Determination of Antioxidant Capacity of Selenium-Enriched Wheat Seeds

By NIHAL OZTOLAN

Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Master of Science

SABANCI UNIVERSITY
June 2009

Determination of Antioxidant Capacity of Selenium-Enriched Wheat Seeds

Approved By

Prof. Dr İsmail Çakmak.....

Doç. Dr. Hikmet Budak .....

Prof. Dr. Zehra Sayers.....

Doç. Dr. Ahu Altınkut Uncuoğlu .....

Doç. Dr. Levent Öztürk .....

Date of Approval: 10.08.2009

Determination of Antioxidant Capacity of Selenium-Enriched Wheat Seeds

Nihal Oztolan

BIO, Master's Thesis, 2009

Thesis Supervisor: Ismail Cakmak

Keywords: Antioxidants, Antioxidant Tests, Biofortification, Selenium, Wheat Seeds

Abstract

Selenium-containing proteins are essential in mammalian systems and have critical biochemical functions. Reduction in oxidative stress, inhibition of uncontrolled cell proliferation and preventing different type of cancers are among the significant functions of selenium (Se) in mammalian systems. Due to the fact that animals cannot synthesize their own seleno-proteins, adequate amount of Se must be taken by daily diets. Consuming Se-rich foods is, thus, a crucial issue for human nutrition and health. Among the foods consumed commonly, wheat represents one of the most important staple foods contributing to the daily Se intake of human-beings. Enrichment of wheat with Se is, therefore, an important research area and public health issue. In literature, there is, however, limited information about the antioxidative effects of wheat enriched with Se by applying Se-fertilizers.

This study basically analyzed antioxidant capacity of Se-enriched wheat seeds by using various colorimetric assays and human cell culture methods. The seeds used in this work were obtained from a TUBITAK-supported Se-fertilizer project conducted in different locations in Central Anatolia. The Se concentration of seed samples used showed a a wide range between 28 ppb and 7168 ppb. In order to test the antioxidant capacity of seeds differing in Se concentrations, hot water, cellulose and methanol/ acetone/ water extracts of seeds were used. The assays applied for measurement of antioxidant capacity

of seeds were i) MTT assay by using mammalian cell culture experiments, ii) DPPH (1,1- Diphenyl-2-picrylhydrazyl) radical scavenging test, and iii) ABTS (2,2-Azinobis (3- ethylbenzothiazoline-6-sulfonic acid) radical-scavenging assay. In all these 3 assays applied, there was no consistent effect of the seed extracts with wide range of Se concentrations on the cell viability measured by the MTT assay or in terms of DPPH radical-scavenging or ABTS scavenging capacity. Expected high antioxidant capacity of seed extracts by increasing Se concentrations was not found under given conditions. The reasons for the ineffectiveness of Se-enriched seeds in improving antioxidant capacity might be related to i) the methods applied and/or ii) to the level of the expected increase in antioxidant capacity by Se. Possibly, the level of the expected increase in antioxidant capacity by Se is too low when compared to the inherent antioxidant capacity of seeds, and this difference could not be detected by the methods applied. In future antioxidant tests, attention should be paid to the isolated Se-proteins from seeds differing in Se concentrations.

Selenyum Bakımından Zenginleştirilmiş Buğday Tohumlarının Antiokisidant Kapasitesinin Belirlenmesi

Özet

Önemli biyokimyasal işlevlere sahip olan selenyum (Se) proteinleri, memeli sistemler için mutlak gereklidir. Oksidatif stresin azaltılması, kontrolsüz hızlı hücre çoğalmasının engellenmesi ve değişik kanser türlerinin gelişiminin önüne geçilmesi Se'un memeli sistemlerdeki en önemli işlevlerinden birkaç tanesidir. Hayvansal organizmalar kendi Se proteinlerini sentezleyemedikleri için mutlaka dışardan gıdalar yoluyla yeterli Se almalıdırlar. Bundan dolayıdır ki, Se'ca zengin gıdaların tüketimi insan beslenmesi ve sağlığı için büyük önem arzetmektedir. Genellikle tüketilen gıdalar içinde buğday, insanların günlük Se alımına katkıda bulunan en önemli gıdalardan biridir. Bu nedenledir ki, buğdayın Se bakımından zenginleştirilmesi önemli bir araştırma alanı ve halk sağlığı konusudur. Ancak, literatürde gübreleme yoluyla Se bakımından zenginleştirilmiş buğdayın antioxidant kapasitesi hakkinda çok sınırlı bilgi bulunmaktadir

Bu çalışmada değişik kolorimetrik yöntemler ve insan hücre kültür testleri kullanılarak Se bakımından zenginleştirilmiş tohumların antioxidant kapasitesi ölçülmüştür. Burada kullanılan buğday tohumları Orta Anadolu'da degişik bölgelerde yürütülen TÜBİTAK-destekli bir Se-gübreleme projesinden sağlanmıştır. Testlerde kullanılan tohumların Se konsantrasyonları 28 ppb'ile 7168 ppb arasında geniş bir varyasyon göstermektedir. Selenyum bakımından farklı olan tohumların antioxidant kapasitelerini ölçmek için, tohumların sıcak su, selüloz ve de metanol/ aseton/ su ekstraktları kullanılmıştır. Tohumların antioxidant kapasitenin ölçümünde i) memeli hücre kültürlerinde MTT hücre canlılık testi, ii) DPPH (l,l-Diphenyl-2-picrylhydrazyl) radikali ve iii) ABTS (2,2-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radikali detoksifikasyonu testleri kullanılmıştır. Analizlerde kullanılan bu 3 yöntemde de, Se konsantrasyonları farklı tohum ekstraktları, ne hücre canlılık testlerinde ne de DPPH radikali veya ABTS radikalininin yok edilişine dayanan testlerde tutarlı bir antioxidant kapasitesi gösterebilmiştir. Bu tez çalışmasının koşullarında, Se zenginleştirilmesiyle buğdayın antioksidant kapasitesinde beklenen artış bulunamamıştır. Selenyumca zenginleştirilmiş

w

tohumların antioksidant kapasitelerinin artışındaki yetersizlik, i) testlerde kullanılan yöntemlerle veya ii) Se'lu tohumların antioksidant kapasitesinde umulan artışın düzeyiyle ilişkili olabilir. Olasılıkla, Se zenginleştirilmesiyle tohumların antioksidant kapasitesinde umulan artışın düzeyi, teste tabii tutulan tohumların doğal antioksidant kapasitesine göre, mevcut yöntemlerle farklılığı ölçemeyecek kadar, çok düşüktür. Gelecekteki antioksidant testlerinde, Se konsantrasyonları farklı tohumlardan izole edilen selenyum-proteinleriyle çalışılmasına önem verilmelidir.

To My Mom, Dad, Didem, grandmother and Emre...

ACKNOWLEDGEMENTS

I am grateful to my supervisor Ismail Cakmak, whose advise, support, patience, encouragement, ideas and enthusiasm no doubt influence me to orient my interest towards plant sciences.

I would like to express my appreciation to Hikmet Budak for being always there to listen and help me.

I would like to give my special thanks to the members of my thesis committee: Hikmet Budak, Levent Ozturk, Zehra Sayers, and Ahu Altinkut Uncuoglu for spending their time for me and giving valuable advises to me.

I am deeply thankful to Ozge Ozdemir and Ozgur Gokmen for creating an enjoyable laboratory environment. I would also give my special thanks to Ilcim Ozlu, Derin Demiroglu, Yekta Yamaner and Sumeyye Yar for their valuable friendships.

Yekta, Funda, Ethem, and Didem Oztolan, you are always with me. I always feel your love and support wherever I am.

And Emre, my all, since I met you I became the happiest person in the world. Thank you for being always with me.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....viii

1 Introduction.....1

2 Overview.....5

2.1 Historical background of Se and its chemistry..... 5

2.2 Se as a nutritive agent..... 6

2.3 The availability of Se in soil and its transportation to biological systems 9

2.4 The metabolic activities of Se in both plants and animals 10

2.5 The health impacts of Se 13

3 Materials and Methods.....16

3.1 Materials 16

3.2 Methods 17

3.2.1 Measurement of Antioxidant Capacity of Seeds 17

3.2.2 Preparation of Seed Extracts.....19

3.2.3 Preparation of H2O2 solutionfor MTT Assay 20

3.2.4 Application of Antioxidant Tests 20

3.2.4.1 MTT Cell Viability Tests.....20

3.2.4.2 Scavenging capacity of seed extracts for DPPH free radicals..... 21

3.2.4.3 Detoxification capacity of seed extracts for ABTS cationic free radicals 22

4 Results.....24

4.1 Effect of wheat seed extracts differing in Se concentrations on viability of HeLa cells.....24

4.1.1 Optimization tests on seed extracts andH202 concentrations 24

4.1.2 Effect of wheat seed extracts having different Se concentrations on HeLa cell viability.....26

4.2 Scavenging DPPH free radical 28

4.2.1..... DPPH radical scavenging activity of ascorbic acid..... 28

4.2.2..... DPPH radical scavenging activity of wheat extracts..... 29

4.3 ABTS free radical scavenging activity of Se-enriched wheat.....31

4.3.1 ABTS scavenging capacity of the cellulose extracts.....31

4.3.2 ABTS scavenging capacity of methanol / acetone / water extracts..... 32

6 CONCLUSION.....39
7. REFERENCES:.....40
APPENDIX.....52
Chemicals.....52
Equipment.....52

TABLE OF ABREVIATIONS

Se: Selenium

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium

bromid DPPH: a,adiphenil-P-picrylhydrazyl

ABTS: 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid

GPX: Glutathione peroxidase

Na2Se03: Sodium selenate

SePP: Selenoprotein P

kDa: kiloDalton

DMEM: Dulbecco's Modified Eagle Media

H2O2: Hydrogen peroxide

dPBS: Dulbecco's Phosphate Buffered Saline

SFM: Serum Free Media

EDTA: Ethylenediaminetetraacetic acid

nm: nanometer

DMSO: Dimethyl Sulfoxide

Abs: Absorbance

ppb: parts per billion

TEAC: Trolox equivalent antioxidant capacity pg: Microgram mg:

Milligram

HPLC: High performance liquid chromatography ICP-MS:

Inductively coupled plasma mass spectrometry

LIST OF FIGURES

Figure 1: Selenium metabolism in animals and Se-metabolites (Finley, 2005)..... 7

Figure 2: Metabolic pathways of Se in plants (Whanger, 2004)..... 12

Figure 3: Selenium metabolism in animal cells (Schomburg *et al.*, 2004) 13

Figure 4: Effect of increasing amount of wheat extracts (mg (seed) / ml (water)) on HeLa cell viability. Control indicates the samples without treatment with wheat extracts. In this test wheat seeds were used which contained 894 ppb Se. All results were expressed as relative to the control values (100 %)..... 25

Figure 5: Effect of increasing concentration of H2O2 on HeLa cell viability. Control shows the treatments without H2O2 addition. All results were expressed as relative to the control value (100 %)..... 25

Figure 6: Effect of wheat seed extracts differing in Se concentration on HeLa cell viability. In the tests 20 mg (seed) /ml (medium) wheat extracts were used which were derived from wheat seeds containing 58, 564 and 1521 ppb Se and grown in Izmir. HeLa cells were exposed to H202 after the incubation with wheat seeds. All results were expressed as relative to the control value (100 %) and shows the means of 3 replications 26

Figure 7: Effect of wheat seed extracts differing in Se concentration on HeLa cell viability. In the tests 20 mg (seed) /ml (medium) wheat extracts were used which were derived from wheat seeds containing 294, 1520 and 3182 ppb Se and grown in Samsun. HeLa cells were exposed to H2O2 after the incubation with wheat seeds. All results were expressed as relative to the control value (100 %) and shows the means of 3 replications 27

Figure 8: Effect of wheat seed extracts differing in Se concentration on HeLa cell viability. In the tests 20 mg (seed) /ml (medium) wheat extracts were used which were derived from wheat seeds containing 123, 2249 and 5870 ppb Se and grown in Eskisehir. HeLa cells were exposed to H2O2 after the incubation with wheat seeds.

Figure 9: Effect of wheat seed extracts differing in Se concentration on HeLa cell viability. In the tests 20 mg (seed) /ml (medium) wheat extracts were used which were derived from wheat seeds containing 36, 390 and 894 ppb Se and grown in Konya. HeLa cells were exposed to H2O2 after the incubation with wheat seeds. All results were expressed as relative to the control value (100 %) and shows the means of 3 replications 28
Figure 10: Effect of different fractions of wheat seeds on antioxidant capacity measured by detoxification of ABTS free radical. The fractions were obtained from wheat seeds differing in Se concentration (e.g. 28, 1902, and 5127 ppb) and grown in Ankara location 34
Figure 11: Effect of different fractions of wheat seeds on antioxidant capacity measured by detoxification of ABTS free radical. The fractions were obtained from wheat seeds differing in Se concentration (e.g. 254, 1510, and 2275 ppb) and grown in Samsun location.....34
Figure 12: Effect of different fractions of wheat seeds on antioxidant capacity measured by detoxification of ABTS free radical. The fractions were obtained from wheat seeds differing in Se concentration (e.g. 104, 1594, 2770, and 7168 ppb) and grown in Eskisehir location 35

LIST OF TABLES

Table 1: Se contents of mostly consumed food classes regarding some countries (Combs Jr, 2001)..... 8

Table 2: Wheat cultivars differing in locations 16

Table 3: Grain Se concentrations of wheat samples collected from the field trials conducted in Eskisehir, Samsun, Izmir, Diyarbakir, Konya and Ankara

Table 4: DPPH radical scavenging activity (%) as affected by ascorbic acid concentration and incubation time. In this test, 0.4, 1.2, 1.6 and 2 pg (ascorbic acid) / ml (DPPH solution) ascorbic acid solutions were used. The results were reported based on every 20 minutes measurements 29

Table 5: Effect of wheat seed extracts on DPPH radical scavening activity at different incubation time. In this test hot water wheat extracts were used. Wheat seeds used were grown in Samsun and Diyarbakir locations, and had 294 ppb (Se-I), 1520 ppb (Se-II), and 3182 ppb (Se-III) Se for the Samsun location and 47 ppb (Se-IV), 837 ppb (Se-V). 2464 ppb (Se-VI) for the Diyarbakir location..... 30

Table 6: Trolox equivalent antioxidant capacity (TEAC) of wheat seeds which were extracted in cellulose and contained different Se concentrations 32

Table 7: Trolox equivalent antioxidant capacity (TEAC) of wheat seeds extracted with methanol, acetone and water, and differing in Se concentrations

1 INTRODUCTION

Since the time when Se was discovered as a vitamin E-replacing agent it is considered as an essential element in human health. Due to its widely known anticarcinogen, anti-viral, and anti-oxidative effects, Se became a popular element and has attracted many researches to conduct research on its health benefits. Existence of Se deficiency in daily food consumption causes to many severe diseases including Keshan disease, which was first discovered by Keshan Disease Research Group in 1979. The disease occurs mainly in children and women in the age of childbearing and causes impairments in cardiac function, cardiac enlargement and arrhythmia The disease is associated with vitamin E deficiency and also Coxsackie B virus (Levander & Beck, 1999, Lyons *et al.*, 2003).

