SELENIUM ON INCREASING LENTIL (*LENS CULINARIS* MEDIKUS.) GRAIN

YIELD

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ABSTRACT

Selenium is an essential element for mammals but it has not been considered as essential for higher plants. The effect of Se application on lentil grain yield, antioxidant activity, nitrogen fixation, and seed Se concentration studied. Experiments were conducted at Carrington Research and Extension Center in 2012 and 2013. Selenium fertilization increased the lentil grain yield by 5 % in 2012 and by 9 % in 2013. Selenium application increased the antioxidant activity (70 % inhibition) of lentils compared to the untreated control (61% inhibition). Percentage nitrogen derived from air was increased with Se fertilization by 17 % and 12 % in 2012 and 2013, respectively, with Se fertilization. Seed Se concentration was significantly higher in selenate treated lentils (1.4 mg/kg) compared to selenite (0.9 mg/kg) and the control (0.6 mg/kg). Overall, application of Se increased lentil grain yield, nitrogen fixation, antioxidant protection, and seed Se concentration.

Key words: Selenium, biofortification, antioxidant activity, nitrogen fixation, yield, lentil

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1. INTRODUCTION

Selenium (Se) is an important element in human and animal nutrition. The importance of Se as an essential element in plants has not been identified yet (Germ et al., 2007). However, Se is known to increase the growth and production of different food crops (Xue et al., 2001; Djanaguiraman et al., 2010). Application of Se increased seed production by 43 % in canola (*Brassica napus* L.) (Lyons et al., 2009) and dry yield by 14 % in lettuce (*Lactuca sativa* L.) (Xue et al., 2001). Selenium fertilization increased seed size (11 %) and seed weight (26 %) in sorghum (*sorghum bicolor* L.) (Djanaguiraman et al., 2004), and growth (69 % leaf increase and 60 % leaf area increase) in soybean (*Glycine Max* L.) (Djanaguiraman et al., 2010).

Selenium helps counteract biotic and abiotic stress in plants (Smrkolj et al., 2006; Lyons et al., 2009; Djanaguiraman et al., 2010). Selenium-treated wheat (*Triticum aestivum* L.) plants showed an increased protection from ultra violet radiation and cold stress (Yao et al., 2011; Chu et al., 2010). Selenium protected rapeseed (*Brassica napus* L.) seedlings from drought and salinity-induced damage (Hasanuzzaman and Fujita, 2011; Hasanuzzaman et al., 2011). Hanson et al. (2003) observed higher tolerance to caterpillars, aphids, and common *Fussarium* wilt disease in Se-treated brassica (*Brassica napus* L.) plants.

Selenium is important in biological nitrogen fixation (Hara, 2001). Selenium is important for better health and functioning of Rhizobium bacteria (Giller and Wilson, 1991; Hara et al., 1988). Hara (2001) observed a direct requirement of Se for the metabolism of symbiotic nitrogen bacteria. Selenium plays an important role in hydrogenases (NiFeSe- hydrogenases), which contain Se in their active sites as a selenocysteine residue (Baltazar et al., 2011; Parkin et al., 2008). Milton and Theresa (1989) reported that hydrogenase activity can be maximized by supplementing Se. Selenium application increased the hydrogenase expression and activity by

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133% in autotrophically cultured *Bradyrhizobium japonicum* (Boursier et al., 1988). In addition, selenium increased nodule number by 62 % in alfalfa (*Medicago sativa* L.) (Sekyere at al., 2013). Physiological and field-based studies are important to improve the understanding of Se in the functioning of rhizobia in nitrogen fixation.

Lentil is well known as a nutritious food with high protein (22-30 %), dietary fiber, oligosaccharides, resistant starch, and a range of micronutrients (Bhatty et al., 1976; Thavarajah et al., 2011). Lentil has a range of minerals such as iron (Fe), zinc (Zn), magnesium (Mg), potassium (K), calcium (Ca), and Se. This study was conducted to determine the effect of Se fertilization with response to grain yield, antioxidant activity, nitrogen fixation, and seed Se concentration in lentil plants.

2. LITERATURE REVIEW

2.1. Selenium

Selenium, a metalloid, was first discovered by the German scientist Berzelius in 1817 (Oldfield, 2002). Selenium has an atomic number of 34, weight of 78.94 g/mol, radius of 117 pm, melting point of 220.5°C, and boiling point of 685°C (Martens, 2003). There are six Se isotopes (mass of 74, 76, 77, 78, 80, and 82) in nature. Selenium resembles sulfur (S) in terms of atomic size, bond energies, and ionization potentials as these elements occupy the Group VIA in the periodic table. The main oxidation states of Se are +6, +4, 0, and -2 with analogy to S. Similar to S, Se combines with many elements such as bromine (Br), chlorine (Cl), fluorine (F), hydrogen (H), and phosphorus (P). The affinity of Se for oxygen (O) is lower than S; as a result, it mainly forms two oxides, selenite (SeO_3^{-2}) and selenate (SeO_4^{-2}).

Selenium exists in both organic and inorganic forms. The primary organic Se forms are selenocysteine (SeCys) and selenomethionine (SeMet) (Sanmartin et al., 2012). Some organic compounds such as dimethylselenide and dimethyldiselenide are volatile at room temperature (Martens, 2003). The inorganic forms of Se are selenate ($SeO4^{-2}$), selenite (SeO_3^{-2}), selenide (Se^{-2}), and elemental Se, which are mainly found in soil (Sanmartin et al., 2012). The main Se compounds are classified in **Table 2.1.**

Table 2.1. Organic and inorganic Se compounds

Category	Chemical compound
Selenoamino acids	selenomethionine , selenocysteine, selenocystine, methylselenocysteine, selenohomocysteine, se-methylselenocysteine, selenocystamine, selenocystatonine
Se (IV and VI) compounds	selenite, selenate, selenous acid, selenic (VI) acid, selenium dioxide
Selenides	hydrogen selenide, dimethyl selenide, trimethylselenide,
Selenocyanates and other organic compounds	glutathionylselenol, selenodiglutathione , methylselenol

Sources: Sanmartin et al. (2012), Martens (2003)

2.2. Soil Se distribution around the world

Concentration and the distribution of Se are highly variable in soils. Soil Se concentrations generally range from 0.01 mg/kg to 2.0 mg/kg, with an average of 0.4 mg/kg (Fordyce, 2005). Soil Se concentration is mainly determined by the type of parental material (Shamberger, 1981). Volcanic and granite soils are low in Se. Low Se soils are mainly found in the central part of China, New Zealand, Siberia, and Northern Europe including Finland, Sweden, France, Iceland, and Scotland. Mean soil Se concentration in the central part of China is 0.13 mg/kg (Johnson et al., 2010). High Se soils are derived from seleniferous parent materials including shales, sandstones, limestones, slate, and coal series**.** The high Se soils are widespread in mid-western parts of the United States, Canada, South America, northern and southern parts of China, and Russia (Susan et al., 2011). Selenium concentration in high Se soils ranges from 2

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mg/kg to 10 mg/kg (Johnson et al., 2010). Soil Se concentrations are high in the Great Plains of the United States and the Prairie provinces of Canada. Soil Se concentrations in Saskatchewan range from 0.2 to 11.7 mg/kg (Dunn, 1990).

Native substrate of a soil primarily determines the soil Se concentration. However, the plant availability of Se depends on many factors including chemical form of Se, soil physical-chemical properties, weather conditions (e.g., rainfall, temperature), and management practices of soil (e.g., irrigation, aeration, liming, and Se fertilization) (Combs, 2001). The adsorption kinetics of different forms of Se, pH-redox status, moisture content, and the microbial activity in soil greatly influence the availability of Se to plants. Bioavailability of selenate (Se^{+6}) is high in alkaline soils and selenite (Se^{+4}) in acidic soils (Stadlober et al., 2001). Microbes convert both selenite and selenate into reduced forms of Se such as dimethylselenide and dimethyldiselenide, which are volatile (Sager, 2006).

2.3. Uptake, metabolism and transportation of different Se species in plants

Selenium assimilation varies among plants. Plants can be divided into three groups: Se hyperaccumulators, Se nonaccumulators, and secondary Se accumulators (Brown and shrift 1982). Se-hyperaccumulators are *Astragalus, Stanleya, Morinda, Neptunia, Oonopsis,* and *Xylorhiza* that accumulate high levels (thousand to several thousand mg/kg) of Se in their tissues (Brown and shrift, 1982; Ihnat, 1989; Rosenfield and Beath, 1964). Selenium non-accumulators are incapable of assimilating Se at concentration greater 100 mg/kg dry weight of plant tissues (Brown and Shrift, 1982). Mainly, forages and most food crops such as cabbage (*Brassica oleracea* L.), broccoli (*Brassica oleracea* L.), garlic (*Allium sativum* L.), and anion (*Allium cepa* L.) are found in this category. In addition, some plants assimilate Se in moderate levels (up to 1000 mg/kg) (Brown and Shrift, 1982; Ihnat, 1989; Rosenfield and Beath, 1964). These plants

are referred to as secondary accumulators of Se. Examples of the secondary accumulators are *Aster, Triplex, Castilleja, Comandra, Grayia, Grindelia*, and *Machaeranthera* (Brown and Shrift, 1982).

Uptake, metabolism, and translocation of different Se species varies in plants. Selenium uptake is associated with the S assimilation pathway (Sors et al., 2005). But Se-hyperaccumulators assimilate Se over S (Bell et al., 1992). Selenate is absorbed and transported actively whereas selenite is transported passively across the cortex (Sors et al., 2005). After absorption, both selenate and selenite enter the S reaction pathway where selenate is reduced to selenite. Afterward, selenite undergoes reduction reactions, producing selenide, which will assimilate into seleno-amino acids. Selenium translocation in plants depends on the plant species, growth stage, and the Se forms applied (Terry et al., 2000). Selenium hyperaccumulators translocate Se in young leaves during the vegetative stage and in seeds during reproductive stage. Selenium is mainly concentrated in roots and seeds and only small amounts of Se in stems and leaves in Se non-accumulators (Terry et al., 2000). Selenate translocates mostly in shoots, while selenite and other organic forms of Se accumulate mostly in roots. Selenite is rapidly converted nto organic Se compounds such as SeMet and enters into the amino acids profile. As a result, the accumulation of selenite is limited in shoots.

