micrograms per gram of ground wheat.

5.2.3. Determination of antioxidant activity

Antioxidant activity was determined in terms of the free radical scavenging capacity of wheat extracts in 100% methanol against the stable $DPPH^{\bullet}$ as previously described (Mpofu et al 2006). The absorbance (A) of the methanolic extracts was determined at 0 and 30 min using a spectrophotometer at 515 nm. Methanol was used as a blank and antioxidant activity (AOA) calculated as percent discoloration:

$$\text{Percent discoloration} = (1 - [A \text{ of sample}_{t=30}/A \text{ of control}_{t=0}]) \times 100.$$

The extractions and tests were all carried out in duplicate.

5.2.4. Determination of phenolic acid composition

The wheat samples were hydrolyzed with 4 M NaOH for 4 h at room temperature, acidified with cold 6 M HCl, and extracted with ethyl acetate as described previously

(Mpofu et al 2006). The ethyl acetate was evaporated to dryness at 35°C and the residue re-dissolved in 50 % methanol then filtered through a 0.45 μm nylon filter. The filtrate was stored in the dark at -20°C until it was analyzed using HPLC. HPLC analysis was carried out as described previously (Mpofu et al 2006). Briefly, reverse-phase HPLC with a Waters μBondapak C18 column (3.9 × 300 mm) was employed. Phenolic acids were separated using a linear gradient elution program with mobile phase A (water containing 1% (v/v) HAc) and mobile phase B (100% methanol). The solvent gradient was programmed from 15 to 100% of mobile phase B in 33 min with a flow rate of 1.5 mL/min. Phenolic acids in the eluents were monitored at 280 nm using a photodiode array detector. The following phenolic acids were quantified: Gallic acid (GA), p-Hydroxybenzoic (PHBA), Vanillic acid (VA), Caffeic acid (CA), Syringic acid (SA), p-Coumaric acid (PCA), Ferulic acid (FA) and o-Coumaric acid (OCA). Identification of the phenolic acids was accomplished by comparing the retention times of peaks in wheat samples to those of phenolic acid standards. The HPLC analyses were carried out in duplicate.

5.2.5. Statistical Analysis

The data were analyzed by analysis of variance (ANOVA). The ANOVA was performed with the general linear model (GLM) of the SAS software package (release 8.2) (SAS Institute, Cary, U.S.A.) using the split plot design and analyzing genotype and environment as fixed effects. Genotype (G), environment (E) and G × E effects were determined using replicates as blocks with measurements from duplicate analyses for each plot. Comparison of means was done at the 5% significance level using Duncan's

multiple range test. Correlation analyses were performed with the PROC Corr procedure of the SAS software package using the Pearson correlation test.

5.3. Results

5.3.1. Genotype and environmental effects

There were significant differences ($p<0.05$) among the six genotypes for total phenolic content (TPC) (Fig. 5.1). TPC averaged by genotype over all locations ranged between 2116 and 2404 μg ferulic acid equivalents (FAE)/g. Five out of the six genotypes, Neepawa, AC Barrie, Snowbird, AC Elsa and Superb had similar TPCs. AC Vista had the lowest TPC, its TPC was however similar to that of Neepawa, but significantly different ($p<0.05$) from the rest of the genotypes.

No significant difference was detected between the antioxidant activities (AOAs) of the samples when averaged by genotype (Fig. 5.2). The AOAs ranged between 13.3 and 14.9%. The concentrations of each of the phenolic acids tested averaged by genotype are presented in Table 5.2. The genotypes differed significantly ($p<0.05$) in concentration of VA, SA, FA, PCA and OCA. Concentrations of GA, PHBA as well as CA were similar among the genotypes.

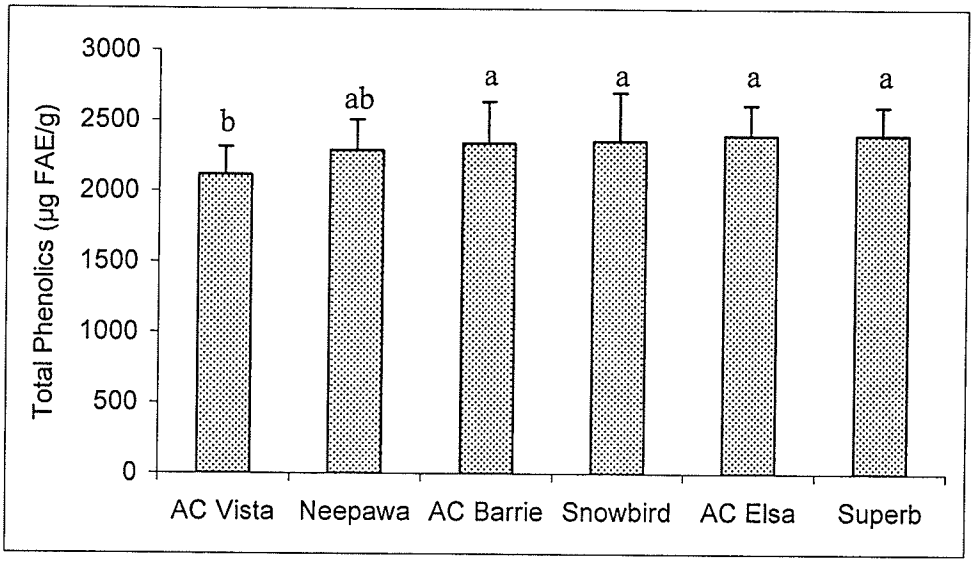

Figure 5.1. Mean total phenolic contents (µg ferulic acid equivalents (FAE)/g) of six wheat genotypes. The vertical bars represent the standard deviation ($n = 9$). Mean values marked by the same letter are not significantly different ($p < 0.05$)

AC Elsa had the highest concentration of SA, FA and OCA, and its concentration of VA (9.22±2.51) was not significantly different from that of AC Barrie (9.25±1.46) which was the highest (Table 5.2.). Superb had the lowest concentrations of SA, PCA and FA, and its concentrations of VA and OCA were similar to those of AC Vista which had the lowest concentration of both.