As indicated above, its anti-carcinogenesis, antioxidative and antiinflammatory effects have made Se an attractive key element for many experiments, which mainly investigated inhibition of tumor generation and growth, and reduction of oxidative stress. In many epidemiological and animal studies, cancer incidences could be alleviated by Se intake. For instance, consumption of Se-rich diets is effective in inhibition of tumorigenesis and Se supplementations in form of selenite exhibit cancer reduction (Medina *et al.,* 2001; Jiang *et al.,* 2002). Besides anticarcinogenesis effects, antioxidant activity of Se is one of the well-documented effects in cells. In both plants and animals, this element behaves like a reducing agent of oxidative stress. Selenomethionine in plants and selenocysteine in animals are known as essential amino acids of seleno-proteins (Sandalova *et al.,* 2001; Zhou *et al.,* 2009). Glutathione peroxidase represents an important Se-containing enzyme, and has been extensively studied in literature (Michiels *et al.,* 1994; Sies *et al.,* 1997; Imai and Nakagawa, 2002). Its major function is to detoxify hydrogen peroxide (H202) and thus to prevent H202-involved lipid peroxidation and DNA damage, and consequently cardiovascular disease and cancer (Redman *et al,* 1998; Ratnasinghe *et al.,* 2000; Seo *et al.,* 2002; Blankenberg *et al.,* 2003). According to a study conducted

by Zhang *et al.* (2002), anti-inflammatory effects of Se contribute to inhibition of tumor necrosis factor-a-induced expression of adhesion molecules, which promote inflammation.

Based on well-documented importance of Se-rich diets in health, currently, humans pay increasing attention to foods containing high concentrations of Se Main sources for daily Se intake include cereals, meat and fish (Combs Jr, 2001). According to Lyons *et al.* (2003) bread is the second important Se source for the human beings in USA. Dairy products and eggs seem to be not a good source for Se intake. Also, vegetables and fruits are low in Se. Combs (2001) claims that 50% of Se intake is supplied with five foods which are beef, white bread, pork, chicken and eggs. It is widely believed that wheat has high potential to cover daily Se intake of human populations. For example, Se rich countries like US, Canada and Australia export wheat high in Se to Europe and other countries, where Se level is low in soils and foods. Several reports are available showing that wheat grown in many European countries (especially in UK) contains 10- to 50-fold lower Se in grain than the wheat grown in North America (Adams *et al.,* 2002). Therefore, many European countries discuss and implement Se fertilization approaches in order to improve grain Se concentrations (Adams *et al.,* 2002; Broadley *et al.,* 2006)

Improving food crops with high Se concentration is a high priority research topic and is of great importance for human nutrition. There are various strategies to improve Se intake of human beings such as food fortification, livestock supplementation, use of Se-enriched fertilizers and finally plant breeding (Oldfield, 1997; Chen *et al.,* 2002; Welch and Graham, 2002; Yalcintas and Saldamli, 2005). Daily needed Se amount is around 40 pg / day for an adult so that commonly consumed foods should provide this amount of Se to prevent development of Se-dependent diseases in body (Combs Jr, 2001). Currently, in many countries enrichment of foods with Se is being widely applied from beverages to cereals. High-Se Brussels sprouts, broccoli, Brassica, garlic, onion, celery, mint, chamomile, tea, vinegar, beer, yeast, mushrooms and mussels are results of such type of Se enrichment studies. Besides fortification of foods with Se plant scientists also focus on plant breeding strategy in order to develop new plant genotypes with high Se concentration in their edible parts (Lyons *et al.,* 2005). Another approach being used

today is the application of genetic engineering approach to modify food crops to accumulate high amounts of Se (Berken *et al.*, 2002).

Improving soil fertility is another way to achieve Se enriched food crops as well. Most countries around the world have soils low in Se, and therefore Se-containing fertilizers should be used to increase Se uptake capacity by crops to contribute human Se intake. The well-known Se-fertilization initiative was taken by Finland nearly 25 years ago. Finnish Ministry of Agriculture and Forestry decided to add selenium into the commonly-applied fertilizers since the level of Se in cereal-based foods was too low. In the past 20 years, Se concentrations of food crops increased sufficiently (up to 10-fold) after enrichment of fertilizers with Se and application of Se-enriched fertilizers nationwide (Ekholm *et al.*, 2005). Broadley *et al.* (2006) reported that when crops accumulate Se from fertilized soil, Se concentration in plasma and blood shows an according increase.

During Se-enrichment programs by using fertilizer strategy in Finnish soils, animal feeds, basic foodstuffs and human blood and plasma were periodically analyzed to monitor the changes in Se concentrations. There were desirable increases in Se concentrations of the analyzed samples during enrichment program (Aspila, 2005; Broadley et al., 2006). Such increases in Se concentration were also found in muscle and liver of pigs and cattle following feeding trials in which Se-enriched foods was used (Venalainen, 1997).

Another Se application method is the application of Se-containing fertilizers to foliar. Due to leaching and transformation of Se to poorly available inorganic forms, plants cannot absorb adequate amount of Se by their roots when Se was applied into soils. Therefore, foliar application of Se fertilizers seems to be an effective way in improving food crops with Se (Marschner, 1995; Gissel-Nielsen, 1998; Fageria et al., 2009). Fageria *et al.* (2009) described the mechanisms of microelement absorbtion through leaves. Unlike root surface, leaves are covered with cuticular membrane, which permeates both organic and inorganic ions and also undissociated molecules (Fang *et al.*, 2008; Fageria *et al.*, 2009). Even though it is said to be permeable, the ion uptake by plants depends on charge, ion radius and absorbability to cell walls (Fageria *et al.*, 2009). Because of very high increases in grain Se after foliar

application of Se-fertilizers (Curtin *et ai,* 2006), it can be suggested that Se absorption through leaf cells and its translocation into seeds is sufficiently high in contrast to many other micronutrients such as iron, manganese and zinc (Marschner, 1995).

This MSc study focuses on the analysis of the total antioxidant capacity of Se-enriched wheat that has been fertilized with foliar applications at different rates in Central Anatolia. The tests used for analysis of the antioxidant capacity include both MTT assay in mammalian cell culture (Fotakis and Timbrell, 2006) and free radical scavenging capacity of seeds by using the DPPH and ABTS assays (Mensor *et al.,* 2001; Ozgen *et ai,* 2006). To our knowledge, there is only one study dealing with the role of foliarly applied Se on the antioxidant capacity of cereal seeds such as rice (Xu and Hu, 2004). In the study conducted by Xu and Hu (2004), it has been shown that enrichment of seeds with Se was effective in improving antioxidant capacity of the ethanolic extracts of rice seeds. In the current study, attention will be given to wheat seeds differing in Se concentrations.

2 OVERVIEW

2.1 Historical background of Se and its chemistry

A Swedish chemist Jons Jacob Berzelius was the first person who discovered Se element in 1817 (Tinggi, 2003). Then, Se has been classified as a metalloid that has a position between sulphur and tellurium in Group VIA, and also between arsenic and bromine in Period 4 of the periodic table. In addition, scientists also discovered that Se chemically resembles sulphur (S) in terms of atomic sizes, bond energies, ionization potentials and electron affinities (Tinggi, 2003). On the other hand, there are also differences between Se and S; for example, while Se exists as reduced quadrivalent form, S exists as oxidized quadrivalent form. Moreover, their acidic strengths are also different from each other; selenium hydride is known to be a strong acid, whereas sulphur hydride is close to neutral.

In the late 1950s, Se was recognized as an essential trace element that could replace vitamin E (Combs Jr, 2001). The principle effect of Se, in the case of replacement of vitamin E, was to prevent liver necrosis in the rat when there was a vitamin E deficiency. Schwarz and Foltz (1957) found that a dietary agent Factor 3 could prevent not only liver but also heart, kidney and muscle necrosis. When they tried to characterize this agent, they realized that Se was an essential part of Factor 3. Starting from this point onwards, they worked on *in vivo* experiments, and finally concluded that feeding rat with inorganic or organic selenium was compensating vitamin E deficiency.

Subsequently, in the early 1970s, Se was found to be the essential element of an antioxidative enzyme called as glutathione peroxidase (Rotruck *et al,* 1973). Later on, several other Se-containing enzymes have been identified such as at least five GPX isoforms, three iodothyronine 5'-deiodinases, three thioredoxin reductases and selenophosphate synthetase (Allan *et al.,* 1999). Selenium also incorporates into amino acid selenocysteine (SeCys) with the modification of tRNA-bound serinyl residues at the loci where UGA codons encode. Moreover, these codons contain

SeCys-insertion sequences in their 3'-untranslated region. Translation of these codons contributes to the production of selenoproteins (Combs Jr, 2001).

2.2 Se as a nutritive agent

Selenium intake by humans is mainly provided by the daily diet. For each sex, age, region and country, a recommended dietary allowance for Se is available. Pedrero and Madrid (2009) reported that the recommended dietary allowance (RDA) for Se was estimated between 50 and 200 pg per day for adults in USA. Later, they defined that RDA should be around 55 and 77 pg of Se per day for women and men respectively. Currently, a RDA of 55 pg Se per day is widely accepted level for both sexes (Rayman, 2000).

The beneficial effects of Se on human depend on its concentration. The concentrations that exceed 1000 ppb may cause cellular toxicity; on the other hand the concentrations that remain below 100 ppb might be the deficient for cellular systems (Pedrero & Madrid, 2009). The range of Se exists in commonly-eaten foods vary substantially such as in fish 0.1-0.60 mg/kg, in cereals 0.05-0.6 mg/kg, in red meats 0.05-0.3 mg/kg and in fruit and vegetables 0.002-0.08 mg/kg (Combs Jr, 2001). The important point is that the foods high in Se contribute to synthesize Se-containing *proteins such as selenomethionine. Although some food systems do not* provide enough Se to synthesize selenocysteine and selenomethionine containing enzymes, both supplements and natural providers of Se are needed to compensate required amount of Se for human beings.

Natural providers of Se, such as broccoli (Finley, 1998), radish, garlic (Carvalho *et al.,* 2003; Pedrero & Madrid, 2009) and ramps (Whanger *et al.,* 2000) contribute to production of Se-containing amino acids as selenomethionine. The intake of selenomethionine from plant-based foods is then followed by conversion to seleno-proteins. Selenium- deficient animals generally take these kinds of providers for biosynthesis of selenocysteine in transulfuration pathway as shown in Figure 1. Selenium-containing amino acid is, then, converted to seleno-proteins like glutathione

peroxidase. Selenium exists also in high amounts in livestock (like in fish) in the form of selenocysteins.

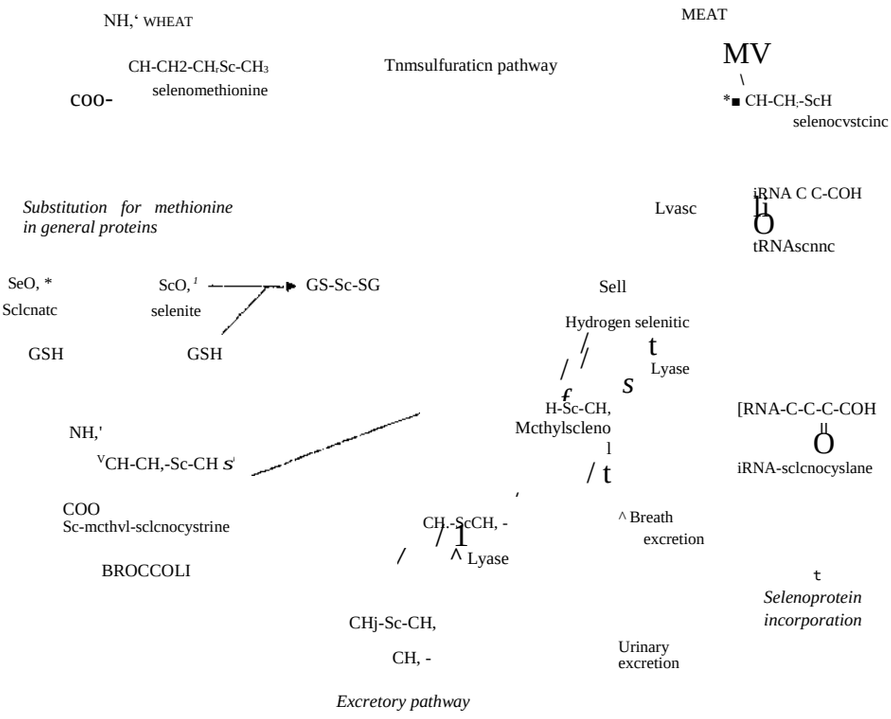

Figure 1: Selenium metabolism in animals and Se-metabolites (Finley, 2005)

Most of food systems cannot synthesize enough seleno-cysteine enzymes due to low Se accumulation. This may induce Se deficiency-dependent health problems including chronic diseases of heart and lungs, and also cancer. To avoid the severe health impacts of Se deficiency, Se enhancement of food systems is an important strategy. Since animals cannot accumulate Se, they should take this mineral from animal feeds. These feeds are supplied with Na2SeC>3 and this application is commercially spread around the world, especially in North America and Europe within the last 25 years (Combs Jr, 2001). Therefore, enrichment of Se in animals leads to conditionally increase in Se intake from animal-based foods (Combs Jr & Combs, 1986b).