2.4. Physiological role of Se in plants

Selenium has a positive effect on grain yield in different food crops (Lyons et al., 2009; Djanaguiraman et al., 2010; Hu et al., 2003). A summary of recent research on the effects of Se in plant growth and development is presented in **Table 2.2.**

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Table 2.2. Selenium effect on increased growth and development of plants

Crop	Response of Se treated plants compared to untreated plants
Canola	43 % higher seed production (Lyons et al., 2009)
Lettuce	14 % higher dry yield (Xue et al., 2001)
Sorghum	26 % increase in filled seed weight, 11 % increase in seed size (Djanaguiraman et al., 2010)
Alfalfa	125 kg/hm^2 yield increase (Hu et al., 2010)
Soybean	69 % more leaves, and 60 % increase in leaf area (Djanaguiraman et al., 2004)
Tea	4 g m^{-2} of total yield increase (Hu et al., 2003)
Potato	40 % increase in tuber yield (Turakinen et al., 2004)

Several studies have shown that Se increases the antioxidant protection in plants. Selenium treated wheat plants exposed to ultra violet radiation showed significantly higher protection than the untreated control (Smrkolij et al., 2006). Similar results have been observed in soybean, buckwheat, white clover (*Trifolium repens* L.), and sorghum (Djanaguiraman et al., 2005, Smrkolij et al., 2006; Wang, 2011; Djanaguiraman et al., 2010). Meanwhile, those plants treated with Se produced higher grain yield and biomass under stressful conditions than the control plants. This emphasizes the fact that Se increases the antioxidant activity and growth of plants. The increase of antioxidant activity of plants was associated with the inhibition of lipid peroxidation due to increased activity of antioxidant enzymes such as Glutathione peroxidase (GPX), Superoxide dismutase (SOD), Ascorbate peroxidase (APOX), and Glutathione reductase (GR) (Djanaguiraman et al., 2005; Hartikainen, et al., 2000; Wang, 2011).

Boyed (2007) reported that a low dosage of Se increased protection from pests and diseases in Brassica. Selenium-treated plants displayed a higher resistance to caterpillars, aphids, and common Fussarium wilt disease compared to the control plants. Selenium treated plants had unpleasant aroma and taste and caused toxic conditions to insects. Feed trails of moth caterpillars (*Spodoptera exigua*) have shown that they prefer to eat plant parts without Se. Also, plant tissues with Se concentration greater than 50 mg/kg was toxic to moth caterpillars (Trumble et al., 1998; Vickerman and Trumble, 1999). Moreover, plants that belong to *Atriplex* spp have shown resistance to *Spodoptera exigua* and reduced insect growth when treated with Se (Vickerman et al., 2002). In addition to the protective role against invertebrate herbivory, Se protects plants from fungal diseases and mammalian feeding (Hanson et al., 2003; Franke and Potter, 1936.). Therefore, application of Se may be beneficial in protecting plants from some insects and pathogens.

Nitrogen fixation is a process by which atmospheric nitrogen (N_2) is converted to ammonia (NH_3) and provides biological nitrogen to the rhizosphere. Legumes have the ability to develop symbiotic relationship with *Rhizobium* bacteria which are capable of fixing atmospheric N_2. Therefore most legumes have high potential to increase production through increased nitrogen fixation. Knut et al. (2003) reported that lentil symbiotically fixed 154 kg N/ha per growing season. During nitrogen fixation, bacteria secrete two enzymes: nitrogenase and hydrogenase. Nitrogenase helps convert atmospheric N_2 to NH_3 while producing hydrogen (H_2) as a byproduct of the process. Milton and Theresa (1989) identified hydrogenase as a Se-dependent enzyme which oxidizes H_2. Hydrogenase helps in cycling H_2 and reduces energy losses. Therefore, the presence of both enzymes is obligatory for smooth and efficient functioning of nitrogen fixation. However, certain symbiotic bacteria are incapable of producing

hydrogenase, or they produce comparatively less hydrogenase compared to nitrogenase (Baginsky et al., 2005). When this occurs, H_2 accumulates in nodules and slows the nitrogen fixation (Baginsky et al., 2005). In such conditions, some *Rhizobium* species produce more hydrogenase to reduce nodule H_2 concentration (Schubert and Evans, 1976). As a result, hydrogenase increases the efficiency of the nitrogen fixation by recovering energy loss (Maier and Triplett, 1996). Milton and Theresa (1989) reported that hydrogenase activity can be maximized by supplementing Se. Therefore, the application of Se may increase the nitrogen fixation as a result of increased activity of hydrogenase (Baginsky et al., 2005).

2.5. Lentil

Globally, lentil (*Lens culinaris* Medikus) ranks sixth in terms of production among pulses (FAOSTAT, 2012). Canada is the major lentil producer in the world, followed by India, Australia, Turkey, and the United States of America which collectively accounted for 79 % of global production in 2012 (FAOSTAT, 2012). Lentil production is comprised of approximately 70 % red lentils, 25 % green lentils, and 5 % other types including brown lentils. Canada dwarfed other lentil exporting nations by exporting 1.16×10^6 MT (59 %) of lentil in 2012. The top four exporters who collectively account for 92 % of global exports are Canada, Turkey, Australia, and USA where the lentil is grown primarily as an export commodity (FAOSTAT, 2011). The lentils exported are categorized into the following market classes: 60 % red lentils, 35 % green lentils, and 5 % brown lentils and others. Turkey is the major lentil importer accounting for 19 % of total world lentil imports, followed by the United Arab Emirates and Sri Lanka (37 %) (FAOSTAT, 2011). Lentil is also consumed in developing countries such as Bangladesh, Eritrea, Nepal, and Sri Lanka.

Lentil is high in protein (22-30 %), dietary fiber, oligosaccharides, resistant starch, and a range of micronutrients (Bhatty et al., 1976; Thavarajah et al., 2011). The nutritional quality of lentil is presented in **Table 2.3**.

Table 2.3. Nutritional quality of lentils

Characteristic	Market Class	
	Red	Green
Moisture %	8.2	8.5
Protein %	25	25
Starch %	53	52
Fat %	< 1.0	
Fiber %	4.4	
Ash %	3	2.7
Calcium (mg/kg)	418	293
Iron (mg/kg)	79	69
Magnesium (mg/kg)	482	367
Potassium (mg/kg)	7243	6954
Selenium (µg/kg)	503	726
Zinc (mg/kg)	40	34

Sources: US Pulse Quality Survey (2012), Bhatty (1988)

2.6. Selenium fertilization

Selenium fertilization increases Se levels in food crops. Application of Se fertilizer has increased the Se intake of people in many countries including as Finland, New Zealand, Australia, and Canada (Eurola et al., 1990). Selenium concentration of most cereal crops in

Finland has increased from 10 mg/kg (mean Se concentration of all Finnish cereals grains) to 250 mg/kg in spring wheat, 50 mg/kg in winter wheat, and 40 mg/kg in rye, after the initiation of Se fertilization program in 1984 (Eurola et al.,1990). Consequently, the average Se intake of the Finnish population was raised from 0.4-4.0 µg/day in 1976 to 124 µg/day in 1989. Furthermore, Lyons et al. (2005) reported that Se application (4-120 g/ha) increased the wheat Se concentration by 133-fold when Se was sprayed on the soil at seeding and by 20-fold when Se was applied as a foliar spray after flowering. Broadley et al. (2009) demonstrated that the wheat Se concentration increased by 10-fold at Se rate of 10 g/ha. Allan et al. (2012) suggested that Se application (5 g/ha) would increase maize Se concentration in Malawi and increased Se intake up to 26-37 µg/day. To date, many Se-enriched food crops such as broccoli (*Brassica oleracea* L.), garlic (*Allium sativum* L.), onion (*Allium cepa* L.), potato (*Solanum tuberosum* L.), and tea (*Camellia sinensis* L.) have been produced through agronomic biofortification to meet the adequate daily Se intake of consumers whose diets are naturally deficient in Se (Fairweather-Tait et al., 2011).

Foods originating from seleniferous areas are excellent sources of dietary Se. Moreover, the application of Se may increase the production of lentils providing an opportunity, especially, for small scale farmers to expand their production. Ultimately, agronomic biofortification would produce Se-rich lentils while increasing the grain production.

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3. OBJECTIVES AND HYPOTHESES

3.1. Chapter 1

3.1.1. Objective

To determine the effect of Se fertilization on grain yield of lentils.

3.1.2. Hypothesis

Selenium fertilization will affects lentil grain yield

3.1.2.1. Testable hypothesis

1. If selenium affects the plant physiology, selenium fertilization will affects lentil grain

yield.

Ho: Lentil grain yield will be equal for both the control and selenium treated plants.

2. If selenite performs an energy conserving metabolism in plants, selenite application will

have a greater yield response compared to selenate treated lentil plants.

Ho: Lentil grain yield will be equal for both selenite and selenate treated lentils

3.2. Chapter 2

3.2.1. Objective

To determine the effect of Se fertilization on grain yield and nitrogen fixation in lentils.

3.2.2. Hypothesis

Selenium fertilization will affect grain yield and nitrogen fixation in lentils.

3.2.2.1. Testable hypothesis

1. If selenium is important in nitrogen fixation, selenium fertilization will affect the

percentage of nitrogen derived from air ($\%N_{dfa}$).

Ho: $\%N_{dfa}$ of Se-treated plants will be the same as the control plants.

2. If selenite is more effective than selenate in nitrogen fixation, selenite will increase

nitrogen fixation ($\%N_{dfa}$) compared to selenite.

Ho: Application of selenite or selenate will equally effect on nitrogen fixation (%N$_{dfa}$)

in lentil plants

4. PAPER 1. EFFECT OF SELENIUM ON LENTIL (*LENS CULINARIS MEDIKUS*) GRAIN YIELD

4.1. Abstract

Selenium is an essential element for mammals but it has not been considered as essential for higher plants. Lentil (*Lens culinaris* Medikus), a cool season food legume, has been used as a model pulse crop for Se biofortification. The objective of this study was to determine the effect of field application of different Se forms on lentil grain yield, seed Se concentration, and antioxidant activity. The experiment was conducted at Carrington Research and Extension Center in 2012 and 2013 with six lentil genotypes treated with three Se treatments at post plant and again at flowering. Selenite and selenate fertilizer increased the lentil grain yield by 10 % and 4 %, respectively, compared to the control. Selenite fertilization significantly increased the grain yield of CDC Richlea, CDC Viceroy, and CDC Maxim by 26 %, 16 %, and 13 %, respectively. Seed Se concentration was significantly higher in lentils treated with selenate (1.4 mg/kg) compared to selenite (0.9 mg/kg) and the control (0.6 mg/kg). Selenium fertilization increased the antioxidant activity (70 % inhibition) of lentils compared to the control (61 % inhibition). Overall, application of Se increased lentil grain yield, antioxidant protection and seed Se concentration.

Key words: Selenium; lentil yield; antioxidant activity; biofortification.

4.2. Introduction

Selenium (Se) is an essential trace mineral in human and animal nutrition and was first discovered by the German scientist Berzelius in 1817 (Oldfield, 2002; Schwarz and Foltz, 1957). Unlike the essentiality of Se in human and animal nutrition, the role of Se in plants is not clearly identified (Germ et al., 2007). Certain lower plants such as planktonic algae require Se for

growth and reproduction while Se is not required for the growth and development of higher plants.