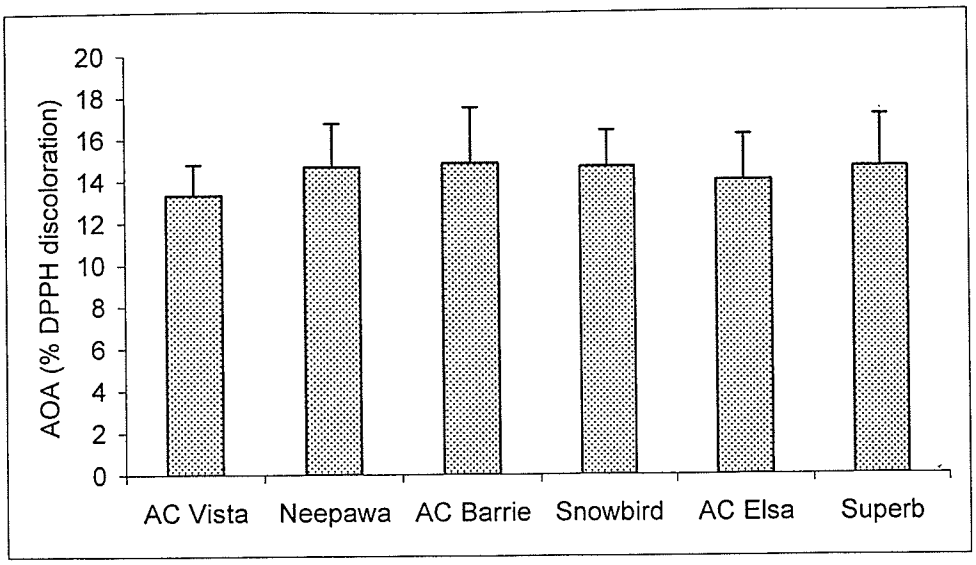

Figure 5.2. Mean antioxidant activities (% DPPH scavenging capacity) of six wheat genotypes. The vertical bars represent the standard deviation ($n = 9$).

Analysis of data per location revealed significant genotype differences in the TPC, and PHBA, VA, PCA and FA concentrations of the genotypes grown in Ca-04. In Re-04, genotypes only differed significantly in their SA concentration. Significant differences in PHBA, VA, SA, PCA and OCA concentrations were detected among genotypes in SC-04. AC Elsa consistently ranked high among the genotypes for all the parameters tested (Table 5.2). This genotype also ranked highly when data was analyzed by location for TPC and concentrations of PHBA, VA and FA in Ca-04. In Re-04, AC Elsa had the highest concentration of SA (15.76 μg/g) and differed significantly from the rest of the genotypes. In SC-04, Neepawa dominated the upper ranks more than AC Elsa for the concentrations of phenolic acids where significant differences were detected among the genotypes (PHBA, VA, SA, PCA and OCA). AC Elsa was however in the upper ranking for SA and PCA concentrations.

Table 5.2. Mean phenolic acid concentrations of wheat genotypes‡*

Genotype	GA (µg/g)	PHBA (µg/g)	VA (µg/g)	CA (µg/g)	SA (µg/g)	PCA (µg/g)	FA (µg/g)	OCA (µg/g)
AC Barrie	2.2±0.5	3.0±1.5	9.3a±2.0	15.8±1.9	10.8b±1.1	22.0±4.2	435.7b±73.3	88.4b±49.7
AC Elsa	2.3±0.6	3.3±1.3	9.2a±2.5	16.0±3.9	12.9a±2.5	23.8ab±8.0	517.1a±177.1	154.5a±85.0
AC Vista	2.1±0.9	2.7±1.1	6.5c±1.9	14.3±3.2	10.2b±1.7	26.6a±6.6	458.9b±74.6	82.6b±21.9
Neepawa	1.9±0.6	3.6±0.8	8.8ab±1.4	14.8±2.0	11.0b±1.6	24.1ab±5.4	447.2b±65.4	112.4ab±48.2
Snowbird	1.9±0.4	3.8±1.1	7.8bc±2.3	15.0±2.8	11.0b±2.0	26.7a±5.7	451.8b±103.2	97.5b±27.6
Superb	2.4±0.6	3.6±1.0	6.6c±1.2	14.3±1.6	9.1c±1.1	17.7c±4.5	419.7b±83.0	123.2ab±69.4

‡ Values expressed as mean ± standard deviation (n=9)

* Means in same column followed by different letters are significantly different ($p<0.05$).

Growing environments differed significantly ($p<0.01$) in all the parameters measured (Fig. 5.3., Fig. 5.4. and Table 5.3.). Wheat grown in Re-04 had the highest levels of each of these parameters, Ca-04 and SC-04 were lower and only differed significantly from each other in concentrations of VA and SA, with wheat from Ca-04 having a lower concentration in both cases (Table 5.3.).

