Promoters:

Prof.Dr.ir. Filip Tack
Department of Applied Analytical and Physical Chemistry
Laboratory of Analytical Chemistry and Applied Ecochemistry
Ghent University

Prof.Dr.ir. Gijs Du Laing
Department of Applied Analytical and Physical Chemistry
Laboratory of Analytical Chemistry and Applied Ecochemistry
Ghent University

Dean: Prof.Dr.ir. Guido Van Huylenbroeck

Rector: Prof.Dr.ir. Paul Van Cauwenberghe

Rama Venkata Srikanth Lavu

Biofortification, speciation and bioaccessibility of selenium in food and feed crops

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

Biofortificatie, speciatie en biobeschikbaarheid van selenium in gewassen voor voeding en veevoeding.

Cover Illustration:

Fertilizing soils with selenium results in increased Se concentrations in food and feed crops. The inset shows the SHIME reactor, a simulator that allows studying selenium bioaccessibility from food crops in the gastro-intestinal tract.

Reference to thesis:

Rama Venkata Srikanth Lavu (2013). Biofortification, speciation and bioaccessibility of selenium in food and feed crops. Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences, Ghent University, Gent, Belgium.

ISBN: 978-90-5989-655-0

Acknowledgement

I wish to express my gratitude to my promotor, Prof. Dr. ir. Filip Tack, for all his support, valuable discussions and for his comments, advice in an attempt to improve the text of my thesis and manuscripts. I was greatly honoured to access advice and expertise from Prof. Filip and I immensely thank him for giving me this rewarding opportunity to work in Laboratory of Analytical Chemistry and Applied Ecochemistry.

I would not have been at this state of completing my doctoral studies without the great support and help of my promotor, Prof. Dr. ir. Gijs Du Laing. His continued interest and constant effort in providing excellent supervision of my PhD studies and in managing the project effectively during all phases of my doctoral studies from organizing experiments, healthy discussions, constructive comments and successful preparation of manuscripts and thesis.

My thesis would be incomplete without bioaccessibility studies and I am indebtedly thankful to Prof. Dr. ir. Tom Van De Wiele for providing an opportunity to use his facilities along with fruitful discussions and support in preparing manuscripts. The valuable practical and theoretical discussions that we had about the encountered problems during the course of practical work helped me work in a better way and to present my work in this thesis. I am thankful to Koen Willekens, Bernaert Nathalie and research group from Instituut voor Landbouw- en Visserijonderzoek (ILVO), Merelbeke, Belgium for their unconditional support throughout field work.

My sincere thanks to Ria Van Hulle and Ann Mills for their help in practical and administrative issues, and useful discussions during my stay in lab.

I would also like to thank my colleagues & friends, Pradeep Alava, Joachim Neri, Martin Meirlaen, Katty Sabo, Evi Michels, Erik Meers, Ivona Sigurnjak, Frederick Van Koetsem, Maja Simpraga, Anirban Biswas, Karel Folens, David Lybaert, Celine Vaneeckhaute, Sofie Demoor, Ellen Dolmans and all other members of our institute for their support and help during my studies.

My greatful thanks to friends in Ghent, Zuzanna Raja yekkala, Lalithya Pratti, Vijaya Sridhar Vepa, Sreeda Krishna CMC, Megha Santhosh Kadam, Ramana Muthuraman, Savitha Vijay

Raj, Tarakchand Ponnala, Chaitanya Nimmala, Ajay Palagani, Swarna Priyabadhini, Vidhya Sundararaman, Srinath Govindarajan, Sriram Balusu, Samrudhi Aseesh, Raoul and special thanks to all my friends Raghuveer Guthikonda, Prabath Vemuri, Harinivesh Donnepudi, Maha Rana Yadavalli, Murali Pendela, Sitaramaraju, Chitta Rajesh, Kishore Lingam for all their help and support. I will never forget your much appreciated kindness and assistance. My very special thanks and respect to Dinesh babu for his constant support. I would like to record my thanks to Prof. Dr. Sathish Chitneni and Dr. Swapna Mallampati, who motivated me into research career.

Financial support from the Fonds Wetenschappelijk Onderzoek (FWO) grant (G.0194.08) is highly appreciated.