Plants show an ability to assimilate and accumulate Se. Certain plant species such as *Astragalus, Stanleya,* and *Morinda* accumulate a thousand to several thousand mg/kg of Se (Se-accumulators) (Brown and Shrift, 1982; Ihnat, 1989; Rosenfield and Beath, 1964). In contrast, Se-non accumulators, which are most forages and crops such as cabbage (*Brassica oleracea* L.), broccoli (*Brassica oleracea* L.), garlic (*Allium sativum* L.), and onion (*Allium cepa* L.) accumulate low levels of Se (25 mg/kg). Secondary Se accumulators accumulate up to 100 mg/kg of Se (Brown and Shrift, 1982). Plants mostly uptake Se as selenate (SeO_4^{-2}) and selenite (SeO_3^{-2}). Metabolism of different Se forms (SeO_4^{-2} and SeO_3^{-2}) in plants is different (Sors et al., 2005). Selenium is assimilated through the sulfur assimilation pathway and is converted into organic compounds such as selenocysteine, selenomethionine, selenocystathionine, and Se-methylselenocysteine.

Recent studies demonstrate that the application of Se increases plant productivity in terms of biomass or grain yield. The application of Se increased seed production (43 %) in canola (*Brassica napus* L.), biomass yield (14 %) in lettuce (*Lactuca sativa* L.), and tuber yield (40 %) in potato (*Solanum tuberosum* L.) (Lyons et al., 2009; Xue et al., 2001; Turakinen et al., 2004).

Application of Se increases the plant ability to tolerate environmental stress conditions including drought, cold, salinity and ultraviolet radiation (Smrkolij et al., 2006; Wang, 2011; Djanaguiraman et al., 2010). Application of Se increased the protection of wheat seedlings from ultraviolet radiation (Yao et al., 2010 a, b) and cold stress (Chu et al., 2010). Selenium protected rapeseed seedlings from drought (Hasanuzzaman and Fujita, 2011) and salinity induced damage (Hasanuzzaman et al., 2011). Boyed (2007) and Hanson et al. (2003) reported that Se-

fertilization increases resistance to caterpillars, aphids, and common *Fussarium* wilt in brassica. However, the mechanisms of the Se-enhanced resistance and/or tolerance of plants to environmental stresses have yet to be studied.

Lentil (*Lens culinaris* Medikus), a cool season food legume, has been used as a model pulse crop for Se biofortification (Thavarajah et al., 2007). Selenium concentration in lentils grown in Canada range between 160 and 720 μg/kg, providing 29-130 % of the recommended daily intake from 100 g of dry lentils (Thavarajah et al., 2007). Lentils are emerging as a major pulse crop in the Northern plains, primarily due to an excellent fit in existing crop rotations. The objective of this study was to determine the effect of Se fertilization on lentil grain yield, seed Se concentration, and antioxidant activity.

4.3. Materials and Methods

4.3.1. Materials

All chemicals (standards and solvents) used in this experiment were high-purity and analytical grades. All chemicals were purchased from VWR International (Radnor, PA, USA) and Sigma-Aldrich Co. (St. Louis, MO, USA). All reagents and solutions were prepared using deionized water (resistivity = 18.2 MΩ cm), which was distilled and deionized from Milli-Q Water System (Milford, MA, USA).

4.3.2. Field Experiment

The field experiment was conducted at the Carrington Research and Extension Center (CREC) (47.317N, 99.024 W), North Dakota, USA, in 2012 and 2013. The field was tilled and treated with herbicide (active ingredient: Ethalfluralin; 2.3 L/ha) prior to planting. Five commonly grown lentil genotypes (CDC Maxim, CDC Richlea, CDC Viceroy, CDC Imigreen, and CDC Impress) were selected. These genotypes were selected based on their high yield,

disease resistance, and consumer acceptability. The plots were planted on 5.29.2013. Each genotype was sown at a rate to achieve 194 plants/m^2 and a seeding depth of 3.5 cm in 1 m×10.4 m plots. Each plot (n=120) consisted of four rows with spacing of 30 cm between rows. Border plots were established between each plot to minimize the Se contamination via lateral movements. Three Se treatments were applied: 30 g/ha of potassium selenate (13 mg/m^3 of SeO$_4^-$^{2}); 30 g/ha of potassium selenite (12 mg/m^3 of SeO$_3^{-2}$); and control (no Se). The first Se application was done on 5.29.2013. The second Se application was done at the same rate on 7.18.2013 at flowering. Weeding was done manually as required. The second application of herbicide (active ingredient: Quizalofop; 17 L/ha) was done on 7.2.2013. All agronomic practices were followed as recommended by the CREC. Average monthly rainfall and temperature during growing season (May-August) in Carrington in 2012 and 2013 are presented in **Table 4.1**. Plants were harvested at physiological maturity (early August). Each plot was harvested separately using a small plot combine. The plot yield was measured and the total production (kg/ha) was calculated based on the size of the plot. Seeds were stored at -18 °C analyzed for antioxidant activity and Se concentration.

Table 4.1. Soil conditions and average monthly rainfall and temperature (May-August) at Carrington, ND, USA, in 2012 and 2013

Soil and climatic conditions		2012	2013
Soil	Nitrogen (NO_3^{-1}) (kg /ha)	107	48
	Phosphorous (PO_4^{-2}) (kg/ha)	167	27
	Potassium (K^+) (kg/ha)	1175	600
	pH	6.6	5.9
	Organic matter %	7.8	5.4
	Electrical conductivity (mmhos/cm)	0.4	0.2
	Texture	Clay Loam	Silt Loam
	Soil type	Fram-Wyard loam	Heimdal-Emrick loam
	Soil Se (mg/kg)	1.1	1.5
[a]Climate	Average monthly temperature (°C)	18	17
	Average monthly rainfall (mm)	59	43

[a] North Dakota Agricultural Weather Network (NDAWN)

4.3.3. Soil physical and chemical quality parameters

Soils were analyzed for nitrogen (NO_3^-), phosphate (PO_4^{-2}) and potassium (K^+) concentration; organic matter (OM) content; electrical conductivity (EC); pH; and soil texture at the North Dakota State University (NDSU) soil analysis lab (North Dakota, USA). Soil NO_3^{-1} concentration was measured using the method described by Vendrell and Zupincic. (1990). Soil PO_4^{-2} concentration was measured using the "Olsen ($NaHCO_3$) Phosphorus Test" described by

Laverty. (1973). Modified Ammonium Acetate method was used to measure the K^+ concentration (Warncke and Brown, 2012). Sand, silt and clay contents of soils were determined by Hydrometer method and the soil type was determined according to the US soil classification. Walkley-Black method was used to estimate the organic matter content of soil (Combs and Nathan, 2012). Soil pH and the electrical conductivity were determined by glass electrode pH meter according to the method described by Peters et al. (2012).

4.3.4. Antioxidant activity

Antioxidant activity of seeds was measured using dipheny-picrylhydrazyl (DPPH) free radical scavenging method (Apostolidis et al., 2007). Seeds were ground in liquid nitrogen using a mortar and pestle. One gram of ground seeds was mixed with 3 mL of water, vortexed for 30 sec, and centrifuged (2,000 rpm for 20 min). The supernatant was separated using a 10 ml syringe. The DPPH stock solution (1 mM) was prepared by dissolving 19.7 mg DPPH (2, 2-Diphenyl-1-picrylhydrazyl) in 50 ml methanol. Stock solution (10 ml) was diluted in methanol (90 ml) to prepare the working solution (0.1 mM). Fifty μl of sample extract was mixed with 3 mL of newly prepared working solution to make the sample solutions. Negative solution was prepared by mixing 3 mL of 0.1 mM DPPH working solution with 50 μl of distilled water. A blank solution was prepared by mixing 3 mL of methanol with 50 μl of seed extract. All sample solutions were kept in a dark place for 30 min at room temperature. After incubation, solutions were centrifuged at 2500 rpm for 10 min and the precipitate was separated. Absorbances were measured at 518 nm and the following formula was used to calculate the antioxidant activity (inhibition %).

$$\text{Inhibition \%} = \left(\frac{A_{\text{Negative}} - A_{\text{Sample}}}{A_{\text{Negative}}}\right) * 100 \qquad \text{(Eq.1)}$$

4.3.5. Seed Se concentration

The seed Se concentration was measured using modified the HNO_3–H_2O_2 method described by Thavarajah et al. (2008). Approximately 500 mg of ground sample was measured into a digestion tube. Samples were digested at 90 °C using programmable and automated digestion system (Questron Technologies Corp, Mississauga, Canada). Samples were digested with 6 mL of concentrated HNO_3 (70 %) for 1 h, followed by the addition of 3 ml of H_2O_2 for 15 min. Samples were further digested with 3 mL of 6 M hydrochloric acid (HCl) for 5 min to ensure the complete digestion. After digestion, samples were cooled to room temperature and filtered (Whatman number 4, Whatman PLC. Maidstone, Kent, UK) using a vacuum system (Gardener Denver Thomas Inc., Welch Vacuum Technologies, LA, USA). Total volume of supernatant was adjusted to 10 mL using deionized water. Selenium concentration was determined using Inductively Coupled Plasma Emission Spectrometry (ICP-EMS; ICP-6500 Duo, Thermo Fisher Scientific, PA, USA). The National Institute of Standards and Technology (NIST) standard reference materials (1573 apple leaves (Se = 0.054±0.003 mg/kg)) were used as external standards to validate the Se measurements. CDC Redberry (Se = 0.5290±0.04 mg/kg) was used as the laboratory reference. Selenium standards (Se = 1000μg/ml ± 0.5 %, micro element mixture, CPI International, USA) were used to develop the standard curve. The detection limit of the ICP-EMS was (3.05 μg/L). Seed Se concentration was determined using the standard curve generated with Se from 0-15 mg/kg.

4.3.6. Seedling emergence, chlorophyll count and seed analysis

Seedling emergence was counted in mid-June when the seedlings had reached the second node. The total number of seedlings in 0.6 m^2 area, in three random locations of each plot was recorded. Chlorophyll content of 2×3 mm^2 leaf area was measured at 50% flowering (mid-July)

using a chlorophyll meter (Minolta SPAD 502DL plus, Spectrum Technologies Inc.). Three leaves from each of 3 plants (a leaflet of the third youngest leaf form branch 3, 5, and 7) were selected randomly to measure the chlorophyll content. Mean chlorophyll content of each genotype treated with selenite, selenate and control was calculated.