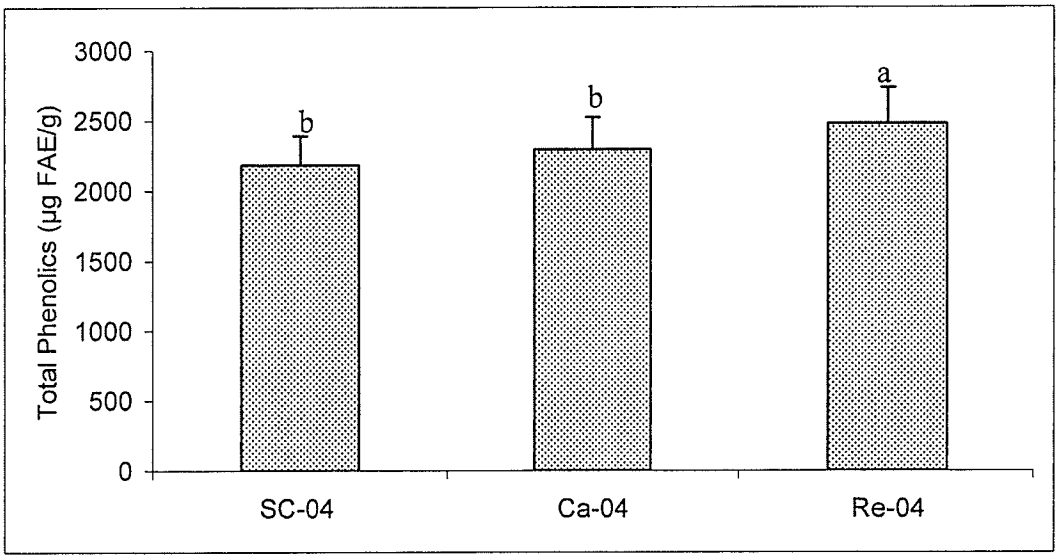

Figure 5.3. Total phenolic contents averaged by growing location. The vertical bars represent the standard deviation ($n = 18$). Mean values marked by the same letter are not significantly different ($p < 0.01$).

There was a significant negative correlation between concentrations of GA and test weight averaged by growing location (r=-0.997). PHBA was similarly correlated to test weight (r=-0.998). An even higher negative correlation was detected between concentration of FA and test weight (r=-0.999). Significant correlations were detected between concentration of VA and mean rainfall (r=0.998) and between the concentration

of SA and mean rainfall (r=0.998). No other correlations were detected between any of the parameters averaged by growing location and the growing environment data reported in Table 5.1. Significant, negative correlations were detected between the degree of *Fusarium* damage and DPPH as well as TPC (r=-0.511 and r=-0.662, respectively) in wheat from Ca-04. In wheat from Re-04, significant and negative correlations were detected between test weight and TPC (r=-0.617). Test weight was also negatively correlated to concentrations of GA, FA, and OCA, (r=-0.439, r=-0.778 and r=-0.490 respectively).

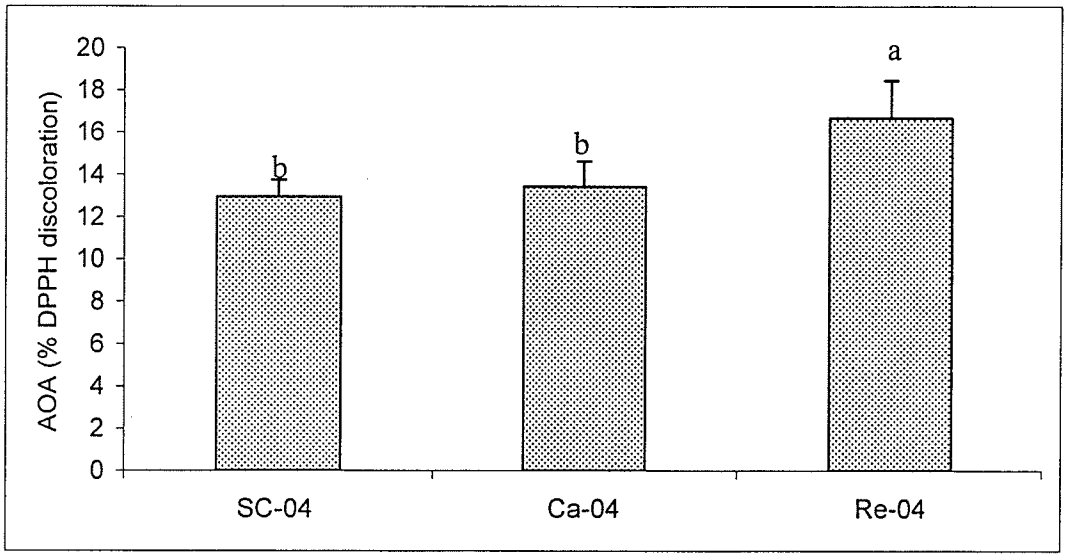

Figure 5.4. Antioxidant activity (% DPPH scavenging capacity) averaged by site-year. The vertical bars represent the standard deviation ($n = 18$). Mean values marked by the same letter are not significantly different ($p < 0.01$)

Table 5.3. Mean phenolic acid concentrations of wheat genotypes‡*

Site-year	GA (µg/g)	PHBA (µg/g)	VA (µg/g)	CA (µg/g)	SA (µg/g)	PCA (µg/g)	FA (µg/g)	OCA (µg/g)
Re-04	2.6a±0.7	4.3a±1.3	9.2a±2.3	17.0a±2.9	12.17a±2.13	29.7a±6.0	563a±108	153a±78
Ca-04	1.9b±0.4	2.8b±0.8	6.9c±1.9	13.5b±1.9	9.65c±1.69	21.2b±3.5	402b±38	77b±24
SC-04	1.9b±0.4	2.9b±0.7	8.0b±1.8	14.5b±1.6	10.7b±1.40	19.5b±3.9	400b±40	99b±20

‡ Values expressed as mean ± standard deviation (n=18)

* Means in same column followed by different letters are significantly different ($p<0.05$).