In addition to Se enrichment of animal feeds, several food crops that are widely consumed by humans should be also enriched with Se, especially in the non-seleniferous areas. As indicated above, Finnish soils are very low in Se concentrations and nationwide use of Se fertilizers in this country resulted in significant increases in

Se content of most foods, and thus dietary Se intake of humans. Reports show that dietary Se intake was increased by four-fold, and Se concentrations of plasma were doubled in Finland after application of Se-containing NPK fertilizers (Combs Jr, 2001).

Table 1: Se contents of mostly consumed food classes regarding some countries (Combs Jr, 2001)

				Fnland			China, by So-area			
Food Class	USA't	England**	Germany*	Pro-1984*§	Post-1984§	New Zealand*	Levi^ |	Moderate*	Highl	Venezuela*
Cereai products	0-06-0-66	002-0-53	0-03-038	0X5-0-12	0-01-0-27	0004-009	0-005-002	0017-0-11	1 06-6 9	0-123-0-51
Vegetables	0-001-0-14	0 01-0-09	0-04-0-10	0-X1-0-02	0-01-0-02	0X1-0 02	0-X2-002	0002-0-09	0-34-4S7	0-G02-2-98
Fruits	0 005-0 06	0X5-0-01	0-002-0 04	0X2-0-03	-	0X1 -0X4	0-X1-0 003	0X5-004	-	0-005-0-06
Red meats	0-08-0-50	0-C5-0-H	0-13-0-28	0-05-0-10	0-27-0-91	0-01-0 04	0-01-0-03	0-05-0-25	-	0-17-0-83
Poultry	0-01-0-26	0 C5-0-15	0-05-0-15	0-05-0-10	-	0 05-0-10	0 02-006	005-0-10	-	0-10-0-70
Fish	0-15-1 48	0-10-0-61	0-24-0 53	0-18-0-98	-	0-03-0 31	0-03-0-20	0-10-0-60	-	032-0-93
Milk products	001-0-26	0 01-0-08	0-01-0-10	0-01-0-09	0-01-0-25	0-003-0 025	0-X2-0 01	0-01-0 03	-	0 11-0-43
Eggs	0-06-0-20	005-0-2	0-05-0-20	0 C5-0-20	0-02-0-15	0-24-0 98	002-006	005-0-15	-	0-50-1-5

In Table 1, the mostly consumed food classes are shown for different countries having distinct types of soil. In Finland after 1984, when the Se-fertilization programs started, the ranges of the Se contents in foods significantly increased. Before 1984, Se concentration of cereal grains was 0.01 mg/kg or less, and after starting the Se-enrichment program of fertilizers, it increased to about 0.25 mg/kg. Consequently, daily Se intake in Finnish population greatly enhanced. For instance, daily Se intake between 1986 and 1989 increased from 20-30 pg to 80-90 pg (Lyons, 2005; Ekholm et ah, 2005). As soon as these Se- supplemented products have been served to the Finnish people, the rates of cardiovascular diseases and incidence of certain cancers have decreased among the Finnish people.

Increasing number of reports available showing that wheat is an important source of Se for human beings. For instance, in a study conducted in Russia it was found that Se level in serum of blood donors correlated very closely with Se level in wheat flour used in bread making (Golubkina & Alfthan, 1999). In USA, bread represents the second important source of Se, and in Australia bread consumption meets the 1/3 of the daily Se intake of children (Lyons et al., 2003). These results indicate that consumption of wheat-derived products, such as bread and pasta, contributes greatly to Se intake by humans.

In a global view, the Se contents of wheat changes in accordance with the geography and soil conditions. Selenium values in soils range from 0.001 mg/kg in southwest Western Australia to 30 mg/kg in highly selemferous areas of South Dakota (Lyons *et al.,* 2003). Soils that contain high Se concentration are found in Canada and USA, and they contain around 0.2-0.6 mg Se /kg. On the other hand, in New Zealand and Eastern Europe these ranges fall down to 0.028 mg/kg as reported by Mihailovic *et al.* (1996). Due to the such huge variability in Se concentration of soils the Se content of wheat also shows a high variation and ranges (e.g., between 0.02 to 0.6 mg/kg.

To overcome the low Se levels in wheat, Se-containing fertilizers are applied either to soils or onto foliar (shoot). The field trials in New Zealand showed that both soil and foliar applications of Se fertilizers were highly effective in increasing grain Se concentration in wheat (e.g., from 30 ppb upto 500 ppb) (Curtin *et al.,* 2006). The effect of the Se fertilization depends on the Se form applied, soil characteristic, method of basal application and time of foliar application. It has been estimated that selenate form of Se is much more effective than selenite form in increasing Se concentration of plants (Ylaranta, 1983*a,b).* For wheat grown on the clay soils, foliar selenate fertilization leads to higher accumulation of Se than the soil Se fertilization. For example, 10 g/ha of foliar selenate fertilization rose the Se content of wheat from 16 ppb up to 168 ppb on the clay soil; while it just rose to 77 ppb when 9 g/ha of basal selenate fertilization was applied into soil (Ylaranta, 1984). Currently, selenate fertilization of the plants, either by foliar applications or soil applications, becomes a widely applied fertilization approach in many countries.

2.3 The availability of Se in soil and its transportation to biological systems

Selenium is unevenly distributed in various regions, since its availability in soil depends largely on the weathering of Se-containing rocks. Consequently, in some places Se exists at low levels, in other places it exists in very high concentrations. The seleniferous areas with high Se concentrations are widespread in the Great Plains of the USA and Canada; Enshi County, Hubei Province, China; and some parts of

Ireland, Columbia and Venezuela (Combs Jr, 2001). In contrast, the non-seleniferous areas extend from Northern and Eastern Europe, such as Denmark, Finland and Siberia, to some parts of Australia, including a belt from northeast to south-central China and also Zaire in Africa (Diplock, 1993; Combs Jr, 2001; Lyons *et al.*, 2003). The highest Se is naturally found in Niobara and Pierre shales of the USA as 90 mg/kg, on the other hand, non-seleniferous areas possess less than 2 mg/kg of Se level due to low Se granites and metamorphic sandstones (Combs Jr, 2001).

The cycle of Se in plants and microorganisms starts with the absorption of the Se from soils and its transport into their tissues and then continues with the conversion into either functional proteins or volatile metabolites, which are then lost in the atmosphere. These volatile metabolites later return to soils but little parts of Se enter into the biological cycle (Terry and Zayed, 1994). The chemical availability and root uptake of Se is affected mainly by soil pH and moisture. The effect of pH in soils determines the conversion of inorganic Se to selenate or selenite. In the alkaline conditions, the inorganic Se is converted into selenate (Se^{+6}) and then it is hardly fixed in the soil. Acidic soil conditions favor the selenite form (Se^{+4}), which tends to adsorb to clays and then is strongly fixed by iron hydroxides (Gissel-Nielsen, 1998). Therefore, both in high and low pH, the bioavailability of Se remains 10 times at lower levels since the fixation by iron hydroxides causes to insoluble selenium complexes (Cary & Allaway, 1969).

2.4 The metabolic activities of Se in both plants and animals

Involvement of Se in metabolic activities in plant and animal cells has attracted many scientists to discover functional seleno-compounds. Whanger (1989) summarized several seleno-compounds, which have been discovered as important seleno-compounds in biological systems. These include selenate, selenite, selenocystine, selenomethionine, selenohomocysteine, Se-methylselenocysteine, glutamyl-selenocystathionine, selenomethionine selenoxide, s?-glutamyl-Se-methylselenocysteine, selenocysteineselenic acid, Se-proponylselenocysteine selenoxide, Se-methylselenomethionine, selenocystathionine, dimethyl diselenide,

selenosinignn, selenopeptide and seleno-wax. These compounds are believed to have some protective functions in anti-carcinogen metabolisms of animals and plants.

As illustrated in Figure 2, wheat and broccoli accumulate Se in the form of selenomethionine and Se-methyl-selenocysteine, respectively. According to Whanger (2002) most of the Se accumulator plants, like broccoli and Astragalus species, contain Se in the form of Se-methylselenocysteine. Apart from involvement in functional sites of proteins, some accumulator plants synthesize non-protein amino acids, like Se-methylselenocysteine, selenocystathionine, Se-methylselenomethionine, N?-glutamyl-Se-methylselenocysteine, s^-glutamyl-selenocystathionine, selenopeptides, and selenohomocysteine (Whanger, 2002). Selenate is, first, reduced to selenide by reduced glutathione protein; then, it is converted to hydrogen selenide. Later *O*- acetyl serine reacts with selenide and produces selenocysteine, which is the precursor for the biosynthesis of selenomethionine and other essential proteins (Figure 2).

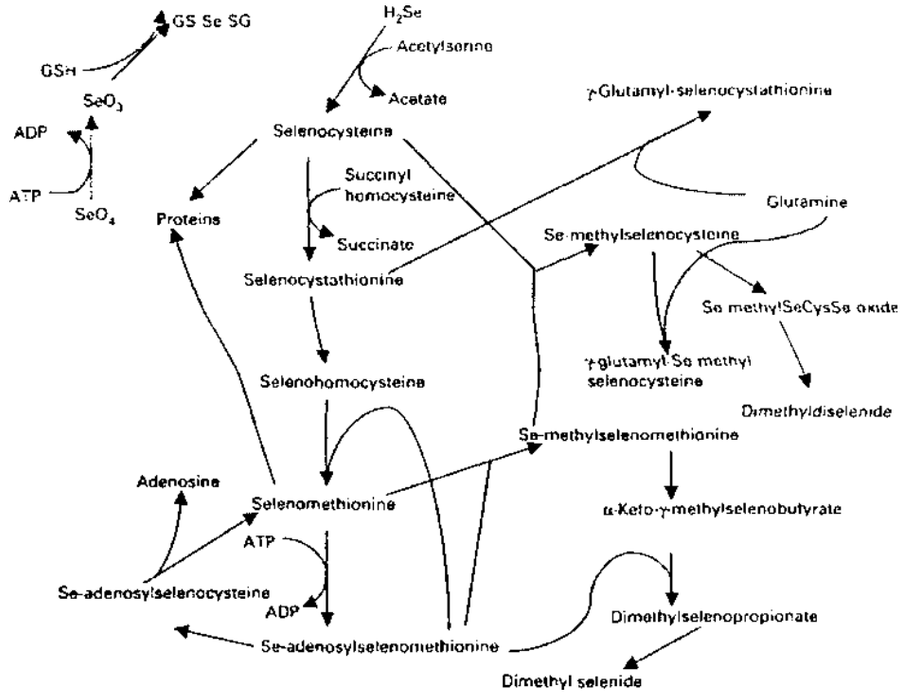

Figure 2: Metabolic pathways of Se in plants (Whanger, 2004).

In animal tissues, Se is present in the form of selenocysteine. After its absorption, animal cells spontaneously transform Se into selenocysteine. In plant tissues, selenomethionine can directly enter the protein metabolism, however, in animal tissues, selenocysteine cannot immediately incorporate into the protein metabolism; it should be first converted into hydrogen selenide (Figure 1). Therafter, the seleno-proteins are produced. Animals can also take up Se in inorganic form, such as selenite and selenate. These inorganic Se forms directly enter the selenocysteine derived enzyme metabolism (Arthur, 2003).

Figure 3 illustrates Se metabolism in an animal cell. In the first step, Se is liberated in the cell after dietary intakes. Subsequently, it is involved in tRNA. In the second step, as marked in the figure, posttranscriptional processes takes place and two different $tRNA^{[Ser]Sec}$ isoforms are generated. Additional factors are displayed in step 4; the role of these factors is to assemble to specific selenosomes. Their functionality is important for the translation of selenoproteins. In the fifth step, as a result of deciphering multiple UGA codons on mRNA, selenoprotein P (SePP) is synthesized.

After the synthesis in step 6, SePP is released into the extracellular space and stays in plasma or reenters into the cell to retrieve Se.