I would like to express my appreciation for my mother, father, sister and brother: Lakshmi, Samba Siva Rao Lavu, Deepthi Billupati and Pratap Lavu. Without my family I would not have become the person who I am today. Their unconditional love and belief in my abilities has allowed me to pursue my dreams. My brother-in-law, Leela Prasad Billupati, has provided me constant guidance and support when needed. They have always been able to bring a smile on my face and show me they care.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AIDS	Acquired Immuno-Deficiency Disease Syndrome
ANOVA	Analysis of Variance
BW	Body Weight
CEC	Cation Exchange Capacity
DRI	Dietary Reference Intake
DW	Dry Weight
EC	Electrical Conductivity
Eh	Redox potential
EPA	Extension Planning Area
FAO	Food and Agriculture Organisation of the United Nations
GPx	Glutathione Peroxidase activity
HIV	Human Immuno-Suppression Virus
HPLC	High Performance Liquid Chromatography
HWC	Hot Water extractable Carbon
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
LSD	Least Significant Difference
OM	Organic Matter
OC	Organic Carbon
PCA	Principal Component Analysis
pe or pE	Negative logarithm of electron activities
pH	Negative logarithm of hydrogen ion activities
RDA	Required Daily Allowance
RNI	Required Nutrient Intake
SCFA	Short Chain Fatty Acids
SHIME	Simulator of Human Intestinal Microbial Ecosystem
WHO	World Health Organisation

Summary

Selenium (Se) is an essential micronutrient for humans and livestock, having an important role in many vital processes. However, its intake by humans and animals varies between countries due to its variable distribution in soils and availability in food and feed crops. Many world regions are considered as Se deficient, but several strategies can be followed to overcome such deficiencies. Agronomic biofortification is considered to be the most feasible option to improve the Se status, as this strategy is able to deliver Se to the entire population effectively, efficiently and in the most suitable chemical form. Although most biofortification strategies and studies focus on supplementing staple food crops with Se, use of vegetables is also an option as they are also consumed by a majority of population. Among the vegetables, *Allium* and *Brassica* species are particularly interesting due to their high potential for Se accumulation and conversion to Se species that are considered as beneficial for human health, such as *γ-glut-cyst,* SeCys and MeSeCys. Moreover, kenaf (*Hibiscus cannabinus*), which belongs to *Malvaceae* family, has much potential as it can be used as food as well as feed ingredient, and has proven to have potential for Se accumulation.

Therefore, we assessed the impact of Se fertilizer type and dose, soil properties, and genetic crop variety on Se uptake by leek (*Allium* ampeloprasum var. porrum) and its Se speciation in greenhouse and field experiments. Moreover, we assessed accumulation and speciation of Se in kenaf and its physiological response to Se, as well as how contents of other micronutrients in kenaf may be affected by Se fertilization. In addition, we studied how liming and supply of organic amendments, such as compost, pig and cow manure, to a sandy loam soil may affect availability of Se, and how this evolves in the first months after application of the Se fertilizer. Finally, we assessed in vitro the bioaccessibility of Se from the Se-enriched food crops and compared it with commercially available food supplements, meanwhile assessing also the role of intestinal microorganisms in the release and biotransformation of Se in the gastrointestinal tract.

The soils in Flanders (Belgium) were observed to be lower in Se levels. When Se fertilizer is added to the soil, the concentration and speciation of Se in leek depend on the form and dose of Se fertilizer used, with use of selenate resulting in the highest accumulation in the crop. Its uptake by the leek ranges between 5-10% and 36-48% of the amount added to the soil for supply of selenite and selenate, respectively. Accordingly, the use of selenite as fertilizer

results in a higher risk for Se accumulation in the soil on longer term. Among 20 different leek cultivars tested in a field experiment, some cultivars seem to be superior in accumulating Se. In a field experiment conducted on different field plots across Flanders with selenite as fertilizer, a negative correlation between soil organic carbon and Se uptake by the leek was observed. Moreover, in a pot experiment, organic amendments were found to decrease Se availability and its concentration in wheat. In the soils amended with cow and pig manure, Se uptake by the plants decreases by 91-95% and 88-89%, respectively, when the soils were spiked with selenite or selenate. Soil liming improves Se concentrations in crops especially when selenate fertilizers are used.

As was observed for leek, the uptake of Se by kenaf was also highest when the soil was fertilized with selenate, whereas also the speciation of Se in kenaf differs when different fertilizers are used, with a higher percentage of organic species being formed in the crop when soils are fertilized with selenite. At higher doses of selenate fertilizer, plant growth was negatively affected, whereas this was not the case when selenite was used at a same Se application dose.

The majority of Se was found to be bioaccessible in the small intestine, and a significant fraction of Se contained in the crops also has good chances to reach the colon, where it seems to be taken up by the microbial community and may also induce positive health effects. However, further research is needed to assess whether this is actually the case. Bioaccessibility of biofortified food crops was found to be quite similar to the bioaccessibility of commercially available food supplements containing Se in the form of selenized yeast or selenomethionine. However, a yoghurt-based food supplement containing Se microparticles showed a very low bioaccessibility (less 26%). These observations highlight the need for assessing Se speciation and bioaccessibility when evaluating the efficacy of new food supplements and fortified food products being brought to the market.