5.3.2. Inter-correlations among total phenolics, antioxidant activity and concentrations of phenolic acids

Inter-correlations among total phenolics, antioxidant activity and concentrations of phenolic acids are presented in Table 5.4. There was a highly significant ($p<0.0001$) correlation between TPC and AOA ($r=0.594$). TPC was significantly correlated ($p<0.01$) to the concentrations of GA, FA and OCA ($r=0.356$, $r=0.386$ and $r=0.477$). There were highly significant correlations between AOA and concentrations of PCA, FA and OCA ($r=0.553$, $r=0.653$ and $r=0.656$ respectively) and significant ($p<0.01$) correlations between AOA and the concentrations of GA, VA, CA and SA ($r=0.309$, $r=0.373$, $r=0.478$ and $r=0.373$ respectively). No significant correlations were detected between concentrations of GA and PA, PCA and FA and between the concentration of PA and that of any other phenolic acid, otherwise concentrations of all the phenolic acids were significantly correlated (Table 5.4.).

Table 5.4. Correlation coefficients between total phenolic content, AOA and phenolic acid concentrations (n=54)

	TPC	GA	PA	VA	CA	SA	PCA	FA	OCA
Gallic acid (GA)	0.356**								
Protocatechuic acid (PA)	0.109								
Vanillic acid (VA)	0.060	0.241	0.173						
Caffeic acid (CA)	0.259	0.477**	0.109	0.732***					
Syringic acid (SA)	0.117	0.334*	0.005	0.767***	0.787***				
p-Coumaric acid (PCA)	0.245	0.383**	-0.041	0.418**	0.592***	0.581***			
Ferulic acid (FA)	0.386**	0.554***	0.043	0.583***	0.774***	0.755***	0.826***		
o-Coumaric acid (OCA)	0.477**	0.520***	-0.096	0.384**	0.512***	0.515***	0.418	0.701***	
AOA	0.594***	0.309**	0.041	0.373**	0.478**	0.373**	0.553***	0.653***	0.656***

* Significant ($p<0.05$)
** Significant ($p<0.01$)
** Highly significant ($p<0.0001$)

5.3.3. Relative influence of genotype, environment and G × E interactions

The relative influences of genotype, environment and G × E interactions on each of the parameters tested in this study are summarized in Table 5.5. The information presented in this table is a comparison of the proportion each mean square contributes to total mean squares for each variance component.

As with the mean difference results for the measured parameters (Tables 5.2. and 5.3., and Figs. 5.1. 5.2., 5.3. and 5.4.), variance due to genotype was significant for TPC, concentrations of VA, SA, FA, PCA and OCA and not significant for AOA as well as for concentrations of GA, PHBA and CA. Variance due to growing environment was significant for AOA, TPC and concentrations of all the phenolic acids tested. Together, genotype and growing environment explained from 74 % (TPC) to 96 % (PCA concentration) of the total variance. The contribution of growing environment to total variation was more than that of genotype for all the parameters tested by between 18% (in concentration of VA) and 85% (in AOA).

For all the parameters tested, except concentration of CA (which was influenced more by G × E interaction than G), the influence of G × E interaction was less than that of both G and E. The influence of G × E interaction on CA concentration was however not significant. Concentrations of PHA, CA and FA were the only response factors significantly influenced by G × E interactions ($p<0.05$). The VA concentration of AC Vista was more influenced by G × E interaction, (CV across locations=29.97%) than that of the other genotypes. G × E interaction was more influential in the SA and FA concentrations of AC Elsa (CV=19.85% and 34.37%, respectively) than for concentration of these two phenolic acids in other genotypes. The concentrations of VA and SA in

Superb were more stable to G × E interaction (CV=11.04% and 5.50%, respectively) than concentrations of these phenolic acids in other genotypes, while concentration of FA, was most stable in Neepawa (CV=13.02%).

Table 5.5. Variance components (% of total mean squares) for genotype (G), growing environment (E), and G × E interaction effects for total phenolic content, antioxidant activity, and phenolic acid composition of six wheat genotypes grown at three locations

Variance components & degrees of freedom:	G (5)	E (2)	G X E (10)	G X Block/E (30)	Block/E (6)
Parameter					
Total Phenolic content (µg FAE/g)	15.26*	58.96**	10.77	5.53	9.48
Antioxidant activity (% DPPH discoloration)	3.73	88.91***	1.51	1.61	4.24
Gallic acid (µg/g)	9.38	72.35**	5.70	7.60	4.97
p-Hydroxybenzoic acid (µg/g)	9.96	75.94**	5.82	6.55	1.73
Vanillic acid (µg/g)	29.71**	48.10**	10.48*	4.10	7.61
Caffeic acid (µg/g)	5.85	72.56***	7.67	5.26	8.65
Syringic acid (µg/g)	28.77***	57.33***	7.28*	2.50	4.11
p-Coumaric acid (µg/g)	14.70***	79.04***	1.60	1.65	3.02
Ferulic acid (µg/g)	5.40*	85.02***	3.44*	1.48	4.66
o-Coumaric acid (µg/g)	15.83*	69.55***	4.25	4.85	5.53

* Significant ($p<0.05$)
** Significant ($p<0.01$)
*** Highly significant ($p<0.0001$)
† Significance is based on genotype × block/E mean square
‡ Significance is based on block/E mean square

Table 5.6. Mean concentrations and coefficients of variation (CV) for VA, SA and FA of six wheat genotypes grown at each of three locations