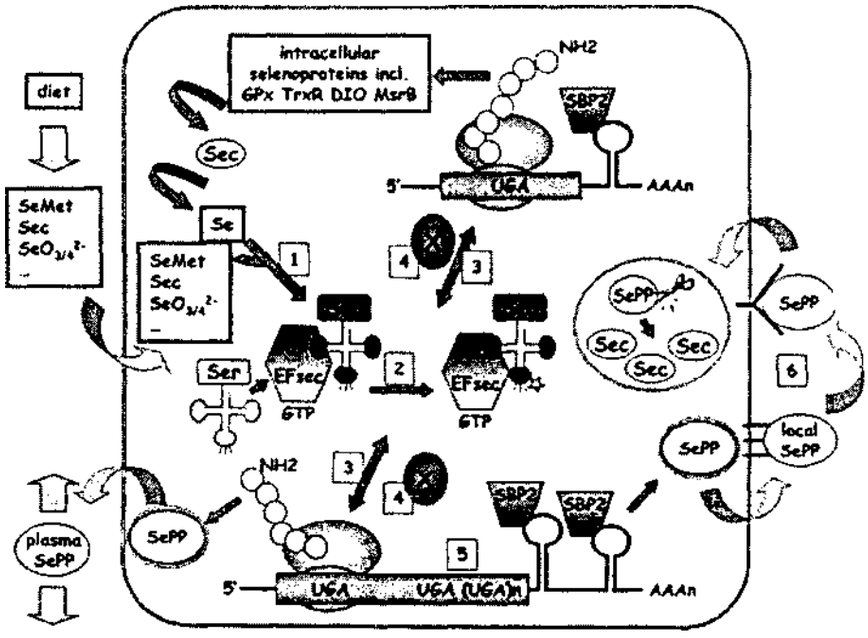

Figure 3: Selenium metabolism in animal cells (Schomburg *et al.*, 2004).

As a result of these steps, Se enters human metabolism to synthesize

')

2.5 The health impacts of Se

With the discovery of Se by Schwarz and Foltz (1957) as a substitutive agent against liver degeneration, it has been recognized as an essential element for human cells. Mainly, Se acts against cancer and oxidative stresses (Ip & Lisk, 1994; Finley, 2005). Since the inorganic forms of Se, selenate and selenite, can easily get into the metabolism, when they are ingested, and then they form hydrogen selenide. This compound is subsequently incorporated into selenoproteins that are the main Se-compounds contributing to health. Some seleno-proteins that have been found to be important in human health are glutathione peroxidases, sperm mitochondrial capsule

seleno-protein, iodothyronine deiodinases, thioredoxin reductases, selenophosphate synthetase, selenoproteins P, selenoproteins W, prostate epithelial selenoproteins (15 kDa), DNA-bound spermatid selenoproteins (34 kDa), 18 kDa selenoproteins (Rayman, 2000).

Glutathione peroxidase is a Se-dependent protein and has many isoforms like GPxl, GPx2, GPx3, and GPx4, which are known as antioxidant enzymes. Basically, they remove hydrogen peroxide and lipid and phospholipid hydroperoxides (Foyer *et al*, 1994). Therefore, these enzymes sustain membrane integrity, modulate eicosanoid synthesis, regulate inflammation and alleviate cell damage resulting from oxidative stress to lipids, lipoproteins and DNA. Sperm mitochondrial capsule seleno-protein protects the developing sperm cells against oxidative damage. The third enzyme, iodothyronine deiodinases, produces and regulates the level of active thyroid hormone T3 from thyroxine T4. Thioredoxin reductases are the enzymes that reduce nucleotides in DNA synthesis, regenerate antioxidant systems and regulate intracellular redox mechanism that is critical for cell viability. Selenophostate synthetase is needed for synthesizing selenophostate, and also known as the precursor of selenocysteine that is an essential amino acid finding in seleno-proteins of animals. Seleno-protein P protects endothelial cells against the damage of peroxynitrite. Another protein, called Seleno-protein W is required for muscle function. Prostate epithelial seleno-protein that is found in epithelial cells of ventral prostate is also in involved in a redox mechanism, which reduces cancer development. DNA-bound spermatid seleno-protein (34 kDa) acts like glutathione peroxidase. The last protein, 18 kDa seleno-protein, has a role to preserve Se for providing during the Se deficiency (Rayman, 2000).

The seleno-proteins mentioned provide various protective effects against cell damage and health defects. For example, in a study conducted by Redman *et al.* (1998), use of 40-50 pM of selenomethionine had the protective effects in breast carcinoma cell lines, melanoma and prostate cancer cells. In another animal study, addition of Se into animal cells was highly protective against prostate cancer (Waters *et al.,* 2003). Moreover, selenomethionine activates the silenced p53 through a redox mechanism (Seo *et al.* 2002). Therefore, selenomethionine can activate p53 protein in tumor cells, and thus, it can hinder tumor growth and so p53 can later repair DNA

damage. These studies support the idea that Se acts as an anti-carcinogen compound in animal cells (Rayman, 2005).

Besides its anti-carcinogenic effect, seleno-proteins improve also the immune system of cells under infection. Hurwitz *et al.* (2007) conducted an experiment, which investigated the effect of Se supplementation on the HIV-1 viral load. This study reported that the HIV related burden was alleviated by adding Se.

The Se acts also protective agent against oxidative stress and is considered as a successful antioxidant. Steinbrenner *et al.* (2006) showed the antioxidative role of Se on cell viability of human astrocytes. In this study, when the selenoproteins were downregulated, the oxidative stress was induced and therefore human astrocytes could not survive. The Se supplementation can also prevent the superoxide-induced damage under UV irradiation (Jilani *et al*, 2007).

In contrast to the cancer preventive and antioxidant effects of Se, there are some studies reporting its poor antioxidant and chemo-preventive effects (Dunstan *et al*, 2007; Lippman *et al, 2009;* Gaziano *et al*, 2009).

In plant tissues, high Se was also found to be beneficial against stress situations (Foyer *et al.*, 1994; Hartikainen *et al.*, 2000). Xu and Hu (2004) showed that enrichment of rice seeds with Se through applying Se to foliar exhibited high antioxidant capacity. To our knowledge, there are no further studies that investigated role of Se-enriched wheat seeds by Se fertilization on the antioxidant capacity of seeds..

In this thesis, three colorimetric assays were employed to collect information about the role of Se-enriched wheat seeds on the antioxidant capacity of seeds. The methods used were MTT cell viability assay (Roche Applied Science, 2005) and the DPPH radical and ABTS cationic radical scavenging assays which were used widely in literature (Sanchez-Moreno *et al.*, 1999; Antolovich *et al*, 2002; Serpen *et al*, 2007; Xu and Hu, 2004; Liyana-Pathirana and Shahidi, 2006).

3 MATERIALS AND METHODS

3.1 Materials

The wheat *(Triticum aestivum*) seeds differing in Se concentrations were the material of this MSc Thesis. The seed samples were received from a TUBITAK project (Nr: 105 O 637) conducted in joint research collaboration between Sabanci University and the Research Institutions of the Ministry of Agriculture and Rural Affairs. The field trials of this TUBITAK project were conducted in Central Anatolia (Ankara, Konya and Eskisehir), Southeastern Anatolia (Diyarbakir), Aegean (Izmir), and Black-Sea (Samsun) regions (Table 2). Wheat has been treated differently by soil and foliar applications of Se fertilizers under field conditions to obtain seeds differing in Se concentrations. In this thesis, the seed samples presented in Table 3 were used in order to measure antioxidant capacity of the seeds.

Table 2: Wheat cultivars differing in locations

Names of wheat cultivars	
Ankara	
Eski$ebir	**Bezostaja**
Konya	**Karahan99**
Diyarbakir	**San?anak98**
Izmir	**G6nen98**
Samsun	**Tahirova2000**

Table 3: Grain Se concentrations of wheat samples collected from the field trials conducted in Eskisehir, Samsun, Izmir, Diyarbakir, Konya and Ankara

	Selenium concentrations of seeds uq/kq					
	Eskisehir	**Samsun**	**Izmir**	**Diyarbakir**	**Konya**	**Ankara**
Se-I	**51** **104** **108** **124**	**254** **294** **301** **353**	**48** **61**	**47**	**36**	**28**
Se-II	**-**	**1124** **1523** **1884**	**956**	**2566**	**390**	**1902**
Se-III	**1594** **1911** **2249**	**1510**	**249**	**837**	**-**	**-**
Se-IV	**2770** **3934**			**1616**		
Se-V	**5871** **6580** **7168**	**2275** **3103** **3310**	**1621** **1955**	**2464**	**894**	**5127**

*Se-I: Control

Se-II: Foliar fertilization two times applied (1st node detectable; boots just swollen)

Se-III: Foliar fertilization two times applied (early milk; early dough)

Se-IV: Foliar fertilization two times applied (boots just swollen; early milk)

Se-V: Foliar fertilization four times applied (1st node detectable; boots just swollen; early milk; early dough)

3.2 Methods

3.2.1 Measurement of Antioxidant Capacity of Seeds

Three assays were considered to measure the effect of Se-enriched seeds on the antioxidant capacity of seeds. The methods used were MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell viability assay (Tim

Mosmann, 1983; Roche Applied Science, 2005) and the detoxification capacity of seeds for the DPPH radical (Sanchez-Moreno *et al.*, 1999; Malencic *et al*, 2000; Xu and Hu, 2004) and the ABTS cationic radical (Serpen et al., 2007). These methods are described in detail below.

MTT (3-[4,5-dimethyIthiazol-2-yI]-2,5-diphenyl tetrazolium bromid) assay is based on the conversion of yellow tetrazolium salt into purple formazan crystals. The amount of the conversion is based on the number of viable cells. At the end, ELISA Microplate Reader measures the ratio of viability using a wavelength between 550- 600 nm.

Determination of radical scavenging capacity of seed extracts was other approach used in this thesis to collect information about the antioxidant capacity of seeds. As indicated above, measurement of the DPPH (a,adiphenil-P-picrylhydrazyl) free radical scavenging capacity was one of the approaches used here to measure antioxidant capacity of seeds. The method was applied according to Sanchez-Moreno *et al.* (1999) that is based on scavenging of the DPPH free radical from a medium after addition of the seed extracts. The results were visualized with the help of color change and were measured at 490 nm via ELISA Microplate Reader.

The detoxification capacity of seed extracts for ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) cationic radical is based on the decolorization of the $ABTS^{-'}$ radical by the addition of the seed extracts. This assay represents a Trolox equivalent antioxidant capacity assay (TEAC) (Huang *et al.*, 2005). The TEAC method used in the present study was applied as described by Serpen *et al. (2007).* Two different media were used for extraction procedures; i) methanol/acetone/water ii) cellulose. In order to involve both polar and apolar compounds of seed in the extract, three kinds of solvent having different polarities were used: methanol, acetone and water. On the other hand, cellulose and seed mixture provided the antioxidative capacity measurement of both soluble and insoluble parts of seeds. Besides the antioxidant measurements in whole seeds, also antioxidant assays were conducted in different seed section such as embryo, endosperm and bran. These seeds fractions may be different in antioxidant activity. There are some reports showing differential

antioxidant activity of the mentioned seed fractions (Adorn *et ai.* 2005; Liyana-Pathirana CM and Shahidi, 2007).

3.2.2 Preparation of Seed Extracts

Depending on the methods used, hot water, cellulose and methanol, acetone and water extracts have been used in the assays described. Hot water extracts were used in MTT cell viability and DPPH assays; whereas cellulose and methanol, acetone and water extracts were applied in the ABTS assays. For the MTT cell viability assays hot water extracts have been used as following. First, in order to optimize the level of Se containing extracts, a hot water extraction was prepared as stock solution. In this test wheat seeds were used which contained 894 ppb Se. Ground seed samples of about 500 mg were suspended in 10 ml water; so, the final concentration of stock solution became 50 mg (wheat) / ml (water). After shaking about 1 hour, the extract was incubated at 60°C for 1 hour. Thereafter, in the case of precipitating cell debris, the extract was centrifuged at 4600 rpm for 30 minutes. Finally, the seed extract was filtered with a sterile filter having pores 0.22 pm. The filtered extract was mixed with the complete DMEM solution containing 10% FBS in a ratio of 1 (extract) / 8 (DMEM). By using this stock solution, six different hot water extractions were prepared: 30, 20, 10, 5, 2 and 1 mg (wheat) / ml (water). As a result of optimization procedures, the optimum Se-enriched seed concentration was found as 20 mg (wheat)/ml (medium) that was used in the seed extractions described below. For DPPH assays, nearly 1 g ground seed samples were used by mixing in 10 ml water (see below).

Cellulose extraction methods that were applied in ABTS assays have been described as following. Cellulose and seed solution was prepared in a ratio of 1 (wheat) / 3 (cellulose) and this mixture was used in the ABTS assays as described below. The second extraction procedure for ABTS assays was methanol/ acetone/ water extraction in a ratio of 7 (methanol) / 7 (acetone) / 6 (water). 100 mg ground seed sample was mixed with 1 ml of methanol/ acetone/ water solution. The mixture was centrifuged at 10000 rpm for 5 minutes; then, the supernatant was transferred into

a new falcon tube. The remaining part was resuspended into the methanol acetone/ water *solution and centrifuged again.* This *procedure was* repeated *three times. Then* the collected supernatants were used in the ABTS assays as mentioned below.

3.2.3 Preparation of H_2O_2 solution for MTT Assay

In MTT cell viability assays, cells have been treated with H2O2 to generate an oxidative stress in medium as following: First, a stock solution of H202 has been prepared containing 200 **mM** H202. Then, six different H202 concentrations were prepared from this solution by mixing with complete DMEM at following rates: 0.5, 1, 1.3, 1.5, 1.7, and 2 pi (H_20_2) /ml (DMEM). As a result of optimization tests, 1 mM H_20_2 was used in the cell viability assays as described below.