Weight of 100 seeds was measured by using a top loading electric balance and multiplied by 10 to get the 1000 seed weight. Selenium yield (mg/ha) was calculated using the grain yield (kg/ha) and seed Se concentration (μg/kg) of each genotype. Seeds were ground using a top-loading UD grinder (Unholtz Dickie Corporation, USA) and protein content was measured using the Kjeldhal method (AOAC official method 2011.01).

4.3.7. Statistical analysis

The experimental design was a randomized complete block design (RCBD) with four replicates, five genotypes, three Se treatments for two years (n=120). Replicates, genotypes and years were considered as random factors. Replicates, years and genotypes were included as class variables. Data from both years were combined and analyzed using general linear model procedure (PROC GLM) mixed model (SAS (9.3) Institute, 2011). Individual analysis of variance by year, treatment, and genotype were conducted. Fisher's Least Significant Difference (LSD) at ≤ 0.05 was performed for mean separation. Correlations (Pearson correlation coefficients) among grain yield, 1000 seed weight, seed Se, Se yield, antioxidant activity and chlorophyll content were determined.

4.4. Results

Combined analysis of variance for lentil grain yield, seed Se concentration, antioxidant activity, 1000 seed weight (SW), and chlorophyll content of lentil genotypes is presented in **Table 4.2**.

Table 4.2. Combined analysis of variance for lentil grain yield, seed Se concentration, Se yield, antioxidant activity, 1000 seed weight (SW), and chlorophyll content for lentil genotypes grown at Carrington, ND, USA

Source	df	Yield	Seed Se	Se yield	Antioxidant activity	1000 SW	Chlorophyll
Year	1	NS	*	*	*	*	**
Geno	4	NS	NS	NS	NS	NS	NS
Trt	2	**	**	**	**	**	NS
rep	3	NS	NS	NS	NS	NS	NS
year×geno	4	**	NS	*	**	**	*
trt×geno	8	**	NS	NS	*	**	*
year×trt	2	**	NS	**	**	*	NS
Error		6	32	46	0.4	0.1	0.3

*, ** = Significant at $p < 0.1$ and $p < 0.05$, respectively. NS, not significant

The effect of Se application was significant on all variables except the chlorophyll content at $p < 0.05$ **(Table 4.2)**. Growing year was significant for seed Se concentration, Se yield, antioxidant activity, and 1000 SW at $p < 0.1$. Year × genotype interaction effect was significant for grain yield, antioxidant activity, 1000 SW at $p < 0.05$ and chlorophyll content and Se yield at $p < 0.1$. Interaction of treatment x genotype was significant for grain yield and 1000 SW at $p < 0.05$.

Lentil grain yield increased with application of Se compared with the untreated control in 2012. Grain yield of both selenite (1437 kg/ha) and selenate (1418 kg/ha) treated plots were higher than control plots (1356 kg/ha) (**Table 4.3**). Selenate-treated lentils showed an increased Se yield (2827 mg/ha), and antioxidant activity (61%) than those lentils treated with selenite and control at $p < 0.1$ in 2012. Selenate-treated lentils showed an increased seed Se concentration

(2057 µg/kg) compared to the control (576 µg/kg). Thousand seed weight of lentils treated with selenate (48 g) and control (48 g) was significantly higher than selenite (47 g) in 2012. Selenium application did not affect lentil chlorophyll content. In 2013, grain yield was significantly higher in selenite (1363 kg/ha) compared to selenate (1236 kg/ha) and control (1195 kg/ha) (**Table 4.3**). Selenate increased seed Se concentration (1260 µg/kg), Se yield (1580 mg/ha), and 1000 seed weight (42 g) than the selenite and control. Both selenite and selenate treated lentils showed an increased antioxidant activity (70 %) compared to the control (60 %) (**Table 4.3**).

Table 4.3. Response to Se fertilizer application of lentil grain yield, seed Se concentration, Se yield, antioxidant activity, 1000 seed weight (SW), and chlorophyll content of lentils grown at Carrington, ND in 2012 and 2013

Year	Treatment	Yield (kg/ha)	Seed Se (µg/kg)	Se yield (mg/ha)	Antioxidant activity (% inhibition)	SW (g)	Chlorophyll (SPAD)
2012	Selenite	1437 a	925 b	1325 b	57 b	47 b	47
	Selenate	1418 a	2057 a	2827 a	61 a	48 a	48
	Control	1356 b	576 c	780 c	56 b	48 a	49
	SE	38	103	142	0.7	0.3	0.4
	Mean	1404	1186	1644	58	48	48
2013	Selenite	1363 a	665 b	912 b	70 a	41b	42
	Selenate	1236 b	1260 a	1580 a	70 a	42 a	41
	Control	1195 c	400 c	478 c	60 b	40 b	40
	SE	27	57	81	0.9	0.5	0.5
	Mean	1265	775	989	67	41	41

[a] Means within a column and year followed by different letters are significantly different at $p < 0.05$

Selenium application significantly increased the grain yield of lentils at $p < 0.05$.

Selenite treatment significantly increased yield of CDC Maxim (1550 kg/ha), CDC Richlea (1523 kg/ha) and CDC Viceroy (1410 kg/ha) compared to their controls at $p < 0.05$ (**Figure 4.1**).

Se application did not influence the grain yield of CDC Imigreen or CDC Impress.
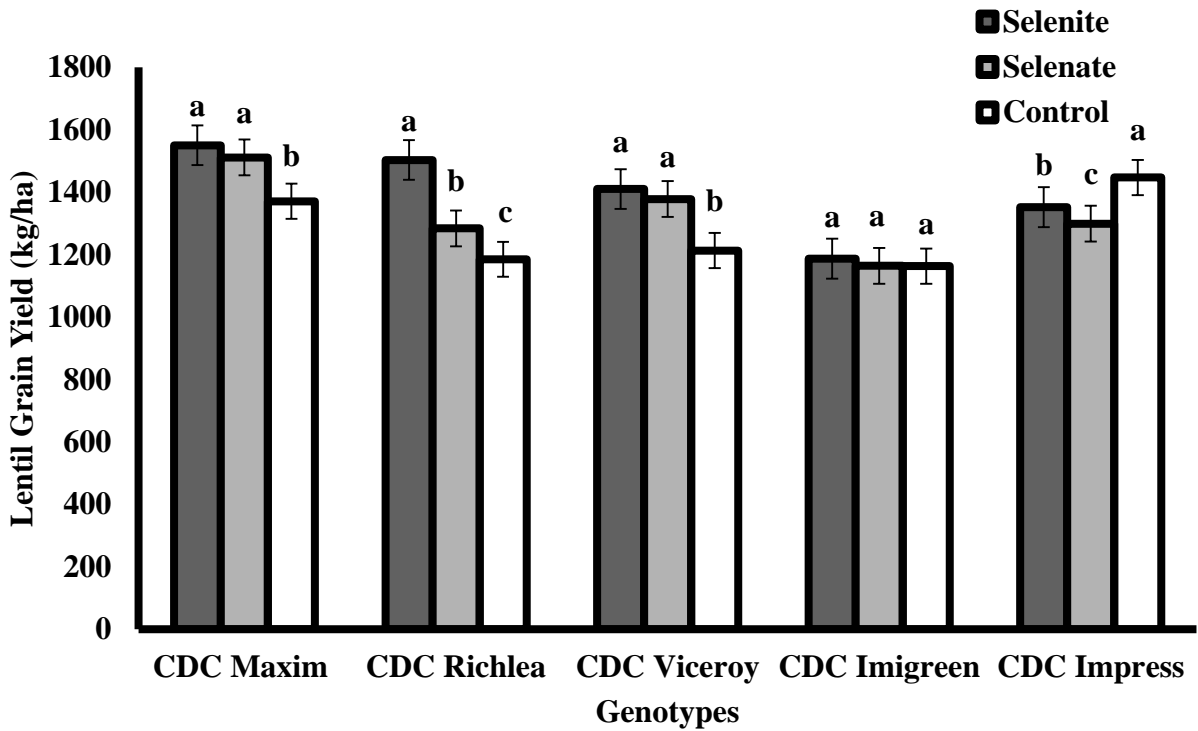
Figure 4.1. Effect of Se application on grain yield of lentil

Percentage recommended daily allowance (RDA %) of 25 g of lentils is presented in **Table 4.4.** Lentils (25 g) grown in 2012 and 2013 can provide 54 % and 35 %, respectively, of the recommended daily Se allowance of consumers in USA, respectively. Average seed Se concentration in 2012 was higher than in 2013. Soil pH was much favorable for selenate bioavailability in 2012 than in 2013 resulting higher Se accumulation in seeds in 2012.

Table 4.4. Seed Se concentration and percentage recommended daily allowance (RDA %) from 25 g of lentils

Year	Genotype	Seed selenium (µg/kg)	% RDA from 25 g
2012	CDC Imigreen	1335	61
	CDC Impress	1286	58
	CDC Maxim	1026	47
	CDC Richlea	1327	60
	CDC Viceroy	959	44
	SE	103	1
	Mean	1186	54
2013	CDC Imigreen	724	33
	CDC Impress	785	36
	CDC Maxim	800	36
	CDC Richlea	783	36
	CDC Viceroy	784	36
	SE	57	0.2
	Mean	775	35

RDA % from 25 g of lentil is based on the 55 µg RDA % for adults in USA (Monsen, 2000)