	Vanillic acid (µg/g)			
Genotype	Ca-04	Re-04	SC-04	CV (%)
AC Barrie	8.0a±0.8	10.4±3.2	9.3a±0.8	12.78
AC Elsa	8.9a±1.3	11.8±2.4	7.0b±0.7	25.98
AC Vista	4.9b±1.3	8.7±1.1	5.9b±0.4	29.97
Neepawa	8.1a±1.7	8.3±0.6	10.0a±0.9	12.04
Snowbird	5.6b±0.8	8.6±2.5	9.1a±1.7	24.17
Superb	5.8b±1.4	7.2±0.6	6.8b±1.4	11.04

	Syringic acid (µg/g)			
Genotype	Ca-04	Re-04	SC-04	CV (%)
AC Barrie	9.7±0.1	12.0b±0.7	10.7abc±0.4	10.68
AC Elsa	12.3±0.2	15.8a±2.0	10.8abc±0.8	19.85
AC Vista	8.8±1.6	11.8b±1.3	9.9bc±0.4	15.17
Neepawa	9.7±2.1	11.6b±0.3	11.8ab±1.4	10.4
Snowbird	8.8±1.4	12.2b±0.7	12.1a±1.4	17.78
Superb	8.7±1.2	9.6c±1.4	8.9c±0.9	5.5

	Ferulic acid (µg/g)			CV (%)
Genotype	Ca-04	Re-04	SC-04	
AC Barrie	406ab±8	526±50	376±7	18.23
AC Elsa	449a±28	719±165	384±52	34.37
AC Vista	415ab±21	552±38	409 ±26	17.63
Neepawa	403abc±51	513±51	425±42	13.02
Snowbird	383bc±10	543±136	430±60	18.25
Superb	356c±31	525±33	377±3	21.98

* For any of the phenolic acids, means in the same column followed by different letters are significantly different ($p<0.05$)

5.4. Discussion

Phenolic compounds have several roles in plants including protecting the plants from the reactive oxygen species (ROS) generated during periods of stress (Dixon and Paiva 1995). When consumed as part of food, phenolics can contribute to protection of the consumer against several forms of cancer and cardiovascular diseases among other degenerative diseases. Selecting for high phenolic antioxidant levels in a breeding program would be desirable to increase the resistance of wheat to different forms of plant stress, most of which reduce yield and to improve human health benefits. Knowledge of how synthesis of phenolics in wheat genotypes responds to different environmental conditions is essential for breeding. It is also important to know the degree to which genotype and environment interact in synthesis of phenolic compounds. Investigation of these effects was in this case done in wheat that had undergone environmental stress (*Fusarium* head blight and frost damage) and compared with wheat that had grown under normal conditions.

Significant differences ($p<0.05$) were detected among genotypes averaged over all growing environments for TPC and concentrations of VA, SA, PCA, FA and OCA. Similar to our previous report (Mpofu et al 2006), there was no link between genotype color (red vs. white) and any of the antioxidant properties measured. Beta et al 2005 made a similar observation, contrary to the report by Milller and others (2000) that red wheat has superior antioxidant properties. It is, however worth noting that Miller et al (2000) based their conclusions on a comparison of two wheat samples whose genotypes or growing conditions they did not specify. The two white colored wheat genotypes (AC Vista and Snowbird had significantly different TPCs. The TPC of AC Vista was lower

and was the lowest among the six genotypes, but it was similar to that of Neepawa, a red colored genotype. Although significantly different from that of AC Vista, the TPC of AC Snowbird was also similar to that of Neepawa and additionally similar to that of the other three red wheat genotypes, AC Barrie, AC Elsa and Superb, clearly indicating the lack of a link between color and phenolic antioxidant properties.

The influence of growing environment was significant for TPC, AOA and concentrations of all the phenolic acids measured. Growing environment had a greater influence than genotype for all these response factors. Depending on the response factor, growing environment accounted for between 48% (in VA concentration) and 89% (AOA) of total variance while genotype accounted for between 3% (AOA) and 30% (VA concentration) clearly showing how predominant environmental influences were. Compared to our previous report (Mpofu et al 2006), there was a marked reduction in the contribution of genotype to total variation in antioxidant properties, (genotype accounted for between 17% and 65% of total variation in the previous study). The observed difference is likely due to the marked difference in environmental conditions as the wheat from two sites in the present study was affected by frost and *Fusarium* damage, whereas all the wheat investigated previously was all of sound milling quality.

Averaged by growing location, concentrations of GA, PHBA and FA were negatively correlated to the location average test weights reported in Table 5.1. Test weight is a measure of soundness of wheat; it is influenced by any factor that alters size and shape of kernels such as heat stress, drought, frost damage, or disease (Abaye et al 1997). Sound wheat has high test weight, whereas damaged wheat has low values. Interestingly, the wheat from Ca-04 which was *Fusarium* damaged had a similar average

test weight to that from SC-04 which was of sound milling quality. Wheat from Re-04 had a much lower average test weight likely indicating greater damage and lower milling quality than Ca-04 and SC-04 wheat. Average concentration of VA and SA per location was significantly correlated with rainfall (r=0.998 for both). Woodhead (1981) reported lack of significant correlation between rainfall and the concentrations of phenolics in sorghum. Moisture stress (which likely did not occur in the wheat investigated) may, however, elicit antioxidant responses which invariably include synthesis of phenolics (Dixon and Paiva 1995).