3.2.4 Application of Antioxidant Tests

3.2.4.1 MTT Cell Viability Tests

MTT assay was applied in order to determine the ratio of viable HeLa cells. HeLa cells were grown to confluency in complete DMEM containing 10% (v/v) FBS, 2 mM glutamine, 100 U of penicillin/ml, 100 pg/ml of streptomycin, in 5% atmosphere at 37°C in a mammalian incubator. On the day of the experiment, HeLa cells were rinsed with 5 ml of Dulbecco's Phosphate Buffered Saline (dPBS)and dislodged from their substratum by addition of 5 ml of Tripsin-EDTA followed by a 5 minute incubation at 37°C in mammalian cell culture incubator . Thereafter, 5 ml of serum free medium (SFM) was was added onto the cells. This mixture was then transferred into a falcon tube for centrifuging at 300rg for 5 minutes. Supernatant occurring at the end of centrifuging has been discarded due to the existence of SFM and Tripsin-EDTA. In the following step, cell counting was conducted by using Hemocytometer. The number of cells in each well should be 10000 per 100 pi complete DMEM solution. Thereafter, HeLa cells were seeded in 96-well plates and incubated in 5% atmosphere at 37°C for 16 hours/

After an overnight incubation, HeLa cells were ready for treatment with the seed extracts. First of all, cell monolayer was washed once with SFM and then incubated with SFM (100 |ulAvell) for 4 hours for synchronization. Later SFM was removed from the cells and cells were treated for 2 hours with prepared seed extracts dissolved in SFM at different concentrations. Following the seed extract pretreatment, 1 mM H2O2 solution was added to HeLa cells for 24 hours in order to determine the antioxidant effect of the seed extracts.

In order to measure the cell viability pf HeLa cells 10 pi MTT labeling solution was added into each 96-well plate. Then the plates were incubated for 4 hours. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazole, which can be reduced to formazan by electron transport chain in living cell. Therefore, only living cells could reduce the MTT reagent to formazan and produce purple colour, which can then be measured with spectrophotometer. At the end of incubation, 100 pi of solubilization solution was added. Later on 96-well plates were kept in the same incubator for overnight. After checking with inverted microscopy whether formazan crystals were dissolved or not, the ratio of the viable cells to the control cells was measured by ELISA Microplate Reader at 570 nm.

3.2.4.2 Scavenging capacity of seed extracts for DPPH free radicals

First, 0.018 gram DPPH radical was dissolved in 60 ml of DMSO (Dimethyl Sulfoxide), and then the solution was kept under dark conditions at room temperature.

Before analyzing the antioxidant capacity of seed extracts, ascorbic acid was used as a control scavenger to compare the antioxidative capacity of both ascorbic acid and seed extracts. In the tests with ascorbic acid, 0.4 mg ascorbic acid was added into 10 ml water. After shaking, increasing amounts of ascorbic acid solution (1, 3, 4, 5 pi from 0.4 mg ascorbic acid/10 ml) were added 95 pi of DPPH solution containing 0.3 mg (DPPH) /ml (DMSO). As soon as the solution was inserted in 96-well plates, ELISA Microplate Reader measured the results at 490 nm. This measurement was repeated every 20 minutes.

In the case of the seed extracts, first 1 g ground seed sample was stirred in 10 ml water for extraction. After centrifuging, 5 pi supernatant was mixed with 95 pi DPPH solution. Then, the measurements were made by using the ELISA Microplate Reader at 490 nm.

The results were reported based on the calculation of decreasing amount of DPPH radical by 50% of the initial concentration:

% scavenging = {($Abs_{contr}oi$ - $Abs_{samp}ie$) / $Abs_{con}troi$}x 100,

where $Abs_{cont}roi$ = absorbance of DPPH radical + DMSO; $Abs_{samp}ie$ = absorbance of DPPH radical + wheat extracts or ascorbic acid (Antolovich *et al.*, 2002; Huang *et al.*, 2005).

3.2.4.3 Detoxification capacity of seed extracts for ABTS cationic free radicals

In addition to the DPPH assay, ABTS assay was also used for measurement of the antioxidative capacity of seeds (Serpen et al., 2007). For the preparation of ABTS free radical solution, 6.615 mg of potassium peroxidisulfate was dissolved in 10 ml water, and then 38.41 mg ABTS radical was mixed with potassium peroxidisulfate solution. As soon as ABTS was added, the color of the solution turned into dark blue because of the generation of ABTS*" radical. After stirring, the ABTS solution prepared was kept under dark conditions for 16 hours at room temperature. At each assay, always a fresh ABTS solution was prepared.

The results of the ABTS assay have been reported as a Trolox equivalent according to Serpen et al. (2007). About ten different Trolox solutions were prepared for the standard solution tests (e.g., 5, 10, 20, 40, 60, 80, 100, 125, 150, and 175 ppm). A 100 pi sample from each solution was added into 6 ml of ABTS and these solutions were shaked for 30 seconds under dark conditions. As a result, greenish blue color of the free radical ABTS turned into light green with the increasing concentration of Trolox, and the color developed was measured by using a UV-visible spectrophotometer at 734 nm.

To apply the ABTS assay on seed samples, first, methanol, acetone and water mixture was prepared at the ratio of 7:7:6 (methanol/acetone/water) to use for extraction of seed samples. Then, 100 mg ground seed sample was mixed with 1 ml of methanol/acetone/water solution. After the extraction procedure as described above,

100 pi the collected supernatants were added in 6 ml ABTS solution and shaken. The color developed was measured at 734 nm. Alternatively, another seed extraction method was applied by using a cellulose extraction method (see 3.2.2). The ground seed samples were mixed with cellulose in a ratio of 1 (ground seed sample) / 3 (cellulose). Thereafter, 10 mg the solution was added into 6 ml ABTS solution. The mixtures were shaken for 30 minutes under dark conditions and centrifuged at 12500 G for 2 minutes in order to precipitate the residuals. Finally, the color developed was measured via spectrophotometer at 734 nm.

e

4 RESULTS

4.1 Effect of wheat seed extracts differing in Se concentrations on viability of HeLa cells

4.1.1 Optimization tests on seed extracts and H202 concentrations

Prior to determining the effect of Se-enriched wheat extracts, optimization tests have been conducted on seed extracts and H2O2 concentration for using in the MTT assay. As a result of the optimization of seed extracts on HeLa cells, it was found that the optimum level of wheat extracts that should be used in MTT assays was 20 mg (seed) / ml (water) (Figure 4).

In the optimization tests with H2O2 on HeLa cells, 1 mM H202 was found to be the most suitable concentration for using in MTT assays that reduced cell viability nealy by 50 to 60 % (Figure 5).

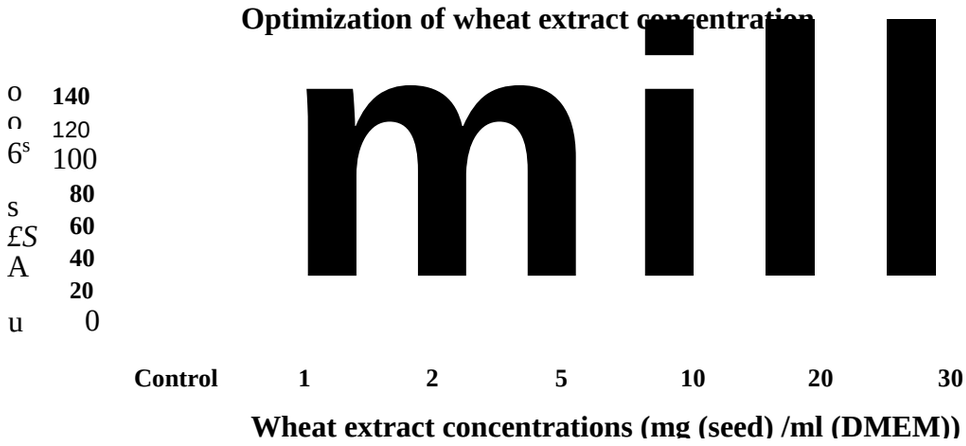

Figure 4: Effect of increasing amount of wheat extracts (mg (seed) / ml (water)) on HeLa cell viability. Control indicates the samples without treatment with wheat extracts. In this test wheat seeds were used which contained 894 ppb Se. All results were expressed as relative to the control values (100 %).

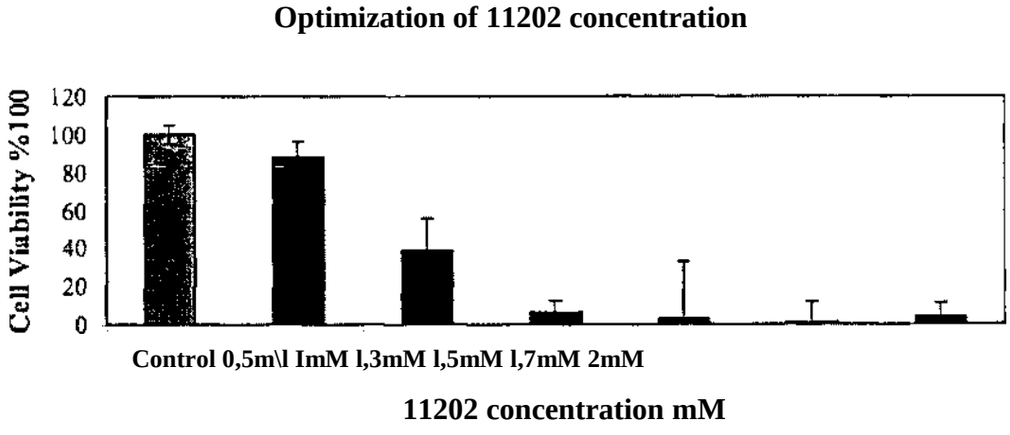

Figure 5: Effect of increasing concentration of H2O2 on HeLa cell viability. Control shows the treatments without H2O2 addition. All results were expressed as relative to the control value (100 %).

4.1.2 Effect of wheat seed extracts having different Se concentrations on HeLa cell viability

Seed samples from Izmir, (Figure 6), Samsun (Figure 7), Eskisehir (Figure 8) and Konya (Figure 9) were used in MTT assays. As can be seen in the mentioned Figures, seeds used were substantially different in Se concentrations (nearly 100-fold ranging between 58 ppb to 5870 ppb). Despite such great variation in seed Se concentrations there was no positive and consistent effect of the seed samples with various Se concentrations on cell viability (Figures 6-9). At very high concentrations of seed Se, there was a clear tendency for reduction in cell viability.

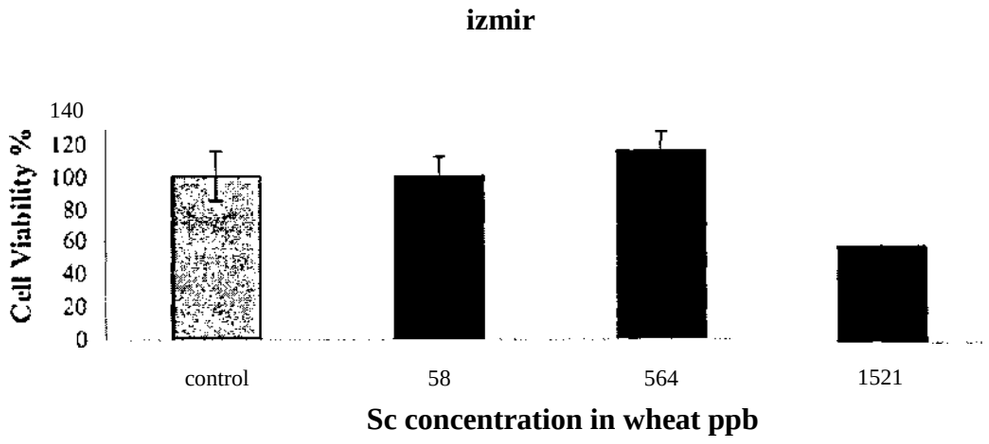

Figure 6: Effect of wheat seed extracts differing in Se concentration on FleLa cell viability. In the tests 20 mg (seed) /ml (medium) wheat extracts were used which were derived from wheat seeds containing 58, 564 and 1521 ppb Se and grown in Izmir. HeLa cells were exposed to H202 after the incubation with wheat seeds. All results were expressed as relative to the control value (100 %) and shows the means of 3 replications.

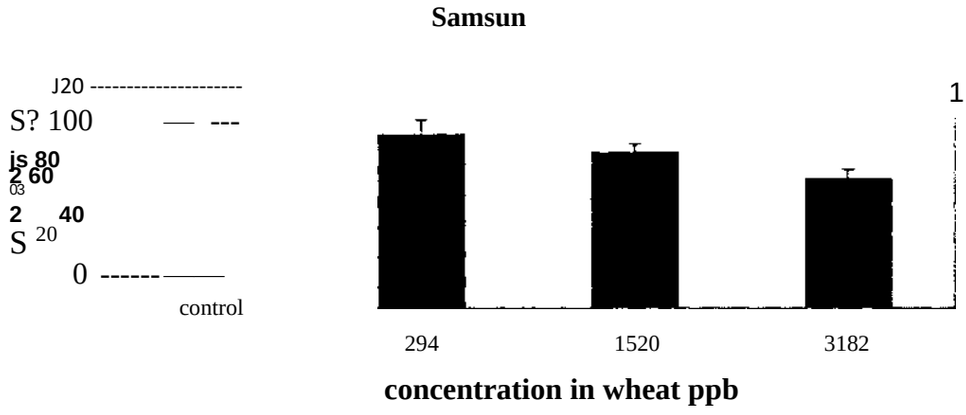

Figure 7: Effect of wheat seed extracts differing in Se concentration on HeLa cell viability. In the tests 20 mg (seed) /ml (medium) wheat extracts were used which were derived from wheat seeds containing 294, 1520 and 3182 ppb Se and grown in Samsun. HeLa cells were exposed to H2O2 after the incubation with wheat seeds. All results were expressed as relative to the control value (100 %) and shows the means of 3 replications.