Antioxidant activity increased with Se application for all genotypes except CDC Maxim (**Figure 4.2**). Selenate significantly increased the antioxidant activity (73 %) of CDC Imigreen compared to selenite (68 %) and control (60 %). Both Selenate and selenite significantly increased the antioxidant activity of CDC Viceroy, CDC Richlea and CDC Impress compared to the control treatment.
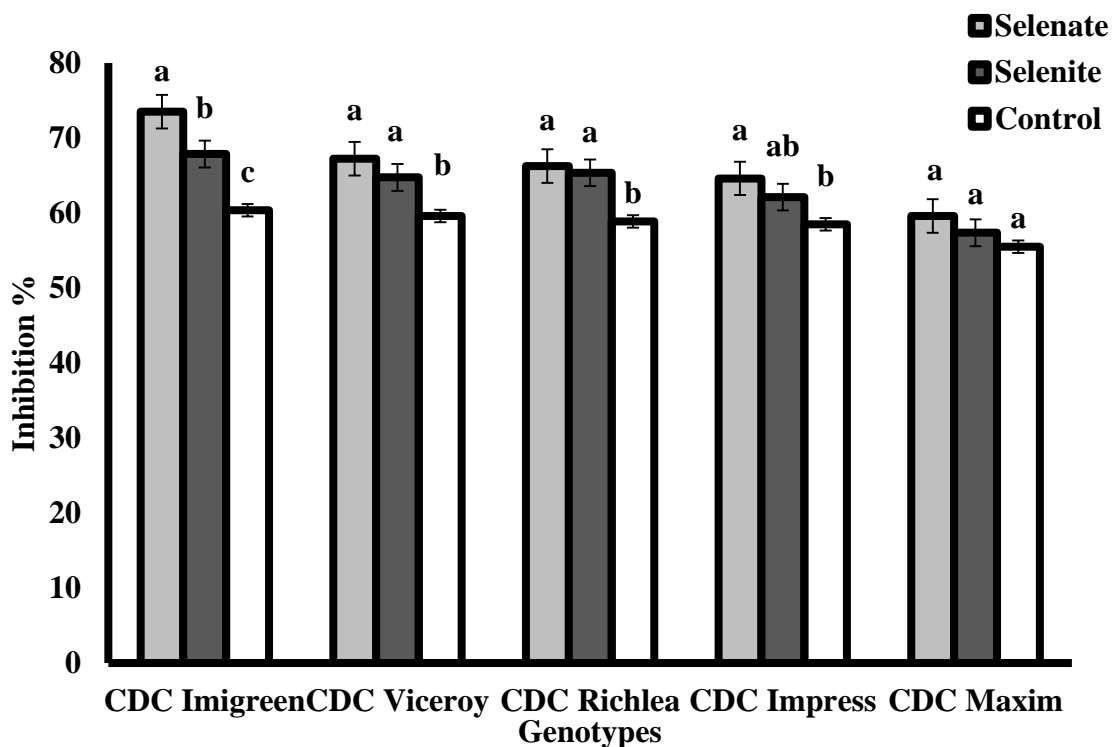
Figure 4.2. Antioxidant activity of lentils treated with different Se forms in 2012 and 2013 (combined analysis)

Grain yield and seed Se concentration were negatively correlated at $p < 0.05$. Grain yield and chlorophyll content were positively correlated at $p < 0.1$. Thousand seed weight was positively correlated with chlorophyll content, seed Se concentration, and Se yield (**Table 4.5**). Furthermore, a positive correlation was observed in seed Se concentration and Se yield at $p < 0.05$. Antioxidant activity and chlorophyll content were negatively correlated at $p < 0.05$.

Table 4.5. Pearson correlation coefficients for grain yield, 1000 seed weight, seed Se, Se yield, antioxidant activity and chlorophyll content of lentil grown at Carrington, ND, USA

Variables	Grain yield	1000 seed weight	Seed Se	Se yield	Antioxidant activity	Chlorophyll content
Grain Yield		0.22	-0.36**	0.47**	-0.07	0.24*
1000 Seed weight	0.22		0.23*	0.26*	-0.39**	0.77**
Seed Se	-0.36**	0.23*		0.93**	0.09	0.1
Se yield	0.47**	0.26*	0.93**		0.01	0.18
Antioxidant activity	-0.07	-0.39**	0.09	0.01		-0.37**
Chlorophyll content	0.24*	0.77**	0.10	0.18	-0.37**	

Values indicate Pearson correlation coefficients (r^2). *$p < 0.1$; **$p < 0.05$

4.5. Discussion

Selenium application increased grain yield of lentils. Selenium-treated lentils showed 5.3 % and 8.7 % increase in grain yield in 2012 and 2013, respectively (**Table 4.3**). The effect of Se application on plant growth in different plants has been reported (Lyons et al., 2009; Xue et al., 2001; Djanaguiraman et al., 2010). Selenium application resulted in 28% increase in rice (*Oryza sativa*) grain yield (Wang et al., 2013).

Seed Se concentration of lentils increased with Se fertilization. Average lentil seed Se concentrations were 1186 µg/kg and 775 µg/kg in 2012 and 2013, respectively. Soil Se concentration ranged from 626 to 839 µg/kg across both years. Seed Se concentration of naturally grown lentils in Saskatoon, Canada was from 160 to 720 µg/kg (Thavarajah et al., 2007). Soils in the Canadian prairies are naturally high in Se (> 1000 µg/kg). Seed Se concentration of lentils grown in Carrington, ND, USA with Se fertilization was higher than lentils grown in Canada.

Antioxidant activity of lentils increased with Se fertilization. Selenium increased the antioxidant activity of lentils by 3 % and 10 % in 2012 and 2013, respectively. A high concentration of antioxidant bioactivities has been reported in Se-enriched rice grains (Wang et al., 2013). Elevated inhibition of oxidation (89.6 %) was observed in rice plants when treated with Se (Wang et al., 2013). These results suggest that the application of Se bolsters the antioxidant activity, perhaps by increasing the pool of antioxidant bioactive compounds such as selenoglutathione (Se-GSH) (Sors et al., 2005; Zhu et al., 2009). An increase in antioxidant activity of plants may partially explain the grain yield increase obtain with Se fertilization.

Selenium significantly increased the antioxidant activity in all lentil genotypes except CDC Maxim (**Figure 4.2**). Selenate increased the antioxidant activity compared to selenite in

CDC Imigreen. In the roots, selenite is more easily and readily converted to organic Se forms such as selenomethionine and selenocistine than selenate (Zayed et al., 1998). As a result, selenate is prevalent at active sites where organic compounds are synthesized. The production of selenoenzymes involved in antioxidant activity increases with the application of selenate. Increased antioxidant activity in plants is associated with increased activity of antioxidant enzymes such as Glutathione peroxidase (GPX), Superoxide dismutase (SOD), Ascorbate peroxidase (APOX), and Glutathione reductase (GR) (Wang, 2011; Djanaguiraman et al., 2005; Hartikainen, et al., 2000). It is suggested that the application of selenate increases antioxidant protection of certain lentil genotypes. Selenium increase the antioxidant of lentil plants in stressful environmental conditions. Antioxidant activity of lentil plants was higher in 2013 than 2012 as environmental conditions were not favorable for lentil growth in 2013 (**Table 4.1**).

Different genotypes showed differential yield responses to selenite and selenate applications. Selenite (30 g/ha) increased the grain yield of CDC Richlea, CDC Viceroy, and CDC Maxim by 26 %, 16 %, and 13 % respectively. Selenite metabolism may be specific in plant growth. Sors et al. (2005) explain the difference of selenite and selenate metabolism in plants. Selenite uptake and transportation is different from selenate and organic Se. Plants uptake selenite passively across the cortex via simple diffusion while selenate and organic Se are assimilated actively via the S assimilation path way. Selenate uses most of the enzymes involved in sulfate reduction (Sors et al., 2005). In contrast the final reduction step from selenite to selenide appears to occur non-enzymatically and consume no energy (Sors et al., 2005). Based on these points, selenite metabolism may need comparatively low energy compared to selenate. Therefore, selenite presence would have conserved energy for increased grain production in lentils than selenate.

A significant increase of 1000 seed weight was observed with the application of selenate compared to selenite. If so, grain yield should be high in selenate-treated lentil plants. However, grain yield was high in selenite-treated lentil plants. This could be due to the increase seed number with the selenite application. Selenium fertilization affected the number of seeds in soybeans (Djanaguiraman et al., 2005). Chlorophyll content of lentil plant was not affected by the Se application. Thus, it is speculated that the increased yield production was not associated with increased photosynthesis but increase seed number. Similar results were observed in brassica (Lyons et al., 2009). Highest grain yield was observed in CDC Maxim, which is a small red lentil genotype. Small seeded genotypes have been bred with the purpose of increasing the lentil yield by increasing the number of seeds per plant. Therefore, it is suggested that, Se application increased the number of seeds in lentils and ultimately, increased grain yield.

Lentil grain yield was negatively correlated with seed Se concentration. Increased biomass production dilutes the Se concentration in lentil seeds. A positive correlation between chlorophyll content and 1000 seed weight was observed. When the chlorophyll content (photosynthesis) increased seed weight also increased as a result of increased photosynthesis. Antioxidant activity and the 1000 seed weight were negatively correlated. With the favorable environmental conditions, plant increased the photosynthesis but not the antioxidant activities.

Environment and soil conditions were more favorable for lentil growth in 2012 than 2013 (**Table 4.1**). Lentil grain yield was greater in 2012 than in 2013. Nutrient concentration and organic matter content in the soil also were greater in 2012 than in 2013. These variations in soil nutrient concentration affected the grain yield of lentils in 2012. Soil pH was not a critical factor for selenite and selenate assimilation as it was in favorable range for Se assimilation. Alkaline, oxidized soils (pH 7.5-8.5) are dominant with soluble forms of Se such as selenate and selenite

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(Sager, 2006). Average monthly rainfall during May-August in 2012 was higher than in 2013 and affected the grain yield. Therefore, environment effect also may have contributed to the increased grain yield in 2012.

4.6. Conclusion

Selenium increased grain yield of lentils. Selenium applied as selenite was more effective in increasing increased the grain yield more than Se applied as selenate. Selenium fertilization increased lentil antioxidant activity and seed Se concentration. Selenate increased the antioxidant activity and seed Se concentration more than selenite. Each genotype responded to Se fertilization differently. Therefore, further studies on genetic effect on Se metabolism in lentil plants are recommended.

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5. PAPER 2. EFFECT OF SELENIUM APPLICATION ON LENTIL (*LENS CULINARIS* MEDIKUS) NITROGEN FIXATION

5.1. Abstract

Lentil (*Lens culinaris* Medikus) is a cool season food legume mainly cultivated as a rotational crop in dry land cereal-based cropping systems. This study was conducted to determine the effect of Se fertilization on nitrogen fixation, grain yield, and seed Se concentration in lentils. The experiment was carried out at the Carrington Research and Extension Center, North Dakota, USA in 2012 and 2013. Six lentil genotypes and a reference crop (flax, cu. Omega) were cultivated with three Se treatments applied at both seeding and at flowering. Selenium fertilization increased the percentage of nitrogen derived from air (% N_{dfa}) by 17 % in 2012 and 12 % in 2013. Selenium increased lentil grain yield by 25 % and 26 % in 2012 and 2013, respectively. Seed Se concentration of Se-treated plants was 1.3 mg/kg in 2012 and 0.9 mg/kg in 2013 compared to control plants with 0.6 mg/kg and 0.1 mg/kg in 2012 and 2013, respectively. Application of Se increased nitrogen fixation, grain yield, and seed Se concentration. Selenate increased % N_{dfa} and seed Se concentration more than selenite. Selenite increased the grain yield more than selenate. Further studies on genetic contribution to Se metabolism and nitrogen fixation in lentil plants are recommended.