Levels of all the response factors measured in wheat from Ca-04 and SC-04 were quite comparable. Significant differences between Ca-04 and SC-04 wheat were only detected in average concentrations of VA and SA. In both cases, Ca-04 wheat had lower average concentrations. *Fusarium* damage therefore on average seems not to alter net levels of phenolic compounds. Correlation analyses, however, revealed significant, negative correlations between the degree of *Fusarium* damage and AOA (DPPH scavenging capacity) as well as TPC (r=-0.511 and r=-0.662 respectively), in wheat from Ca-04 indicating that *Fusarium* resistant genotypes had higher levels of TPC and antioxidant activity than susceptible ones. Since no significant correlation was detected between *Fusarium* damage and concentration of any of the phenolic acids, the observed correlations likely indicate accumulation of phenolic compounds that were not characterized in this study. According to McKeehen et al (1998), accumulation of ferulic acid from anthesis until ~20 days after anthesis is likely related with cultivar resistance to *Fusarium*, however, in mature wheat, concentrations of ferulic acid in the grain of resistant and susceptible cultivars are similar. It is therefore highly likely that towards

maturity, the ferulic acid in resistant wheat gets converted to other phenolic compounds as it would have served its purpose of inhibiting infection since susceptibility to *Fusarium* infection is greatest at anthesis. Ferulic acid is synthesized through the phenylpropanoid pathway and like other simple phenylpropanoids, it rarely accumulates to high levels in plant cells, tending instead to get conjugated to sugars, cell wall carbohydrates, or organic acids (Dixon and Paiva 2005). Ferulic acid and other simple phenylpropanoids also serve as precursors for more complex phenolics such as flavonoids, tannins, suberin and lignin (Shahidi and Naczk 2004). It is therefore possible that FA got converted to some of these compounds.

Test weight was negatively correlated with TPC, GA, FA, and OCA in wheat from Re-04. These negative correlations suggest that frost damage resistant wheat accumulated phenolic compounds including GA, FA, and OCA (assuming test weight to be an indication of frost damage, since it was the predominant source of damage in wheat from Re-04). Phenolic compounds accumulate in plants as a result of cold acclimation, a process that results in some changes in plant morphology, anatomy, physiology, and biochemistry (Trunova 1984, Klimov 2001). Growth of soybeans at lowered temperature resulted in the accumulation of phenolic compounds in their roots (Janas 2002). Zagoskina et al (2005) concluded that low temperature activates phenylpropanoid and especially flavonoid biosynthetic pathways in the leaves of wheat plants during cold hardening resulting in accumulation of soluble phenolic compounds, including flavonoids.

Since frost-damaged wheat has higher levels of antioxidant compounds, it can potentially be used in functional foods and for extraction of nutraceuticals. Use of frost

damaged wheat for these purposes would however be complicated by its hardness, which makes it difficult to roller mill (Dexter et al 1985).

The TPC values obtained for wheat harvested from SC-04 (2184±208.6 μgFAE/g) were similar to values obtained for wheat harvested from the same growing location in 2003 (1889±119.1 μgFAE/g) (Mpofu et al 2006). AOA and concentrations of VA, CA, SA, PCA, FA and OCA were also similar for the two harvests from this site indicating the high likelihood of lack of year effects for this growing environment. The trend was different for the 2003 and 2004 harvests from Regina. Wheat from Re-04 had higher TPC, AOA, and concentrations of VA, CA, SA, PCA, FA, and OCA than the 2003 wheat harvested from the same site. The higher levels in Re-04 were likely a result of accumulation of phenolics during cold acclimation of this predominantly frost-damaged wheat as concluded by Zagoskina et al (2005) regarding wheat plant leaves that had undergone cold hardening. The attenuation of starch synthesis due to frost damage might as well have contributed to the increased concentration of antioxidants highlighting the need for further research.

Contrary to our previous report (Mpofu et al 2006), G × E interaction effects were not significant for TPC, but significant for concentrations of VA, SA and FA. For these variables, the contribution of G × E interaction to total variation ranged between 3.4% and 10.5%. Superb was most stable to G × E interaction for concentrations of VA and SA (CV across locations of 11% and 5.5% respectively). For concentration of FA, Neepawa with a CV=13 % was most stable to interaction. AC Elsa (19.8%<CV<34.4%) was the least stable genotype to influences of G × E interaction for concentrations of all three phenolic acids.

In line with literature findings (Onyeneho and Hettiarachchy 1992, Adom et al 2003, Zhou et al 2004a,b, Zhou and Yu 2004, Mpofu et al 2006), ferulic acid was the predominant phenolic acid. Ferulic acid accounted for between 70 and 76% of the total amount of measured phenolic acids in the wheat samples. In addition to VA, CA, SA, PCA, FA and OCA which were detected in the 2003 wheat samples (Mpofu et al 2006), GA and PHBA were detected in consistent amounts in this study. GA accounted for between 0.3 and 0.4%, whilst PHBA accounted for 0.4 to 0.6% of the total amount of phenolic acids. Phenolic acids present in wheat include ferulic, p-coumaric, vanillic, caffeic, chlorogenic, gentisic, syringic, and *p*-hydroxybenzoic acids (Onyeneho and Hettiarachchy 1992).

As reported in literature (Adom and Liu 2002, Adom et al 2003, Zhou et al 2004, Beta et al 2005, Li et al 2005, Mpofu et al 2006), TPC and concentration of FA were highly correlated with AOA; this likely indicates that these two parameters are good predictors of AOA. The correlation between FA and TPC (r=0.386) was less than the previously reported correlation (r=0.842) (Mpofu et al 2006), likely indicating greater contribution to TPC by other phenolic compounds for most of the wheat samples assayed. Apart from FA and other phenolic acids, the phenolic compounds present in wheat which potentially contribute to TPC include alkylresorcinols, flavanoids, phenolic acid diacyl glycerols, phenolic aldehydes and ferulates (Shahidi and Naczk 2004). Significant correlations were observed between most of the phenolic acid concentrations, most likely because these compounds are synthesized via the same biosynthetic pathway; the phenylpropanoid pathway and can therefore accumulate in ways that are related.