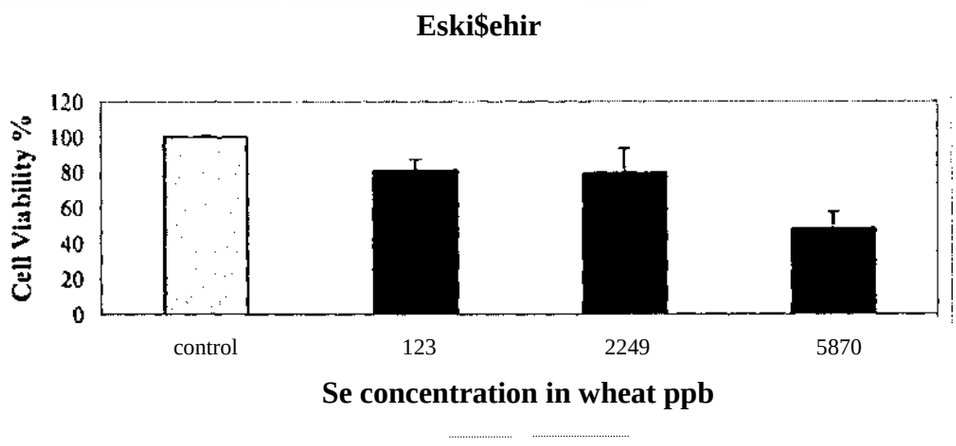

Figure 8: Effect of wheat seed extracts differing in Se concentration on HeLa cell viability. In the tests 20 mg (seed) /ml (medium) wheat extracts were used which were derived from wheat seeds containing 123, 2249 and 5870 ppb Se and grown in Eskisehir. HeLa cells were exposed to H2O2 after the incubation with wheat seeds. All results were expressed as relative to the control value (100 %) and shows the means of 3 replications.

Konva

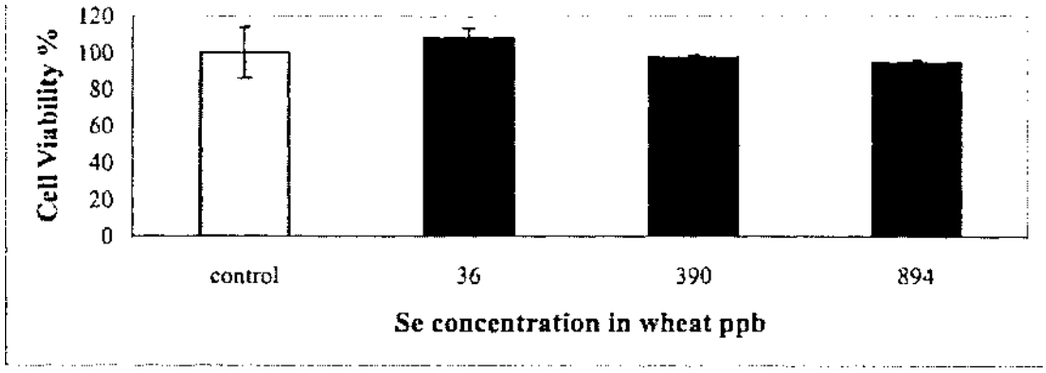

Figure 9: Effect of wheat seed extracts differing in Se concentration on HeLa cell viability. In the tests 20 mg (seed) /ml (medium) wheat extracts were used which were derived from wheat seeds containing 36, 390 and 894 ppb Se and grown in Konya. HeLa cells were exposed to H2O2 after the incubation with wheat seeds. All results were expressed as relative to the control value (100 %) and shows the means of 3 replications.

4,2 Scavenging DPPH free radical

4.2.1 DPPH radical scavenging activity of ascorbic acid

DPPH radical scavenging activity was firstly tested with increasing concentrations of ascorbic acid in order to justify the method. The results are summarized in Table 4. The results obtained showed that the antioxidant activity of 0.4 pg ascorbic acid / ml (ascorbic acid / DPPH solution) did not reduce DPPH free radical by more than 50%. In contrast, starting from 1.2 pg/ml ascorbic acid concentration, the ascorbic acid was found to detoxify DPPH free radical by more than 50 % (Table 4). By further increases in ascorbic acid concentration DPPH radical detoxification was further enhanced which justifies use of DPPH assay in the antioxidant tests.

Table 4: DPPH radical scavenging activity (%) as affected by ascorbic acid concentration and incubation time. In this test, 0.4, 1.2, 1.6 and 2 pg (ascorbic acid) / ml (DPPH solution) ascorbic acid solutions were used. The results were reported based on every 20 minutes measurements.

	Scavenging activity of Ascorbic Acid			
Concentration (jig/ml)	0.4	1.2	1.6	2
Time (minutes)				
0	32	63	81	89
20	37	79	86	86
40	38	82	86	86

4.2.2 DPPH radical scavenging activity of wheat extracts

As shown in Table 5, increases in Se concentrations of wheat seeds both from Samsun and from Diyarbakir did not DPPH radical scavenging. There was no any effect or even tendency on DPPH radical detoxification capacity of seeds despite large differences in seed Se concentration. Only in the case of the seeds from Samsun, there were some increases in radical scavenging activity over incubation time; but these changes were independent on seed Se (Table 5).

Table 5: Effect of wheat seed extracts on DPPH radical scavening activity at different incubation time. In this test hot water wheat extracts were used. Wheat seeds used were grown in Samsun and Diyarbakir locations, and had 294 ppb (Se-I), 1520 ppb (Se-II), and 3182 ppb (Se-III) Se for the Samsun location and 47 ppb (Se-IV), 837 ppb (Se-V). 2464 ppb (Se-VI) for the Diyarbakir location.

DPPH scavenging activity of wheat seeds*

incubation time minutes	**Samsun**		
	Se-I	**Se-II**	**Se-III**
30	**1 ± 0.02**	**2 ± 0.01**	**7 ± 0.05**
90	**7 ± 0.01**	**9 ± 0.01**	**14 ± 0.01**
120	**8 ± 0.01**	**9 ± 0.01**	**15 ± 0.01**
	Diyarbakir		
	Se-IV	**Se-V**	**Se-VI**
30	**25 ± 0.02**	**28 ± 0.02**	**23 ± 0.02**
90	**32 ± 0.02**	**32 ± 0.02**	**29 db 0.03**
120	**22 ± 0.02**	**23 ± 0.02**	**19 ± 0.03**

*Se-I: Control

Se-II: Foliar Se fertilization two times applied (1st node detectable; boots just swollen)

Se-III: Foliar Se fertilization four times applied (1st node detectable; boots just swollen; early milk; early dough)

Se-IV: Control

Se-V: Foliar Se fertilization two times applied (early milk; early dough)

Se-VI: Foliar Se fertilization four times applied (1st node detectable; boots just swollen; early milk; early dough)

4.3 ABTS free radical scavenging activity of Se-enriched wheat

4.3.1 ABTS scavenging capacity of the cellulose extracts

As summarized in Table 6, the increases in Se concentration of seeds from different locations in Turkey did not result in an increase in the antioxidant capacity. The only difference found was related to locations. The seeds from Esktyehir and Samsun locations tended to exhibit the highest and the lowest antioxidant activity irrespective seed Se concentrations.

Table 6: Trolox equivalent antioxidant capacity (TEAC) of wheat seeds which were extracted in cellulose and contained different Se concentrations.

Location	Se concentration in wheat ppb	TEAC %	corresponded Trolox mg
Samsun	**254**	**12.1 ± 1.19**	**4**
	1510	**12.5 ±0.68**	**5**
	2275	**14.4x0.10**	**5**
Ankara	**28**	**17.4 ±0.88**	**6**
	1902	**17.2 ±0.67**	**6**
	5127	**17.7 x 1.14**	**6**
Eskisehir	**51**	**25.6 ±2.06**	**13**
	108	**28.3 ± 1.84**	**14**
	1594	**27.3 ±3.28**	**13**
	1911	**26.8 x 1.83**	**13**
	2770	**23.3 ± 1.91**	**12**
	3934	**25.8 ±3.08**	**13**
	6580	**25.5 ±2.76**	**13**
	7168	**14.7 ± 1.60**	**8**
Izmir	**48**	**20.2 ± 1.72**	**8**
	61	**23.6x0.36**	**10**
	1621	**19.5 ± 1.75**	**8**
	1955	**21.9 ±0.21**	**9**
Diyarbakir	**47**	**23 ± 1.26**	**10**
	837	**20.8 ± 1.08**	**9**
	2464	**18.8 ±0.83**	**8**

4.3.2 ABTS scavenging capacity of methanol / acetone / water extracts

Extracting seeds in methanol-acetone-water solution also did not result differential antioxidant capacity in seeds with wide range of Se concentrations in the ABTS assay (Table 7). Compared to the cellulose extracts mentioned above (Table 6),

this methanol, acetone and water extraction procedure did not yield higher amounts of antioxidants.

Table 7: Trolox equivalent antioxidant capacity (TEAC) of wheat seeds extracted with methanol, acetone and water, and differing in Se concentrations.

Location	Se concentration in wheat ppb	TEAC %	corresponded Trolox mg
Samsun	254	3.8 x 0.66	1
	1510	4 x 0.67	1
	2275	3.9 ± 0.02	1
Ankara	28	4.9 x 0.86	2
	1902	4.5 x 0.05	2
	5127	5.1 x 0.86	2

4.3.3 Antioxidant capacity of embryo, bran and endosperm parts of seeds

All antioxidant tests described above were conducted on whole seed samples. Since seed parts such as endosperm and bran could be different in concentration of bioactive compounds such as antioxidants (Moore et ah, 2006; Hung and Morita, 2008) we wanted to analyze antioxidant activity of endosperm, embryo and bran fractions of seeds. Seeds with different Se concentrations from Ankara, Samsun and Eskisehir were selected to separate their endosperm, embryo and bran fractions for analyses of total antioxidant activity by using the ABTS assay. Like with whole seeds, also different fractions of seeds with wide range in Se concentrations did not affect the antioxidant activity (Figures 10, 11 and 12). In the case of Samsun (Figure 11) and Eskisehir (Figure 12) locations, the bran fractions of seeds showed higher antioxidant activity than the embryo and endosperm.

Ankara

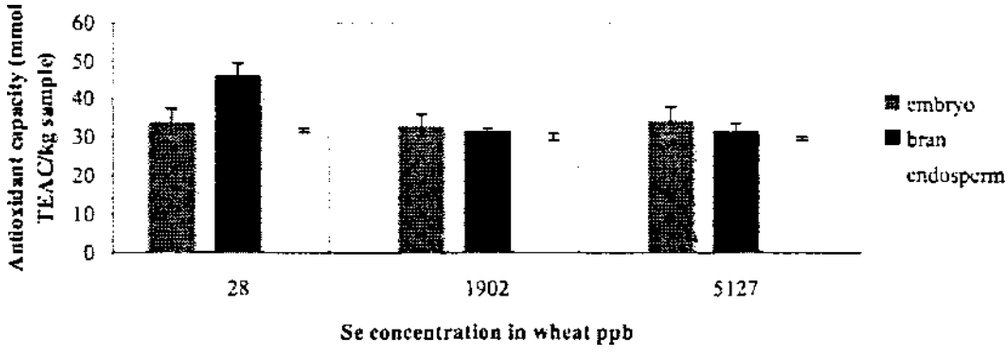

Figure 10: Effect of different fractions of wheat seeds on antioxidant capacity measured by detoxification of ABTS free radical. The fractions were obtained from wheat seeds differing in Se concentration (e.g. 28, 1902, and 5127 ppb) and grown in Ankara location.

Samsun

. 1

M embryo ■
bran
endosperm

Se concentration in wheat ppb

Figure 11: Effect of different fractions of wheat seeds on antioxidant capacity measured by detoxification of ABTS free radical. The fractions were obtained from wheat seeds differing in Se concentration (e.g. 254, 1510, and 2275 ppb) and grown in Samsun location.

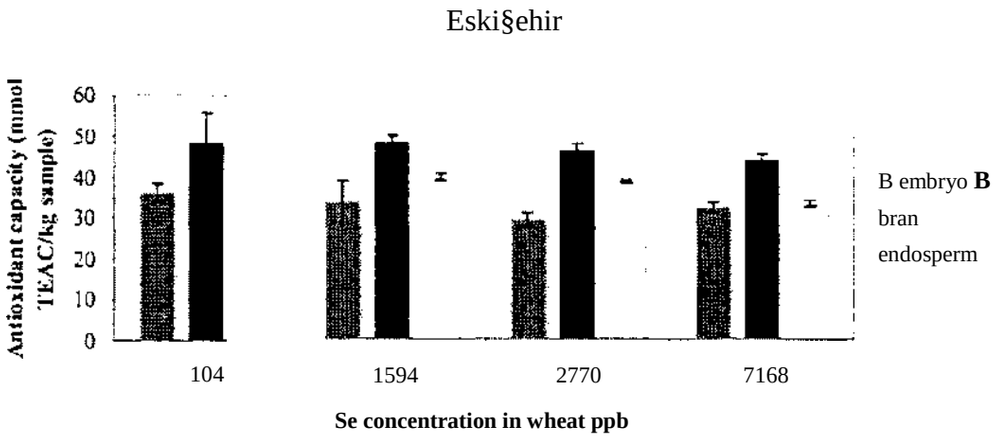

Figure 12: Effect of different fractions of wheat seeds on antioxidant capacity measured by detoxification of ABTS free radical. The fractions were obtained from wheat seeds differing in Se concentration (e.g. 104, 1594, 2770, and 7168 ppb) and grown in Eskisehir location.

5 DISCUSSION

In this Thesis, as experimental materials, seed samples were used which were widely different in Se concentrations showing a variation with approximately 250 fold (Table 3). Therefore, the seed samples of the present study were very useful for studying the antioxidant activities of the varied Se concentrations in seeds. Three assays were used for determination of the antioxidant activity of the seed samples which were extracted by using different extracting solutions. The assays applied on the seeds include i) MTT assay conducted in mammalian cell cultures, ii) DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging test, and iii) ABTS (2,2-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical-scavenging assay.