Key words: Selenium, yield, nitrogen fixation, lentil

5.2. Introduction

Nitrogen fixation is a biological process by which atmospheric nitrogen (N_2) is converted to ammonia (NH_3) by symbiotic nitrogen-fixing bacteria, *Rhizobium* (Kent et al., 2009). Symbiotic bacteria secrete hydrogenase and nitrogenase, oxygen sensitive enzymes, which convert atmospheric N_2 to NH_3 and encourage the H_2 recycling (Baginsky et al., 2004; Maier and

Triplett, 1996; Ruiz-Argueso et al., 2000). Hydrogenase is an important enzyme for legume nitrogen fixation (Schubert and Evans, 1976). Hydrogenase increases efficiency of nitrogen fixation by recovering energy loss (Maier and Triplett, 1996). Selenium plays an important role in hydrogenases (NiFeSe- hydrogenases) which contain Se in their active site as a selenocysteine residue (Baltazar et al., 2011; Parkin et al., 2008). Milton and Theresa (1989) reported that hydrogenase activity can be maximized by supplementing Se. Selenium application increased hydrogenase expression by 133 % in autotrophically cultured *Bradyrhizobium japonicum* (Boursier et al., 1988). Application of Se may increase nitrogen fixation as a result of increased activity of hydrogenase (Baginsky et al., 2005).

Leghemoglobin is an oxygen carrying protein in legumes and contributes to increase oxygen activity inside nodules, where the nitrogen fixation is occurred (Dalton, 1995). Leghemoglobin also functions to increase activated oxygen species in the nodules. Activated oxygen species can reduce the function of nitrogenase and hydrogenase (Dalton, 1995). Increased antioxidant activity can reduce the activated oxygen species and protects enzymes in the nodules that are involved in nitrogen fixation in nodules. Selenium application increased the antioxidant protection in plants such as wheat, canola and sorghum (Smrkolij et al., 2006; Wang 2011; Djanaguiraman et al., 2010). Therefore, Selenium application could increase the protection of enzymes involved in nitrogen fixation and increase the nitrogen fixation in lentils.

Lentil is a cool season food legume, has been used as a rotational food crop. Knut et al. (2003) reported that lentil symbiotically fixed 154 kg N/ha. Elevated nitrogen fixation through Se fertilization could increase the grain yield of lentils; benefit soil microflora and next season crop. The objective of this study was to determine the effect of Se fertilization on lentil nitrogen fixation, grain yield and seed Se concentration.

5.3. Materials and Methods

5.3.1. Materials

High-purity, analytical grade chemicals and Se standards used in the experiment were purchased from VWR International (Radnor, PA, USA) and Sigma-Aldrich Co. (St. Louis, MO, USA). Deionized water (resistivity = 18.2 MΩ cm) was distilled and deionized with a Milli-Q Water System (Milford, MA, USA).

5.3.2. Field Experiment

The experiment was carried out at the Carrington Research and Extension Center (CREC) (47.317N, 99.024 W), North Dakota, USA, in 2012 and 2013. Field was tilled and treated with herbicide (Active ingredient: Ethalfluralin) at a rate of 2.3 L/ha prior to planting. The experimental site was divided into 1m ×10.4 m size plots (n=72). Border plots were established between each treatment plot to reduce the contamination through soil leaching. The experimental design was randomized complete block design with five lentil genotypes, flax, three Se fertilizer treatments and four replicates (n= 72). Lentils (CDC Richlea, CDC Viceroy, CDC Maxim, CDC Imigreen, CDC Impress) and flax (cu 'Omega') were sown at 194 seeds/m^2 and 3.5 cm seeding depth using a plot seeder. All lentil seeds were inoculated with a commercially available *Rhizobium legusarium* strain (Novozymes: liquid pea/lentil rhizobium) prior to seeding. Each plot consisted of four rows 30 cm apart. Selenium treatments were 1) 30 g/ha of potassium selenate (13 mg/m^3 of SeO$_4^{-2}$); 2) 30 g/ha of potassium selenite (12 mg/m^3 of SeO$_3^{-2}$); and 3) untreated control (without Se). Selenium was applied twice at 9 days after seeding (5.29.2013) and at flowering (7.18.2013). Weeding was done manually as required. A second application of herbicide (active ingredient: Quizalofop) was done on 7.2.2014 at a rate of 14 L/ha. Climatic and soil conditions at Carrington in 2012 and 2013 are presented in **Table 5.1**. At physiological

maturity, plants were harvested using a plot combine, air dried and stored at room temperature.

Harvested lentil samples were transported to the Pulse Quality Laboratory at NDSU and stored at

$- 18\,^0C$ until further analysis. The plot yield was measured and the total production (kg/ha) was

calculated based on the size of the plot (1 m×10.4 m). Seeds were analyzed for Se concentration.

Table 5.1. Soil conditions and average monthly rainfall, temperature (May-August) at
Carrington, ND, USA in 2012 and 2013

Soil and climatic conditions		2012	2013
Soil	Nitrogen (NO_3^{-1}) (kg /ha)	130	81
	Phosphorous (PO_4^{-2}) (kg/ha)	65	11
	Potassium (K^+) (kg/ha)	908	335
	Sulfur (SO_4^{-2}) (kg/ha)	27	9
	pH	6.9	6.5
	Organic matter %	6	3
	Salt (mmhol/cm)	0.3	0.4
	Texture	Clay Loam	Silt Loam
	Soil type	Heimdal loam	Heimdal loam
	[b] Soil Se (mg/kg)	0.6-0.8	0.6-0.8
[a]Climate	Average monthly temperature (°C)	18	17
	Average monthly rainfall (mm)	59	43

[a] North Dakota Agricultural Weather Network (NDAWN)
[b] US Department of Interior/ US Geology Survey 2013

5.3.3. Soil physical and chemical quality parameters

Chemical nitrogen (NO_3^{-1}), phosphate (PO_4^{-2}), potassium (K^+) and physical (organic

matter (OM) content; electrical conductivity (EC); pH and soil texture of soil were determined

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at the North Dakota State University (NDSU) soil analysis lab (North Dakota, USA). Soil samples were collected at 0-6 and 6-24 inch depth using a soil auger prior to seeding. Three samples from each plot were randomly collected, cleaned, and mixed to prepare a composite sample. Soils were air dried at 40 °C for 48 h using an oven. Dried soils were sieved through a 2 mm plate, and stored in polythene bags at -40 °C until further analysis. Soil NO_3^{-1} concentration was quantified according to the method described by Vendrell and Zupancic. (1990). The Olsen ($NaHCO_3$) Phosphorus test protocol was followed to measure the Soil PO_4^{-2} concentration as described by Laverty. (1973). Modified Ammonium Acetate method was used to measure the K^+ concentration (Warncke and Brown, 2012). The hydrometer method was used to analyze the sand, silt and clay content of soil. Soil type was determined according to the US soil classification. Organic matter concentration was measured using Walkley-Black method (Combs and Nathan, 2012). A glass electrode pH meter was used to measure the pH and the electrical conductivity of soil using the method described by Peters et al. (2012).

5.3.4. Percentage nitrogen derived from air (%N$_{dfa}$)

The percentage of nitrogen derived from air in lentils was calculated using the isotope comparison method described by Rennie and Kemp (1984). Seeds were analyzed for δ15N using an elemental analyzer (Costech ECS4010, Costech Analytical Technologies Inc., Valencia, CA 91355 - USA) coupled to a Delta V mass spectrometer with Conflo IV interface. Seeds were crushed in liquid nitrogen using a mortar and pestle. Approximately, 0.5 g of ground seed was encapsulated in an aluminum container and sent to the Stable Isotope lab, University of Saskatchewan, Saskatchewan, Canada for isotope measurements. Flax was used as the non-nitrogen-fixing plant control. Nitrogen fixation was calculated using the following formula.

$$\%\text{N}_{dfa} = \frac{\delta15\text{Nref} - \delta15\text{Nfix}}{\delta15\text{Nref} - \delta15\text{N}} \times 100 \qquad (\text{Eq.2})$$

Where:

- % N_{dfa} = percentage nitrogen derived from air

- $\delta^{15}Nref$- $\delta15N$ = value of reference plants (Flax)

- $\delta^{15}Nfix$- $\delta^{15}N$= value of the total N in the lentil plants which both Atmospheric N and N from other sources are included

- $\delta^{15}N$- $\delta^{15}N$= value of soybean plant grown in N-free soil conditions

Isotopic discrimination value was calculated as follows

$$\delta^{15}N = \frac{\frac{15N}{14N}(\text{sample}) - \frac{15N}{14N}(\text{standard})}{\frac{15N}{14N}(\text{standard})} \times 1000\ ^0/_{00}\ ^{15}N \qquad \text{(Eq.3)}$$

Where:

- $^{15}N/^{14}N_{standard}$- ^{15}N= natural abundance of atmospheric N_2 (0.3663 atom % 15N)

- $^{15}N/^{14}N$ (sample) - ^{15}N= natural abundance of N_2 obtained from sample (soybean, lentil or flax).

5.3.5. Seed Se concentration

Seed Se concentration was measured using inductively coupled plasma atomic emission spectrometry (ICP-AES) according to the modified HNO_3–H_2O_2 method described by Thavarajah et al. (2008). Samples were digested in nitric acid (HNO_3), hydrochloric acid (HCL) and hydrogen peroxide (H_2O_2) to solubilize organic and inorganic compounds. Samples were digested using programmable and automated digestion system (Questron Technologies Corp, Mississauga, Canada). Approximately 0.5 g of ground seeds was measured into a digestion tube and digested at controlled temperature (90 °C) with the following consecutive chemical and time combinations: 6 mL of concentrated HNO_3 (70 %) for 1 h; 3 mL of H_2O_2 for 15 min; 3 mL of 6 M hydrochloric acid (HCl) for 5 min. Sample solutions were shaken during digestion. After

digestion, samples were cooled to room temperature. The supernatant was filtered using Whatman number 4 filter papers (Whatman PLC. Maidstone, Kent, UK) under a vacuum. The volume of the supernatant was adjusted to 10 mL using deionized water. Selenium concentration of digested sample was determined using ICP-AES (ICP-6500 Duo, Thermo Fisher Scientific, PA, USA). Selenium measurements and instrument accuracy was validated using the National Institute of Standards and Technology (NIST) standard reference materials: 1573a (apple leaves; (Se) = 0.054±0.003 mg/ kg) as an external standards. CDC Redberry (Se) = 0.5290±0.04 mg/kg) was used as the laboratory reference. Selenium standard curve (0-15 mg/kg) was prepared using Se standards (Se = 1000µg/ml ± 0.5 %, micro element mixture, CPI International, USA). The detection limit of the ICP-AES was (3.05 µg/L). Standard curve was used to calculate the Se concentration of digested seed samples.

5.3.6. Seedling emergence, chlorophyll content and seed analysis

Seedling count was taken at the initiation of second node (mid-June). Lentil seedlings in a randomly selected area (0.6 m^2) were counted. Chlorophyll content was measured using a chlorophyll meter (Minolta SPAD 502DL plus, Spectrum Technologies Inc.). The chlorophyll meter was designed to measure the chlorophyll content of 2×3 mm leaf area.