5.5. Conclusions

The effects of genotype, growing environment and genotype × growing environment interaction on the total phenolic content, antioxidant activity and phenolic acid composition of sound, frost-damaged and *Fusarium*-damaged wheat have been documented for the first time. Genotype effects were significant ($p<0.05$) for TPC and concentrations of VA, SA, PCA, FA and OCA. AC Elsa had the highest concentration of SA, FA and OCA, and its concentration of VA was not significantly different from that of AC Barrie which had the highest VA concentration. Superb had the lowest concentrations of SA, PCA and FA, and its concentrations of VA and OCA were similar to those of AC Vista which had the lowest concentration of both. Growing environment effects were significant for TPC, AOA and concentrations of all the phenolic acids measured. Frost-damaged wheat (Re-04) had the highest levels of all the response variables investigated. There was an indication of net accumulation of GA, FA, OCA (and possibly other phenolic compounds not characterized in this study) in frost resistant wheat since TPC and concentrations of GA, FA, and OCA in wheat from Re-04 were negatively correlated to test weight. *Fusarium*-damaged (Ca-04) and sound (SC-04) wheat only differed significantly in average concentrations of VA and SA out of all the parameters measured. *Fusarium*-damaged wheat had lower average concentrations of both VA and SA. Unlike frost damage, *Fusarium* damage (FD) likely does not result in net alteration of phenolic compound levels. FD resistant wheat, however likely accumulates phenolic compounds that were not determined in this study since the degree of FD was negatively correlated to AOA and TPC, but not to concentration of any of the phenolic acids measured.

The effects of interaction between genotype and environment were only significant for concentrations of PHA, CA and FA. For the concentrations of these three phenolic acids, G × E interaction accounted for 3.5% (in concentration of FA) to 10.5% (in concentration of VA) of total variation. Presence of a significant genetic component in the variation of TPC and concentrations of VA, SA, PCA, FA and OCA indicates that these traits can potentially be selected for in a genotype development program. These traits are, however, likely more influenced by environmental factors for the wheat genotypes studied and also significantly influenced by G × E interaction in the case of concentrations of PHA, CA and FA.

Given these observations, it is seemingly apparent that the successful breeding of wheat with high phenolic antioxidants and associated agronomical and human health advantages will depend on identification of a genotype that is more stable to influences of the environment and interactions between genotype and environmental factors. Future studies will target wheat with a more diverse genetic base and additionally quantify the other phenolic compounds present in wheat including flavonoids, tannins, suberin and lignin. Quantification of non phenolic antioxidants including tocopherols and carotenoids will also be targeted in future studies. Multi-year studies will be conducted as well in order to determine the hereditability of antioxidant properties.

GENERAL DISCUSSION AND CONCLUSION

Effects of genotype, growing environment and/or genotype × environment interactions were suggested in several studies of wheat phenolic antioxidant properties (Onyeneho and Hettiarachchy 2003, Zhou and Yu 2004, Zhou et al 2004a,b, Beta et al 2005). At the initiation of this research project, there had been no reports on the relative contributions of genotype and the growing environment of wheat to its phenolic composition and antioxidant activity. This thesis research project was carried out to investigate the effects of genotype, growing environment and genotype × environment interactions on the total phenolic content, phenolic acid composition and antioxidant activities of wheat genotypes grown in the Canadian Prairie region.

Total phenolic content was determined using the Folin Ciocalteu method. Phenolic acid composition was determined using high performance liquid chromatography (HPLC) analysis. Antioxidant activity was determined in terms of DPPH, superoxide and peroxyl radical scavenging capacities using the DPPH method, the photochemiluminescence) assay and the oxygen radical scavenging capacity assay respectively for the 2003 samples. Only the DPPH method was used to determine the AOA of the 2004 samples based on its simplicity, low cost and high correlation with TPC and concentration of FA, the principal phenolic acid in wheat.

Genotype effects were highly significant ($p<0.0001$) for TPC, AOA and concentrations of ferulic acid (FA), o-coumaric acid (OCA), p-coumaric acid (PCA), syringic acid (SA), caffeic acid (CA), and vanillic acid (VA) in 2003 wheat harvest. Highly significant genotype effects were similarly detected in the superoxide scavenging capacities of water (SSCW) and lipid (SSCL) soluble substances, however, in the

hydrophilic (H-ORAC$_{FL}$), lipophilic (L-ORAC$_{FL}$) and total (T-ORAC$_{FL}$) ORAC$_{FL}$ values of the same sample set, genotype effects were not significant. For 2004 samples, genotype effects were significant ($p<0.05$) for TPC and concentrations of VA, SA, FA, PCA and OCA, and not significant for AOA or concentrations of CA, gallic acid (GA) and p-hydroxybenzoic acid (PHBA). Interaction effects were significant for TPC, SSCW and SSCL among the parameters measured in 2003 wheat. In the 2004 wheat, interaction effects were significant for concentrations of VA, SA and FA, a significant change from 2003 samples where interaction was not significant for concentrations of these phenolic acids. Growing environment effects were significant ($p<0.01$) for all the parameters measured in the 2003 and 2004 samples.