Among these tests, MTT assay has been extensively used assay in mammalian cell cultures to characterize antioxidative effectiveness of various biological compounds (Tim Mosmann, 1983; Roche Applied Science, 2005; Fotakis and Timbrell, 2006). As shown in Figures 6, 7, 8 and 9, increases in seed Se concentrations remained ineffective on antioxidant activity of seeds measured by the MTT assay. Even, at higher concentrations of Se in seeds (especially above 1500 ppb) there was an inhibitory effect of Se on cell viability. In Izmir, Samsun and Eskisehir locations, seeds containing higher than 1000 ppb Se had inhibitory effects on cell viability. It is well-documented that at higher concentrations Se might be toxic to cellular systems. The critical concentrations of Se for deficiency and toxicity are very narrow (Combs and Combs, 1986b, Combs, 2001; Pedrero and Madrid, 2009). According to Marschner (1995) the concentrations of Se between 1000-5000 ppb in dry matter are the maximum tolerable level in the diet of animals. Fan *et al.* (1990) reported that 4000 ppb Se has a detrimental effect on growing animals. Therefore, a special attention should be paid to higher concentrations of Se in foods. According to Pedrero and Madrid (2009), Se concentrations exceeding 1000 ppb might be toxic in biological systems. Based on the results it can be suggested that seed Se concentrations should not exceed 1000 ppb in Se fertilization trials. In various reports it has been reported that the most suitable Se concentrations in cereal grains for a

better human nutation should be between 100 to 1000 ppb (Lyons *et al.*. 2003 and 2005; Broadley *et al*., 2006).

Similar results obtained with the MTT assay were also found with the DPPH radical (Sanchez-Moreno *et al.,* 1999; Malencic *et al.,* 2000; Xu and Hu, 2004) and the ABTS cationic radical (Serpen et ah, 2007) scavenging assays. These assays were successfully used assays in literature to measure antioxidative capacity of cereal grains (Zielinski and Kozlowska, 2000; Serpen *et al.,* 2007; Serpen *et al.,* 2008; Xu and Hu, 2004; Perez-Jimenez and Saura-Calixto, 2005; Liyana-Pathirana and Shahidi, 2006). Seeds with large variation in Se concentrations from Samsun and Diyarbakir (Table 6) did not behave differently in their total antioxidant activity based on the DPPH radical scavenging activity. Also in the case of the ABTS assay (Tables 6 and 7) seeds differing in Se concentrations were similar in their antioxidant activity. Having low or high Se concentrations below 1000 ppb did not result in different antioxidative potential. These results with DPPH and ABTS radical scavenging assays were in agreement with the results obtained with the MTT assay (Figures 6, 7, 8 and 9), indicating that Se has no any antioxidative effect in wheat seeds under given conditions.

In literature there are number of reports indicating high antioxidative role of Se or Se-compounds in cellular systems (Combs Jr, 2001; Pedrero and Madrid, 2009 and the references given in the Introduction section). By contrast, the results presented in this thesis did not provide any indication for an antioxidant role of Se in wheat seeds. One of the reasons for the ineffectiveness of Se on antioxidant capacity of the wheat extracts might be related to the extraction method applied on wheat seeds. The extraction method used for DPPH radical scavenging was the same applied by Xu and Hu (2004) who showed higher antioxidant activity in rice seeds with elevated Se concentrations. Use of different seed materials (e.g., rice vs wheat) might be one reason for such differential results between 2 studies.

Possibly, wheat seeds do not release adequate amount of Se-compounds with high antioxidative activity during the extraction process so that seeds remained ineffective on antioxidant potential of seeds. Adorn and Lui (2002) reported that nearly 90 % of the antioxidants in wheat seeds are bound. In the case of com, rice and

oat, the proportion of the bound antioxidants were 87 %, 71% and 58 %, respectively. However, in the present study, 3 various extraction methods used to test antioxidant potential of seeds: i) hot water, ii) cellulose and iii) methanol, acetone and water. These solutions are often used for extracting antioxidants from different biological samples (Naczk and Shahidi, 2006; Serrano *et al.*, 2007; Yu, 2008; Hassas-Roudsari *et al.*, 2009). Even with these different extraction methods, seeds with varied Se concentrations were same in their antioxidant potential.

Other alternative for the explanation of the ineffectiveness of Se on antioxidant potential of seeds could be related to the inherent antioxidant activity of wheat seeds. Possibly, wheat seeds contain very high inherent antioxidant capacity due to high levels of phytoactive compounds such as flavonoids, phenolics, lutein, tocopherols etc (Yu, 2008). So, it is supposed that the level of the expected increase in antioxidant capacity through the enrichment of seeds with Se is too low compared to the inherent antioxidant capacity of wheat seeds used in the trials so that the resulted increase in the antioxidant capacity of seeds after Se enrichment could not be detected under given conditions. Xu and Hu (2004) showed higher antioxidant activity after enrichment of seeds with Se. It would be interesting to test antioxidant capacity of rice and wheat seeds to determine whether wheat has more antioxidative capacity than the rice seeds. According to the results of Adorn and Lui (2002), wheat seeds contain 2-fold more antioxidant activity than the rice seeds

It might be also possible that enrichment of seeds with Se has, indeed, no influence on the antioxidant capacity of seeds. There are some published data in literature showing that Se compounds have no antioxidant role in mammalian cells. Dunstan *et al.* (2007) reported that in 54 adults having allergic diseases, Se supplementation increased greatly Se concentration of the individuals but Se did not result in any influence on the immune system of the affected people. In another study conducted by Lippman *et al.* (2009), it was determined whether selenium could prevent prostate cancer, and the results showed that Se had no effect on development of prostate cancer. There are also further studies showing the ineffectiveness of Se for the prevention against carcinoma and cardiovascular diseases (Clark *et al.*, 1996; Duffield-Lillico *et al.*, 2002; Hercberg *et al.*, 2004; Stranges *et al.*, 2006).

6 CONCLUSION

The results presented in this study clearly indicate that seeds differing greatly in Se concentrations behaved similarly in their total antioxidant activity. In contrast to our expectations, Se did not affect antioxidant activity of seeds in positive direction in different antioxidant assays and extraction mediums tested. The reason for the ineffectiveness of Se on antioxidant potential of seeds could not be understood, although some papers are available supporting the results of this study, as discussed above. In order to achieve a reliable conclusion about the antioxidative role of Se in wheat seeds further tests are needed such as tests by using isolated Se-compounds (selenoproteins). Measurement of Se-proteins in Se-enriched seeds (with ICP-MS: Inductively Coupled Plasma - Mass Spectrometer) is also important. It would be also important to measure soluble and bound antioxidants in wheat seeds differing in Se concentrations. Having information about the proportion of Se-compounds within the bound antioxidants could be helpful in explanation of the results presented in this thesis.

7. REFERENCES:

1. Adams ML, Lombi E, Zhao F-J, McGrath SP, 2002. Evidence of low selenium concentrations in UK bread-making wheat grain. *Journal of the Science of Food and Agriculture,* 82 (10), 1160-1165.

2. Adorn KK, Sorrells ME, Liu RH, 2005. Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. *Journal of Agricultural and Food Chemistry*, 53, 2297-2306.

3. Adorn K and Liu R, 2002. Antioxidant activity of grains, *Journal of Agriculture and Food Chemistry,* 50, 6182-6187.

4. Allan CB, Lacourciere GM, Stadtman TC, 1999. Responsiveness of selenoproteins to dietary selenium. *Annual Reviews of Nutrition,* 19, 1-16.

5. Antolovich M, Prenzler PD, Patsalides E, McDonald S, Robards K, 2002. Methods for testing antioxidant activity. *The Analyst,* 127, 183-198.

6. Arthur JR, 2003. Micronutrient Group Symposium on 'Micronutrient supplementation: when and why?'. Selenium supplementation: does soil supplementation help and why?. *Proceedings of the Nutrition Society,* 62, 393-397.

7. Aspila P, 2005. History of selenium supplemented fertilization in Finland. In: Eurola M (ed) *Proceedings Twenty Years of Selenium Fertilization*, pp 8-1. Agrifood Research Reports 69.

8. Berken A, Mulholland MM, LeDuc DL, Terry N, 2002. Genetic engineering of plants to enhance selenium phytoremediation. *Critical Reviews in Plant*

9. Blankenberg S, Rupprecht HJ, Bickel C, Torzewski M, Hafner G, Tiret L, Smieja M, Cambien F, Meyer J, Lackner KJ, 2003. Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease. *New England Journal of Medicine,* 349 (17), 1605-1613.

10. Broadley MR, White PJ, Bryson RJ, Meacham MC, Bowen HC, Johnson SE, Hawkesford MJ, McGrath SP, Zhao FJ, Breward N, Flarriman M, Tucker M, 2006. Biofortification of UK food crops with selenium. *Proceedings of the Nutrition Society,* 65, 169-181.

11. Carvalho KM, Gallardo-Williams MT, Benson RF, Martin DF, 2003. Effects of selenium supplementation on four agricultural crops. *Journal of Agricultural and Food Chemistry,* 51, 704-709.

12. Cary EE & Allaway WH, 1969. The stability of different forms of selenium applied to low-selenium soils. *Soil Science Society of America Journal,* 33, 571-574.

13. Chen L, Yang F, Xu J, Hu Y, Hu Q, Zhang Y, Pan G, 2002. Determination of selenium concentration of rice in China and effect fertilization of selenite and selenate on selenium content of rice. *Journal of Agricultural and Food Chemistry,* 50, 5128-5130.

14. Clark LC, Combs Jr GF, Turnbull BW et al., 1996. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. *The Journal of the American Medical Association,* 276(24), 1957-1963.

15. Combs GF Jr & Combs SB, 1986b. Selenium in foods and feeds: In *The role of Selenium in nutrition,* pp 41-126. NY Academic Press, New York.

16. Combs GF Jr, 2001. Selenium in global food system. *British Journal of Nutrition,* 85, 517-547.

17. Curtin D, Hanson R, Lindley TN, Butler RC, 2006. Selenium concentration in wheat (*Triticum aestivum*) grain as influenced by method, rate, and timing of sodium selenate application, *New Zealand Journal of Crop and Horticultural Science,* 34, 329-339.

18. Diplock AT, 1993. Indexes of selenium status in human populations. *Am J Clin Nutr,* 57, 256S-258S.

19. Duffield-Lillico AJ, Reid ME, Turnbull BW, Combs Jr GF et al., 2002. 2002-Baseline characteristics and the effect of selenium supplementation on cancer incidence in a randomized clinical trial: A summary report of the nutritional prevention of cancer trial. *Cancer Epidemiology, Biomarkers & Prevention*, 11,630-639.

20. Dunstan JA, Breckler L, Hale J, Lehmann H, Franklin P, Lyons G, Ching SYL, Mori TA, Barden A, Prescott SL, 2007. Supplementation with vitamins C, E, 13-carotene and selenium has no effect on anti-oxidant status and immune responses in allergic adults: a randomized controlled trial. *Clinical and Experimental Allergy,* 37, 180-187.

21. Ekholm P, Eurola M and Venalainen ER, 2005. Selenium content of foods and diets in Finland. In Proceedings Twenty Years of Selenium Fertilization. Ed: M. Eurola. Agrifood Research Reports 69.

22. Fageria NK, Filho MPB, Moreira A, Guimaraes CM, 2009. Foliar fertilization of crop plants. *Journal of Plant Nutrition,* 32, 1044-1064.

23. Fan A.M., Kizer K.W, 1990. Selenium Nutritional, Toxicologic, and Clinical Aspects. *The Western Journal of Medicine,* 153, 160-167.

24. Fang Y, Wang L, Xin Z, Zhao L, An X, Hu Q, 2008. Effect of foliar application of zinc, selenium, and iron fertilizers on nutrients concentration and yield of rice grain in china. *Journal of Agricultural and Food Chemistry,* 56, 2079-2084.

25. Finley JW, 1998. The absorption and tissue distribution of selenium from high-selenium broccoli are different from selenium from selenite, sodium selenate, and selenomethionine as determined in selenium-deficient rats. *Journal of Agricultural and Food Chemistry,* 46, 3702-3707.

26. Finley JW, 2005. Proposed criteria for assessing the efficacy of cancer reduction by plant foods enriched in carotenoids, glucosinolates, polyphenols and selenocompounds. *Annals of Botany,* 95, 1075-1096.

27. Fotakis G and Timbrell JA, 2006. In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters,* 160 (2), 171-177.

28. Foyer CH, Descourvieres P, Kunert KJ, 1994. Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. *Plant, Cell and Environment,* 17, 507-523.

29. Gaziano JM, Glynn RJ, Christen WG, Kurth T, Belanger C, MacFadyen J, Bubes V, Manson JE, Sesso HD, Buring JE, 2009. Vitamins E and C in the prevention of prostate and total cancer in men: The physicians' health study II randomized controlled trial. *Journal of American Medical Association,* 301(1), 52-62.

30. Gissel-Nielsen G, 1998. Effects of selenium supplementation of field crops: In *Environmental Chemistry of Selenium,* pp 99-112. Marcel Dekker New York, NY.

31. Golubkina NA, Alfthan GV, 1999. The human selenium status in 27 regions of Russia. *Journal of Trace Elements in Medicine and Biology,* 13, 15-20.

32. Hartikainen H., Xue T., Piironen V, 2000. Selenium as an anti-oxidant and pro-oxidant in ryegrass. *Plant and Soil,* 225, 193-200.

33. Hassas-Roudsari M, Chang PR, Pegg, RB, Tyler RT, 2009. Antioxidant

capacity of bioactives extracted from canola meal by subcritical water, ethanolic and hot water extraction. *Food Chemistry,* 114(2), 717-726.