It is concluded that Se-enriched leek or kenaf can be used to increase intake of Se by humans and animals from suboptimal levels to levels which have been reported to promote beneficial health effects. However, long-term field studies monitoring Se mobility and bioavailability in soils amended with Se fertilizers are needed to be able to outweigh the risk for Se accumulation in the soil against the benefit of supplying Se to the crop.

Samenvatting

Selenium (Se) is een essentiële micronutriënt voor mens en dier, die een belangrijke rol speelt in veel vitale processen. Zijn inname door mens en dier varieert echter tussen verschillende landen omwille van zijn variabele distributie in de bodem en beschikbaarheid in voedings- en voedergewassen. Veel werelddelen kunnen beschouwd worden als zijnde selenium-deficiënt. Verschillende strategieën kunnen gevolgd worden om dergelijke deficiënties tegen te gaan. Agronomische biofortificatie wordt beschouwd als de meest realistische optie om de seleniumstatus te verbeteren, gezien deze strategie in staat is de hele populatie op een veilige, effectieve en efficiënte wijze van Se te voorzien, alsook Se in de meest geschikte vorm aan te leveren.

Hoewel de meeste biofortificatie-strategieën en –studies zich richten op het supplementeren van basisvoedingsgewassen met Se, is gebruik van groenten tevens een optie, gezien deze ook geconsumeerd worden door het merendeel van de bevolking. Onder de groenten zijn in het bijzonder *Allium* en *Brassica* gewassen interessant omwille van hun hoog potentieel om Se te accumuleren en om te zetten in vormen die beschouwd worden als gunstig voor de menselijke gezondheid, zoals γ-glut-cyst, SeCys and MeSeCys. Bovendien is Kenaf (*Hibiscus cannabinus*), die behoort tot de *Malvaceae* familie, veelbelovend gezien het gebruikt kan worden als voedings- en voedergewas en reeds bewezen heeft potentieel te hebben om Se te accumuleren.

Daarom werd in deze studie de impact van vorm en dosis van Se-bemesting, bodemeigenschappen en genetische gewasvariëteit op de opname van Se door prei (*Allium* ampeloprasum var. porrum) en diens Se-speciatie ingeschat via serre- en veldexperimenten. Bovendien werden accumulatie en speciatie van Se in kenaf en diens fysiologische respons op Se-toediening bestudeerd, alsook hoe de gehaltes van andere micronutriënten in kenaf kunnen beïnvloed worden door Se-bemesting. Verder werd onderzocht hoe bekalking en toediening van organische amendementen, zoals compost, varkens- en koemest aan een zandleembodem de beschikbaarheid van Se kunnen beïnvloeden, en hoe dit evolueert in de eerste maanden na toedieningen van een Se-meststof. Tenslotte werd in vitro de biotoegankelijkheid van Se in de Se-aangerijkte voedings- en voedergewassen ingeschat en vergeleken met commercieel beschikbare voedingssupplementen, waarbij tevens de rol van

intestinale micro-organismen in de vrijstelling en biotransformatie van Se in het spijsverteringsstelsel werd bestudeerd.

Er werd vastgesteld dat de bodem in Vlaanderen (België) kan beschouwd worden als Se-deficiënt. Wanneer Se-meststof aan de bodem wordt toegediend, hangen de concentratie en speciatie van Se in prei af van de gebruikte vorm en dosis van de Se-meststof, waarbij gebruik van selenaat resulteert in de hoogste accumulatie in het gewas. De opname van Se door de prei varieert tussen 5-10% en 36-48% van de hoeveelheid toegediend aan de bodem, bij gebruik van respectievelijk seleniet en selenaat. Gebruik van seleniet als meststof resulteert dus in een hoger risico voor Se-accumulatie in de bodem op langere termijn. Van de 20 verschillende prei cultivars die getest werden in een veldexperiment, bleken enkele cultivars superieur te zijn in het accumuleren van Se. In een veldexperiment uitgevoerd op verschillende proefvelden verspreid over Vlaanderen met seleniet als meststof, werd een negatieve correlatie tussen organische koolstof in de bodem en Se-opname door de prei waargenomen. Bovendien werd in een potexperiment vastgesteld dat gebruik van organische amendementen resulteert in een afname van de beschikbaarheid van Se en diens concentratie in tarwe. In bodems waaraan koe- en varkensmest werd toegevoegd, nam de Se-opname door de planten af met respectievelijk 91-95% en 88-89% wanneer aan de bodems seleniet of selenaat werd toegediend. Bekalking doet Se-concentraties in de gewassen toenemen, in het bijzonder wanneer selenaat-meststoffen gebruikt worden.