FA was the predominant phenolic acid. It accounted for between 49.7 and 64.8 % of the total amount of measured phenolic acids in the 2003 wheat samples, and it accounted for between 70% and 76% of the phenolic acids in the 2004 samples. FA exists primarily in a bound form with arabinoxylans in the outer layers of cereal grains and constitutes up to 90% of the total phenolic acids in wheat (Sosulski et al 1982). The predominance of ferulic acid among wheat phenolic acids is very apparent in literature and has been reported by Onyeneho and Hettiarachchy (1992), Adom et al (2003), Zhou et al (2004a,b), Zhou and Yu (2004) and Li et al 2005 among other workers. Wheat genotype AC Elsa had the highest ferulic acid concentration of 441.02±45.51μg/g in the 2003 wheat harvest and 517.05±177.14 μg/g in the 2004 harvest. Given its high concentration of FA, AC Elsa would likely be an ideal candidate for breeding wheat with elevated levels of FA, however it was the most unstable genotype to genotype × growing

environment interaction effects on FA concentration in the 2004 crop year (CV across locations = 34.37%).

The ranking order of genotypes for different antioxidant response variables was not consistent. For example, all genotypes of the 2003 wheat had similar hydrophilic (H-$ORAC_{FL}$), lipophilic (L-$ORAC_{FL}$) and total (T-$ORAC_{FL}$) $ORAC_{FL}$ values, but highly significant differences in AOAs (measured in terms of DPPH radical scavenging capacities). Accordingly, the Duncan's multiple range test generated four groupings of the genotypes based on their mean AOAs highlighting the significant differences. Ranking of mean growing environment values for different variables was not consistent either. For example, there were significant differences in the SSCL averages and significant differences in L-$ORAC_{FL}$ for wheat harvested from different locations in 2003. Winnipeg wheat had the highest average SSCL, followed by wheat from Swift Current then Melfort and Regina wheat had the lowest. The ranking was different for average L-$ORAC_{FL}$ per location with Swift Current wheat having a higher L-$ORAC_{FL}$ than wheat from the other locations which all had similar L-$ORAC_{FL}$ values. The observed ranking inconsistencies are likely due to underlying differences in the chemical principles governing the tests. These differences also likely in part account for the differences observed in the inter-correlations between the antioxidant properties and TPC as well as concentrations of phenolic acids. The $ORAC_{FL}$ and PCL assays are based on the hydrogen atom transfer (HAT) mechanism, whereas the DPPH method is based on the HAT and another mechanism, the single electron transfer (SET) mechanism (Prior et al 2005). HAT based methods measure the ability of an antioxidant to quench free radicals by hydrogen donation whereas SET-based methods detect the ability of a

potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals (Wright et al 2001). The methods of extraction and solvents used also likely influence results of antioxidant tests as suggested by Zhou and Yu (2004) limiting the conclusions drawn for this thesis research to the methods, solvents and extraction conditions used. There are currently no standard methods for antioxidant determination although efforts are being made to standardize methods (Prior et al 2005).

Significant genotype effects indicate the possibility of selecting for a trait in a cultivar development program (Emmons and Peterson 2001). Indications from the 2003 sample set were that CA and PCA are influenced more by genotype than growing environment, however in the 2004 wheat, genotype did not have a significant effect on the concentration of CA and the concentration of PCA was more influenced by growing environment (79% of total variance) than genotype (14.7% of total variance). The dominance of growing environment variance over genotype variance in contributing to total variance reflects the role of environmental factors in synthesis of phenolic compounds. The phenylpropanoid pathway for the synthesis of phenolic compounds readily responds to various plant stressors including pathogens, and UV-B radiation (Dixon and Paiva 1995).

In conclusion, this study reported for the first time, relative contributions of genotype, growing environment and genotype × growing environment interaction effects to total variation of total phenolic content (TPC), concentrations of gallic acid (GA), p-hydroxybenzoic (PHBA), vanillic acid (VA), caffeic acid (CA), syringic acid (SA), p-coumaric acid (PCA), ferulic acid (FA) and o-coumaric acid (OCA), and antioxidant

activities (AOAs) determined in terms of DPPH, superoxide and peroxyl radical scavenging capacities.

The relative contribution of genotype and growing environment variances to total variance largely indicated domination of growing environment especially in wheat exposed to environmental plant stressors *Fusarium* and frost damage. Variation in the superoxide radical scavenging capacity of water soluble substances was influenced more by genotype than growing environment whereas that of lipid soluble substances (SSCL) was influenced more by environment. Hydrophilic (H-$ORAC_{FL}$), lipophilic (L-$ORAC_{FL}$) and total (T-$ORAC_{FL}$) $ORAC_{FL}$ were significantly influenced by growing environment and were not under significant genotype influences.

Frost-damaged wheat had a significantly higher TPC, AOA (DPPH scavenging capacity) and higher concentrations of GA, PHBA, VA, CA, SA, PCA, FA and OCA than *Fusarium*-damaged wheat and wheat of sound milling quality. Cold acclimation most probably caused greater net increases in the TPC and concentrations of GA, PHBA and FA in frost resistant than susceptible wheat. *Fusarium* infection likely caused a net increase in the TPC and AOA of resistant wheat, but no net increase in concentration of phenolic acids.

The genotype differences for concentrations of FA, VA and SA detected in wheat from both the harvests studied (2003 and 2004) indicate that it would likely be possible to select for these quantitative traits in a genotype development program. Successful breeding of wheat with high phenolic antioxidant properties and associated agronomical and human heath benefits will most likely depend on identification of a genotype that is

stable to influences of the environment and interactions between genotype and environmental factors.

Extending this study to cover a more diverse range of genotypes and growing environments and conducting it over several years to determine the heritability of the antioxidant properties would be highly desirable. Future research should additionally:

- investigate G × E variation in concentrations of non-phenolic antioxidants particularly lipophilic antioxidants such as tocopherols and carotenoids. Other phenolics found in wheat; alkylresorcinols, flavanoids, ferulates, tannins, suberin and lignin should also be investigated,
- determine effects of processing on phenolic antioxidant properties and
- include *in-vivo* tests so as to elucidate the physiological effects of wheat antioxidants.

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