Selenium yield was calculated using grain yield and seed Se concentration. Seeds were analyzed for protein content using the Kjeldhal method (AOAC official method 2011.01). The weight of 100 cleaned lentil seeds were measured using a top loading balance and extrapolated to thousand seed weight.

5.3.7. Statistical analysis

The experimental design was a randomized complete block design (RCBD) with four replicates, six genotypes, three Se treatments and two years (n=120). Data from both years were

combined and analyzed as a mixed model (PROC GLM) in which replicates, genotypes and years were considered as random factors using SAS software (SAS Institute, 2011). ANOVA by year, treatment, and genotype were conducted. Means were separated according to the Fisher's Least Significant Difference (LSD) at ≤ 0.05. Correlations (Pearson correlation coefficients) among grain yield, 1000 seed weight, seed Se, Se yield, nitrogen fixation and chlorophyll content were determined.

5.4. Results

Combined analysis of variance for percentage nitrogen derived from air (% N_{dfa}), lentil grain yield, seed Se concentration, Se yield, thousand seed weight (1000 SW), protein and chlorophyll content of lentil genotypes presented in **Table 5.2.**

Table 5.2. Combined analysis of variance for percentage nitrogen derived from air (% N_{dfa}), lentil grain yield, seed Se concentration, Se yield, 1000 seed weight (SW), protein, and chlorophyll content of lentil genotypes grown at Carrington, ND, USA in 2012 and 2013

Source	df	% N_{dfa}	Yield	Seed Se	Se yield	1000 SW	Protein	Chlorophyll
year	1	*	NS	*	*	*	NS	**
geno	4	NS	NS	NS	NS	**	NS	NS
trt	2	**	**	**	**	NS	NS	NS
rep	3	NS	NS	NS	NS	NS	NS	NS
year×geno	4	**	**	**	**	*	**	NS
trt×geno	8	*	**	**	**	NS	NS	NS
year×trt	2	**	**	**	**	NS	NS	NS
Error		0.4	8	16	23	0.2	0.06	0.3

*, ** = Significant at $p < 0.1$ and $p < 0.05$, respectively. NS not significant

Application of Se had a significant effect on % N_{dfa}, grain yield, seed Se concentration and Se yield. Genotype effect was significant for 1000 SW at $p < 0.05$. Chlorophyll content of leaves was significantly affected by growing year.

Table 5.3 shows the effect of Se application on percentage nitrogen derived from air (% N_{dfa}), grain yield, seed Se concentration, Se yield, thousand seed weight (1000 SW), protein and chlorophyll content of lentils grown in Carrington, ND in 2012 and 2013.

Table 5.3. Response to Se fertilizer application of percentage nitrogen derived from air (% N_{dfa}), grain yield, seed Se concentration, Se yield, 1000 seed weight (SW), protein and chlorophyll content of lentils grown at Carrington, ND in 2012 and 2013

Year	Treatment	N_{dfa} (%)	Yield (kg/ha)	Seed se (µg/kg)	Se yield (mg/ha)	SW (g)	Protein (%)	Chlorophyll (SPAD)
2012	Selenite	21c	1564a	1329a	2013a	46	24	45
	Selenate	35a	1277b	1359a	1664b	45	25	45
	Control	24b	1133c	937b	1107c	45	24	45
	SE	1.5	40	61	86	1.2	0.1	0.5
	Mean	26	1325	1209	1595	45	24	45
2013	Selenite	46a	1646a	359b	584b	52	23	38
	Selenate	46a	1617a	899a	1442a	51	23	37
	Control	41b	1296b	147c	190c	52	23	38
	SE	0.9	36	42	70	1.2	0.1	0.4
	Mean	44	1520	468	738	52	23	38

Means within a column followed by different letters are significantly different at $p < 0.05$ for each year

Selenate treated lentil showed greater % N_{dfa} (35 %) compared to lentils treated with selenite (21 %) and the control (24 %) in 2012 (**Table 5.3**). Both selenite- and selenate-treated

lentils showed an increased % N_{dfa} (46 %) compared to the control (41 %) in 2013. Lentil yield

increased with application of Se in 2012 (p < 0.05). Grain yield of selenite treated plants (1564

kg/ha) was significantly higher than selenate (1277 kg/ha) treated and control plants (1133 k/ha).

Seed Se concentration of selenate (1359 µg/kg) and selenite (1329 µg/kg) treated lentils was

significantly higher than control (937 µg/kg) plants. Selenium yield was significantly higher in

selenite-treated plants (2013 mg/ha) compared to selenate treated lentils (1664 mg/ha) and the

control (1107 mg/ha) at p < 0.05. Seed Se concentration in 2012 (1209 µg/kg) was higher than in

2013 (468µg/kg). Chlorophyll content (45 SPAD) of lentils grown in 2012 was higher than the

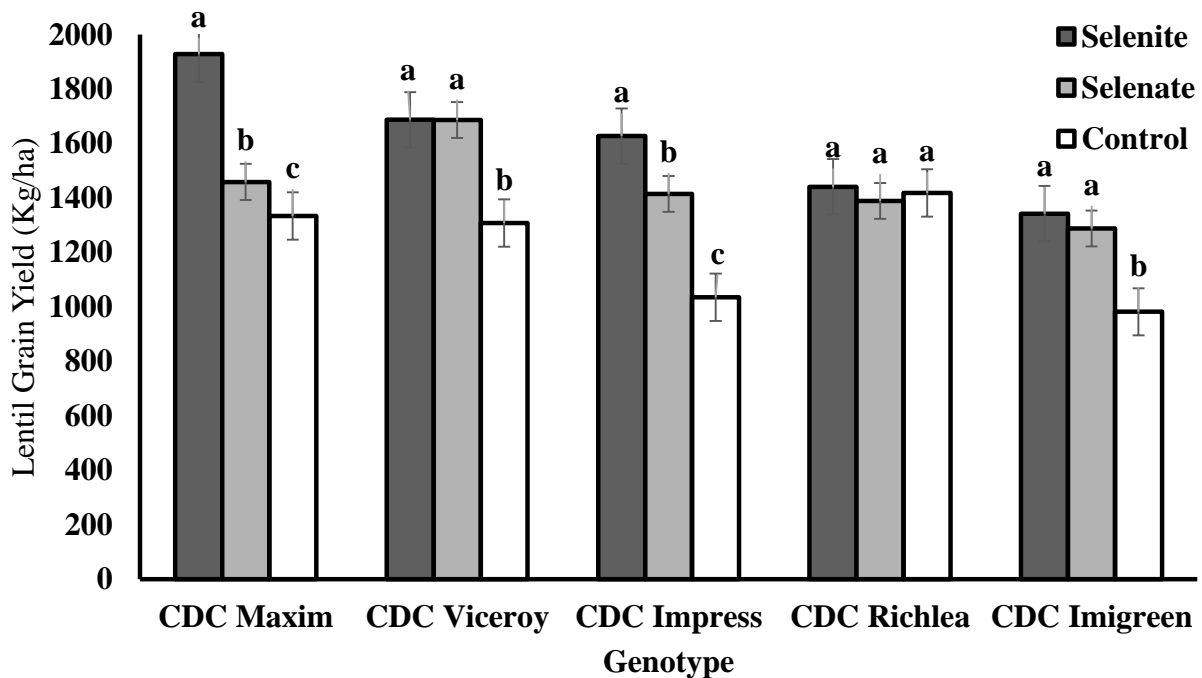mean chlorophyll content of lentils (38 SPAD) grown in 2013.

Figure 5.1. Yield responses of different genotypes for Se treatments in 2012 and 2013 (combined
analysis)

Selenium application significantly improved the grain yield of all lentil genotypes except

CDC Richlea (p < 0.05) (**Figure 5.1**). Selenite application significantly increased the grain yield

of CDC Maxim (1928 kg/ha) compared to both selenate (1458 kg/ha) and the control (1333 kg/ha). A similar trend was observed in CDC Impress in which the grain yield was greater with selenite (1627 kg/ha) than selenate (1415 kg/ha) and the control (1035 kg/ha). Grain yield was higher in plants treated with selenate and selenite in CDC viceroy and CDC Imigreen compared to control plants ($p < 0.05$). Se application did not significantly affect the grain yield of CDC Richlea (**Figure 5.1**).

Seed Se concentration and percentage recommended daily allowance (% RDA) from 25 g of lentils grown in 2012 and 2013 are presented in **Table 5.4**. CDC Imigreen and CDC Richlea have a comparatively high concentration of dietary Se compared to other lentil varieties.

Table 5.4. Seed Se concentration and percentage recommended daily allowance (RDA %) from 25 g of lentils

Year	Genotype	Seed selenium (µg/kg)	% RDA from 25 g
2012	CDC Imigreen	1390	63
	CDC Impress	898	41
	CDC Maxim	1279	58
	CDC Richlea	1654	75
	CDC Viceroy	823	37
	SE	45	2
	Mean	1209	55
2013	CDC Imigreen	504	23
	CDC Impress	468	21
	CDC Maxim	472	22
	CDC Richlea	438	20
	CDC Viceroy	459	21
	SE	3	0.1
	Mean	468	21