34. Hercberg S, Galan P, Preziosi P et al., 2004. The SU.VI.MAX study: a randomized, placebo-controlled trial of the health effects of antioxidant vitamins and minerals. *Archives of Internal Medicine,* 164, 2335-2342.

35. Huang D, Ou B, Prior RL, 2005. The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry,* 53, 1841-1856.

36. Hung PV and Morita N, 2008. Distribution of phenolic compounds in the graded flours milled from whole buckwheat grains and their antioxidant capacities. *Food Chemistry,* 109(2), 325-331.

37. Hurwitz BE, Klaus JR, Llabre MM, Gonzalez A, Lawrence PJ, Maher KJ, Greeson JM, Baum MK, Shor-Posner G, Skyler JS, Schneiderman N, 2007. Suppression of human immunodeficiency virus type 1 viral load with selenium supplementation. *Arch Intern Med,* 167, 148-154.

38. Imai H and Nakagawa Y, 2002. Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. *Free Radical Biology and Medicine,* 34 (2), 145-169.

39. Ip C and Lisk DJ, 1994. Characterization of tissue selenium profiles and anticarcinogenic responses in rats fed natural sources of selenium-rich products. *Carcinogenesis,* 15, 573-576.

40. Jiang C, Wang Z, Ganther H, Lu J, 2002. Distinct effects of methylseleninic acid *versus* selenite on apoptosis, cell cycle, and protein kinase pathways in DU 145 human prostate cancer cells. *Molecular Cancer Therapeutics*, 1, 1059- 1066.

41. Jilani KEB, Panee J, He Q, Berry MJ, Li PA, 2007. Overexpression of selenoprotein H reduces Ht22 neuronal cell death after UVB irradiation by

preventing superoxide formation. *International Journal of Biological Sciences,* 3, 198-204.

42. Keshan Disease Research Group, 1979. Epidemiologic studies on the etiologic relationship of selenium and Keshan disease. *Chinese Medical Journal,* 92(7), 477-82.

43. Levander OA and Beck MA, 1999. Selenium and viral virulence. *British Medical Bulletin,* 55, 528-533.

44. Lippman SM, Klein EA, Goodman PJ, et ah, 2009. Effect of Selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and vitamin E cancer prevention trial (SELECT). *Journal of American Medical Association,* 301(1), 39-51.

45. Liyana-Pathirana CM and Shahidi F, 2006. Importance of insoluble-bound phenolics to antioxidant properties of wheat. *Journal of Agricultural and Food Chemistry,* 54, 1256-1264.

46. Liyana-Pathirana CM and Shahidi F, 2007. Antioxidant and free radical scavenging activities of whole wheat and milling fractions. *Food Chemistry,* 101, 1151-1157.

47. Lyons G, Ortiz-Monasterio I, Stangoulis J, Graham R, 2005. Selenium concentration in wheat grain: Is there sufficient genotypic variation to use in breeding? *Plant and Soil,* 269, 369-380.

48. Lyons G, Stangoulis J, Graham R, 2003. High-selenium wheat: biofortification for better health. *Nutrition Research Reviews,* 16, 45-60.

49. Malencic DJ, Gasic O, Popovic M, Boza P, 2000. Screening for antioxidant properties of *Salvia reflexa* homem. *Phytotherapy Research,* 14, 546-548.

50. Marschner H, 1995. Beneficial Mineral Elements: In *Mineral Nutrition of*

Higher Plants, pp 431. Academic Press London, UK.

51. Medina D, Thompson H, Ganther H, Ip C, 2001. Se-Methylselenocysteine: A new compound for chemoprevention of breast cancer. *Nutrition and Cancer,* 40(1), 12-17.

52. Mensor LL, Menezes FS, Leitao GG, Reis AS, Santos TC, Coube CS, Leitao SG, 2001. Screening of brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytotherapy Research,* 15, 127-130.

53. Michiels C, Raes M, Toussaint O, Remade J, 1994. Importance of SE-glutathione peroxidase, catalase, and CU/ZN-SOD for cell survival against oxidative stress. *Free Radical Biology and Medicine,* 17 (3), 235-248.

54. Mihailovic M, Lindberg P, Jovanovic I, 1996. Selenium content in feedstuffs in Serbia. *Acta Veterinaria Beograd*, 46, 343-348.

55. Moore J, Cheng Z, Su L, Yu L, 2006. Effects of solid-state enzymatic treatments on the antioxidant properties of wheat bran. *Journal of Agricultural and Food Chemistry*, 54, 9032-9045.

56. Mosmann T, 1983.Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity. *Journal of Immunological Methods,* 65 (1-2), 55-63.

57. Naczk M and Shahidi F, 2006. Phenolics in cereals, fruits and vegetables: occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis,* 41, 1523-1542

58. Oldfield JE, 1997. Observations on the efficacy of various forms of selenium for livestock: A review. *Biomedical and Environmental Sciences,* 10 (2-3), 280-291.

59. Ozgen M, Reese RN, Tulio AZ Jr, Scheerens JC, Miller AR, 2006. Modified

2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) method to measure antioxidant capacity of selected small fruits and comparison to ferric reducing antioxidant power (FRAP) and 2,2'-Diphenyl-l-picrylhydrazyl (DPPH) methods. *Journal of Agricultural and Food Chemistry,* 54, 1151-1157.

60. Pedrero Z and Madrid Y, 2009. Novel approaches for selenium speciation in foodstuffs and biological specimens: A review. *Analytica Chimica Acta,* 634, 135-152.

61. Perez-Jimenez J and Saura-Calixto F, 2005. Literature data may underestimate the actual antioxidant capacity of cereals. *Journal of Agricultural and Food Chemistry*, 53, 5036-5040.

62. Ratnasinghe D, Tangrea JA, Andersen MR, Barrett MJ, Virtamo J, Taylor PR, Albanes D, 2000. Glutathione peroxidase codon 198 polymorphism variant increases lung cancer risk. *Cancer Research,* 60, 6381-6383.

63. Rayman MP, 2000. The importance of selenium to human health. *The Lancet,* 356, 233-241.

64. Rayman MP, 2005. Selenium in cancer prevention: a review of the evidence and mechanism of action. *Proceedings of the Nutrition Society,* 64, 527-542.

65. Redman C, Scott JA, Baines AT, Basye JL, Clark LC, Calley C, Roe D, Payne CM, Nelson MA, 1998. Inhibitory effect of selenomethionine on the growth of three selected human tumor cell lines. *Cancer Letters,* 125, 103-110.

66. Roche Applied Science, 2005. “Cell Proliferation Kit I (MTT)”. Retrieved on 2009-08-08.

67. Rotruck JT, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG, 1973. Selenium: Biochemical role as a component of glutathione peroxidase. *Science,* 179, 588-590.

68. Sanchez-Moreno C, Larrauri JA, Saura-Calixto F, 1999. Free radical scavenging capacity of selected red, rose and white wines. *Journal of the Science of Food and Agriculture,* 79(10), 1301-1304.

69. Sandalova T, Zhong L, Lindqvist Y, Holmgren A, Schneider G, 2001. Three-dimensional structure of a mammalian thioredoxin reductases: Implications for mechanism and evolution of a selenocysteine-dependent enzyme. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 9533-9538.

70. Schomburg L, Schweizer U, Kohrle J, 2004. Selenium and selenoproteins in mammals: extraordinary, essential, enigmatic. *Cellular and Molecular Life Sciences,* 61, 1988-1995.

71. Schwarz K and Foltz C, 1957. Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. *Journal of the American Chemical Society,* 79, 3292-3293.

72. Seo Y.R., Kelley M. R., Smith M. L, 2002. Selenomethionine regulation of p53 by a refl-dependent redox mechanism. *PNAS*, 99, 14548-14553.

73. Serpen A, Capuano E, Fogliano, and Gokmen V, 2007. A New Procedure To Measure the Antioxidant Activity of Insoluble Food Components. *Journal of Agricultural and Food Chemistry,* 55, 7676-7681.

74. Serpen A, Gokmen V, Pellegrini N, Fogliano V, 2008. Direct measurement of the total antioxidant capacity of cereal products. *Journal of Cereal Science*, 48(3), 816-820.

75. Serpen A, Gokmen V, Karagoz A, Koksel H, 2008.Phytochemical quantification and total antioxidant capacities of emmer (*Triticum dicoccon* Schrank) and einkom (*Triticum monococcum* L.) wheat landraces. *Journal of Agricultural and Food Chemistry,* 56, 7285-7292.

76. Serrano J, Goni I, and Saura-Calixto F, 2007, Food antioxidant capacity determined by chemical methods may underestimate the physiological antioxidant capacity. *Food Research International* 40, 15-21.

77. Sies H, Sharov VS, Klotz LO, Briviba K, 1997. Glutathione peroxidase protects against peroxynitrite-mediated oxidations- A new fuction for selenoproteins as peroxynitrite reductase. *Journal of Biological Chemistry,* 272 (44), 27812-27817.

78. Steinbrenner H, Alili L, Bilgic E, Sies H, Brenneisen P, 2006. Involvement of selenoproteins P in protection of human astrocytes from oxidative damage. *Free Radical Biology & Medicine,* 40, 1513-1523.

79. Stranges S, Marshall JR, Trevisan M, Natarajan R, Donahue P, Combs GF, Farinaro E, Clark LC, Reid ME, 2006. Effects of Selenium supplementation on cardiovascular disease incidence and mortality: Secondary analyses in a randomized clinical trial. *American Journal of Epidemology,* 163(8), 694-699.

80. Terry N and Zayed AM, 1994. Selenium Volatilization by Plants: Frankenberger WT and Benson S (ed.) In *Selenium in the environment,* pp 343. Marcel Dekker New York, NY.

81. Tinggi U, 2003. Essentiality and toxicity of selenium and its status in Australia: a review. *Toxicology Letters,* 137, 103-110.

82. Venalainen ER, Hirvi T, Him J, 1997. Effect of selenium supplementation on the selenium content in muscle and liver of Finnish pigs and cattle. *Journal of Agricultural and Food Chemistry,* 45, 810-813.

83. Waters DJ, Shen S, Cooley DM, Bostwick DG, Qian J, Combs GF Jr, Glickman LT, Oteham C, Schlittler D, Morris JS, 2003. Effects of dietary selenium supplementation on DNA damage and apoptosis in canine prostate. *Journal of the National Cancer Institute,* 95, 237-244.

84. Welch RM, Graham RD, 2002. Breeding crops for enhanced micronutrient content. *Plant and Soil,* 245, 205-214.

85. Whanger PD, 1989. Proteins and Amino Acids. Selenocompounds in plants and their effects on animals: In *Toxicants of Plant Origin. Vol. Ill,* pp 141- 167. CRC Press.

86. Whanger PD, 2002. Selenocompounds in plants and animals and their biological significance. *Journal of the American College of Nutrition,* 21, 223-232.

87. Whanger PD, Ip C, Polan CE, Uden PC, Welbaum G, 2000. Tumorigenesis, metabolism, speciation, bioavailability, and tissue deposition of selenium in selenium-enriched ramps *(Allium tricoccum). Journal of Agricultural and Food Chemistry,* 48, 5723-5730.

88. Whanger PD, 2004. Selenium and its relationship to cancer: an update. *British Journal of Nutrition,* 91, 11-28.

89. Xu J and Hu Q, 2004. Effect of foliar application of selenium on the antioxidant activity of aqueous and ethanolic extracts of selenium-enriched rice. *Journal of Agricultural and Food Chemistry,* 52, 1759-1763.

90. Yalcintas GS and Saldamli I, 2005. The effect of selenium and zinc fortification on the quality of Turkish white cheese. *International Journal of Food Sciences and Nutrition,* 56(2), 141-146.

91. Ylaranta T, 1983a. Effect of added selenite and selenate on the selenium content of Italian rye grass (*Lolium multiflorum)* in different soils. *Annales Agriculturae Fenniae,* 22, 139-151.

92. Ylaranta T, 19836. Effect of added selenite and selenate on the selenium content of barley (*Hordeum vulgare*). *Annales Agriculturae Fenniae,* 22, 164-

174.

93. Ylaranta T, 1984. Raising the selenium content of spring wheat and barley using selenite and selenate. *Annales Agricidturae Fenniae,* 23, 75-84.

94. Yu L. 2008. Wheat Antioxidants; John Wiley & Sons, Inc. Hoboken, NY 07030.

95. Zhang F, Yu W, Hargrove JL, Greenspan P, Dean RG, Taylor EW, Hartle DK, 2002. Inhibition of TNF-alpha induced ICAM-1, VCAM-1 and E-selectin expression by selenium. *Atherosclerosis*, 161, 381-386.

96. Zhou X, Yuan Y, Yang Y, Rutzke M, Thannhauser TW, Kochian LV, Li L, 2009. Involvement of a broccoli COQ5 methyltransferase in the production of volatile selenium compounds. *Plant Physiology,* 10.1104, 109.142521.

97. Zielinski H and Kozlowska H, 2000. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. *Journal of Agricultural and Food Chemistry,* 48, 2008-2016.

APPENDIX

Chemicals

All chemicals and standart solutions were supplied by
Merck (Germany),

SIGMA (USA),

Fluka (Switzerland)

Equipment

Centrifuge:	Kendro Lab. Prod., Heraeus Multifuge 3 S-R, GERMANY
Distilled water:	Millipore, Elix-S, FRANCE Millipore, MilliQ, Academic, FRANCE
Spectrophotometer:	ELISA Microplate Reader 680 BIORAD Varian Cary 300 UV-Visible Spectrophotometer
Microliter Pipette:	Gilson, Pipetman, FRANCE Eppendorf, GERMANY