RDA % from 25 g of lentil is based on the 55 µg RDA % for adults in USA (Monsen, 2000)
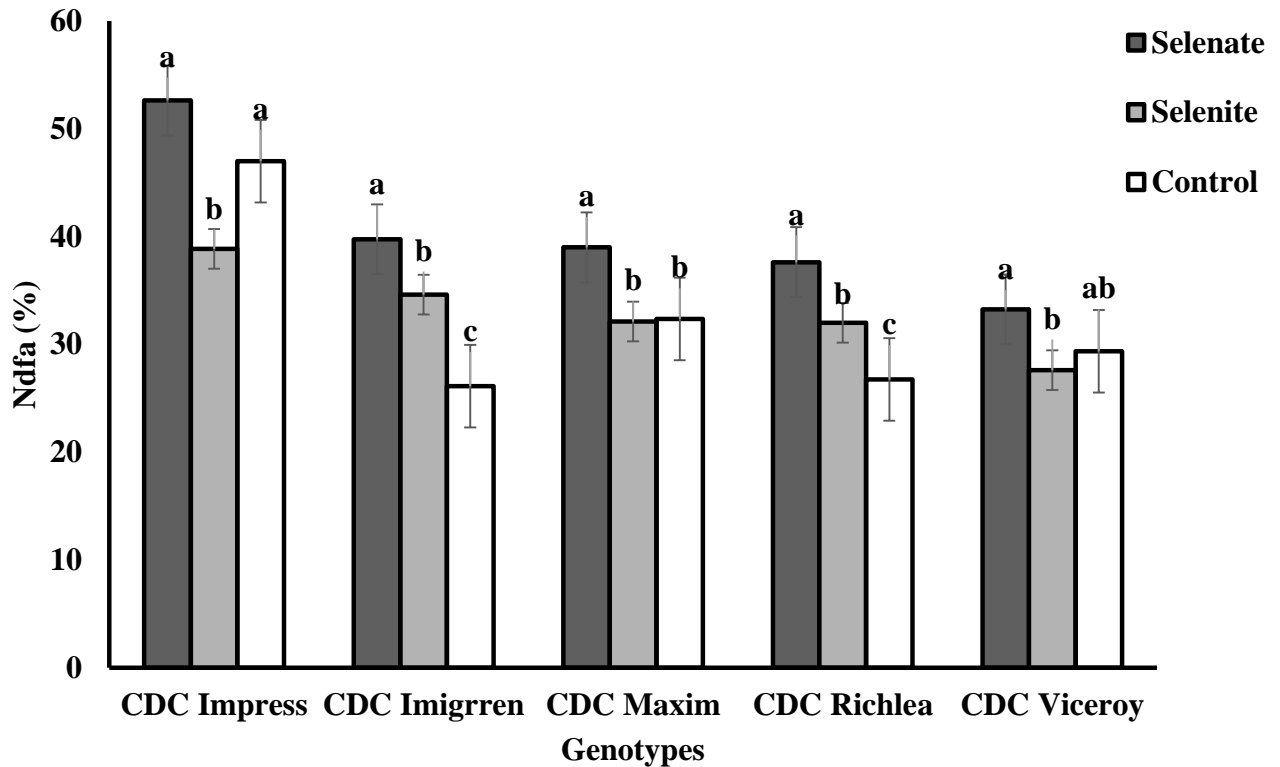
Figure 5.2. Percentage nitrogen derived from air (% N_{dfa}) of lentil treated with Se fertilizer in 2012 and 2013 (combined analysis)

Selenium application increased % N_{dfa} in CDC Imigreen, CDC Maxim and CDC Richlea (**Figure 5.2**). Selenate significantly increased % N_{dfa} of CDC Impress (52 %), CDC Imigreen (40 %), CDC Maxim (39 %), CDC Richlea (38 %) and CDC Viceroy (33 %) compared to selenite and the control at $p < 0.05$.

Correlations among percentage nitrogen derived from air (% N_{dfa}), grain yield, seed Se, Se yield, thousand seed weight (1000 SW), chlorophyll content, protein and chlorophyll content of lentils grown in Carrington, ND, USA in 2012 and 2013 are presented in **Table 5.5**.

Table 5.5. Pearson correlation coefficients for nitrogen derived from air (% N_{dfa}), grain yield, seed Se, Se yield, 1000 seed weight (SW), chlorophyll content, and protein of lentil grown at Carrington, ND, USA in 2012 and 2013

Variables	%N_{dfa}	Grain yield	Seed Se	Se yield	1000 SW	Chlorophyll content	Protein
%N_{dfa}		0.15	-0.47**	-0.43**	0.38**	-0.64**	-0.46**
Grain Yield	0.15		-0.15	0.20*	0.27*	0.23*	-0.10
Seed Se	-0.47**	-0.15		0.91**	-0.08	0.49**	0.20*
Se yield	-0.43**	0.20*	0.91**		-0.19*	0.42**	0.20*
1000 Seed weight	0.38**	0.27*	-0.08	-0.19*		-0.27*	-0.44**
Chlorophyll content	-0.64**	0.23*	0.49**	0.42**	-0.27*		0.50**
Protein	-0.46**	-0.10	0.20*	0.20*	-0.44**	0.50**	

Values indicate Pearson correlation coefficients (r^2). *p < 0.1; **p < 0.05

A positive correlation was observed between % N_{dfa} and 1000 SW at $p < 0.1$ (**Table 5.5**). Percentage nitrogen derived from air was negatively correlated with seed Se concentration, Se yield and chlorophyll content. Lentil grain yield, 1000 SW, Se yield and chlorophyll content were positively correlated at $p < 0.1$. Seed Se concentration was positively correlated with Se yield and chlorophyll content at $p < 0.05$. Selenium yield was positively correlated with chlorophyll content. Thousand SW was negatively correlated with protein content at $p<0.05$. Chlorophyll content and protein content was positively correlated at $p < 0.05$.

5.5. Discussion

Percentage nitrogen derived from air was increased by 17 % and 12 % in 2012 and 2013 respectively (**Figure 5.2**). Increased activity of hydrogenase followings Se application could increase nitrogen fixation in lentil plants. Boursier et al. (1988) reported that Se application increased the hydrogenase expression in autotrophically cultured *Bradyrhizobium japonicum*. Elevated antioxidant concentrations in nodules protect enzymes such as leghemoglobin, nitrogenase and hydrogenase from activated oxygen species (Dalton, 1995). Many studies have shown that Se increase the antioxidant protection in plants such as canola, wheat and sorghum (Smrkolij et al., 2006; Wang, 2011; Djanaguiraman et al., 2010). Increased antioxidant activity with Se fertilization could increase nitrogen fixation in lentil plants. It is anticipated that increased nitrogen fixation in lentil plants could be due to the increased activity of antioxidants.

Selenium application significantly increased the grain yield of lentils (**Table 5.2**). In this study, 25 % and 26 % increase of lentil grain yield was observed in 2012 and 2013, respectively, with the application of Se. Lyons et al. (2009) reported 43 % increased seed production in canola (*Brassica napus* L.) with the application of Se. Wang et al. (2013) reported 28 % increase

in rice (*Oryza sativa* L *ssp japonica*) grain yield with Se fertilization. This result indicates that Se fertilization is an effective approach to increase the grain yield of lentils.

Lentil seed Se concentration significantly increased with Se application (**Table 5.2**). Selenium-treated plants showed 43 % increased seed Se concentration compared to control. Average seed Se concentration of Se-treated and control plants were 1344 µg/kg and 937 µg/kg respectively in 2012. Seed Se concentration of lentil grown with Se and control were 629 µg/kg and 147 µg/kg respectively in 2013. Adams et al. (2002) reported 10-fold increase in seed Se concentration in wheat (*Triticum aestivum* L.) with Se fertilization. Broadley et al. (2010) reported 16-26 ng increase of seed Se for each gram of Se per hectare in wheat. Selenium concentration is high in crops grown in soil with high selenium. Seed se concentration of naturally grown lentils in Saskatoon, Canada was 160-720 µg/kg (Thavarajah et al., 2007). Selenium fertilization increases the Se accumulation in lentil plants.

A variation of % N_{dfa} and grain yield were observed within lentil genotypes with Se fertilization. Selenate was more effective than selenite during nitrogen fixation (**Figure 5.2**). Selenite application significantly increased the grain yield of CDC Maxim and CDC Impress compared to both selenate and control. (**Figure 5.1**). These responses to Se in plants would be mediated by the genetic potential of different lentil genotypes for Se metabolism, nitrogen fixation and grain yield increase with Se fertilization.

Selenate and selenite had different effects on seed Se concentration (**Table 5.3**). Plants treated with selenate had high seed Se concentration compared to the control in both years. In the roots selenite is more easily and readily converted in to organic Se, such as selenomethionine and selenocistine than is selenate (Zayed et al., 1998). Therefore, the translocation of selenate is

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higher than selenite. As a result, accumulation of Se was higher with the application of selenate than selenite.

A positive correlation was observed between % N_{dfa} and 1000 SW at $p < 0.05$. Nitrogen fixation increases the biomass in plants and seed weight. Percentage nitrogen derived from air was negatively correlated with seed Se concentration and Se yield. Increased biomass reduced seed Se concentration due to a dilution effect. In summary, Se can increase the grain yield and % N_{dfa} in lentils. But increased biomass production diluted the Se content in seeds. Even so, overall Se yield increased with the Se application.

Soil and environment conditions also contribute to the grain yield in lentils. Soil fertility was higher in 2012 than 2013. As a result, lentil grain yield was higher in each genotype in 2012 compared to 2013. Soil pH also was preferable for Se assimilation in 2012 (6.9) compared to 2013 (6.5). Alkaline, oxidized soils (pH 7.5-8.5) increase the bioavailability of Se (Sager, 2006). Even though, the clay loam soil could restrict the availability of Se to plant, increase precipitation would have limited the Se bioavailability to plants.

5.6. Conclusion

Selenium fertilization increased symbiotic nitrogen fixation and grain yield in lentils. Selenate increased symbiotic nitrogen fixation and seed Se concentration. Selenite was more effective than selenate with respect to increasing grain yield of lentils. Selenium fertilization in lentils is a successful approach to increase nitrogen fixation, grain yield and seed Se concentration.

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6. SUMMARY

Selenium fertilization increased lentil grain yield, antioxidant activity, symbiotic nitrogen fixation, and seed Se concentration. Lentil grain yield was increased by 5 % in 2012 and 9 % in 2013. Selenium increased the antioxidant activity of lentils by 3 % and 10 % in 2012 and 2013, respectively. Percent nitrogen derived from air was increased by 17 % in 2012 and 12 % in 2013. Selenium increased the seed Se concentration. Grain yield, antioxidant activity, and nitrogen fixation of lentils were also influenced by genotype and Se forms applied. Selenite increased the grain yield of CDC Richlea, CDC Viceroy, and CDC Maxim by 26 %, 16 %, and 13 % respectively. Selenite was more effective in increasing lentil grain yield. In contrast, selenate was more effective than selenite in increasing the nitrogen fixation, antioxidant activity and seed Se concentration in lentils. Consumption of 25 g of lentils can provide up to 54% of the recommended daily allowance of Se.

Our results should be beneficial for farmers, agronomists and breeders. Selenium can be used as a fertilizer to increase grain yield in lentils. However, Selenium may not increase the grain yield of all lentil genotypes. Therefore, each genotype should be evaluated for yield response to Se application. Breeding of lentils could focus on increasing Se accumulation in lentil plants, identification of lentil genotypes with greater yield response to Se application and breeding for increased plant metabolites involved in antioxidant activity. Field studies on the effect of environment, soil and management practices on Se metabolism in plants would be useful.

The response of lentil plants to Se application is mediated by the genetic potential of different lentil genotypes for Se metabolism and environmental factors with respect to growth, nitrogen fixation and antioxidant activity. Therefore, studies on genetic contribution of lentils to

Se metabolism are recommended to strengthen the understanding of the role of Se in lentil. Future studies will mainly focus on evaluating the effect of Se application on photosynthesis, reproduction, metabolism of carbohydrates and protein, and respiration which are related to growth of lentil plants. Metabolites involved in antioxidant activity in lentil plants will be identified. Further, the importance of Se for rhizobium bacteria will be evaluated. Selenium speciation in lentil plants will be evaluated to understand the translocation of Se in lentil plants during different growth stages.