Zoals tevens werd waargenomen voor prei, is de opname van Se door Kenaf ook hoger wanneer de bodem bemest wordt met selenaat, terwijl de speciatie van Se in Kenaf ook verschilt wanneer verschillende meststoffen gebruikt worden. Het hoogste percentage aan organische Se-vormen wordt waargenomen in de gewassen wanneer bodems bemest worden met seleniet. Bij hogere dosissen van selenaat-meststof wordt de plantengroei negatief beïnvloed, terwijl dit niet het geval is wanneer seleniet wordt toegediend aan dezelfde Se dosis.

Het merendeel van het Se aanwezig in de gewassen kan beschouwd worden als zijnde biotoegankelijk in de dunne darm, terwijl tevens een significant deel een goede kans heeft om de dikke darm te bereiken, waar het blijkt opgenomen te worden door de microbiële gemeenschap en positieve gezondheidseffecten zou kunnen induceren. Meer onderzoek is echter nodig om te kunnen inschatten of dit effectief het geval is. De biotoegankelijkheid van aangerijkte voedingsgewassen is gelijkaardig aan de biotoegankelijkheid van commercieel

beschikbare voedingssupplementen die Se bevatten in de vorm van Se-aangerijkte gist of selenomethionine. Een yoghurt-gebaseerd voedingssupplement dat Se micropartikels bevat, vertoonde echter een erg lage biotoegankelijkheid (minder dan 26%). Deze waarnemingen wijzen op de nood om Se speciatie en biotoegankelijkheid in te schatten wanneer de effectiviteit van nieuwe voedingssupplementen en aangerijkte voedingsproducten die op de markt gebracht worden, geëvalueerd wordt.

Er kan besloten worden dat Se-aangereikte prei of kenaf gebruikt kunnen worden om de inname van Se door mens en dier te verhogen van suboptimale niveaus naar niveaus die positieve gezondheidseffecten zouden induceren. Veldexperimenten waarin de mobiliteit en biobeschikbaarheid van Se in bodems gedurende langere termijn opgevolgd worden, zijn echter noodzakelijk om het risico op Se-accumulatie in de bodem te kunnen afwegen tegen het voordeel van Se-toediening aan het gewas.

Chapter 1. General introduction and objectives

1.1 General introduction

Selenium (Se) is a naturally occurring trace mineral. It is an important essential nutrient for humans and livestock. Selenium has an important proven role in many vital processes. Its deficiency was related to the incidence of major human diseases, such as cancer, the Down-syndrome, Alzheimer's disease, etc (Bedwal et al., 1993; Rayman, 2000; Rayman, 2002; Jackson et al., 2004). Soils were found to be Se-deficient in many parts of the world, such as England, Finland, some parts of China and the United states (Borowska, 1998, Gupta and Gupta, 2010). Food crops obtained from these Se deficient soils lead to Se deficiency in the human population. For example, in the UK dietary Se intake has fallen from 60 µg d^{-1} in 1974 to <39 µg d^{-1} in recent years, which is thought to be due to replacement of North American wheat (high in Se) by European wheat (low in Se) as source for bread (Broadley et al., 2006). However, there are also regions where soil has much higher Se concentrations, e.g. in some parts of China, India and Ireland (Dhillon and Dhillon, 1991, Fleming, 1962, Hira et al., 2004). Although Se is an essential element, it can also be toxic when taken up in excess. The narrow gap between its daily requirement and toxic dose ranges between 0.055 and 0.4 mg Se d^{-1} per adult male or female (Department of Health, 1991).

Several strategies can be followed to overcome Se deficiency in the human diet. Se-enriched food supplements are commercially available in many countries (Dumont et al., 2004). They are mainly sold as tablets containing Se-enriched baker's yeast (*Saccharomyces cerevisiae*) having selenomethionine as dominating Se form. Supply of Se-enriched animal products such as Se-enriched milk, meat and eggs, is also an option. Enrichment of these animal products may be obtained by supplying cattle with Se-rich pasture crops or feed supplements containing Se-enriched baker's yeast. A third strategy is based on production of Se-fortified food crops, which is referred to as biofortification. These Se-enriched crops can be obtained through genetic engineering, selective breeding or use of Se fertilizers. The efficacy of using Se-fortified food crops was recognised in Finland in the early 1980s, where Se fertilizers such as sodium selenate and sodium selenite were used to obtain the Se-enriched crops (Ylaranta 1990, Eurola et al. 1991). Among all strategies, agronomic biofortification is considered to be the most feasible option to increase the selenium status as it concerns a food system approach that can deliver dietary selenium to the entire population safely, effectively, efficiently and in the most suitable chemical forms (Welch and Graham, 1999). Although most biofortification

strategies and studies focus on supplementing staple food crops with Se, use of vegetables is also an option as they are consumed by a majority of population. Among the vegetables, *Allium* and *Brassica* species are particularly interesting due to their high potential for Se accumulation and conversion to Se species that may be beneficial for human health, such as *γ-glut-cyst*, SeCys and MeSeCys (Pyrzynska, 2009).

To obtain Se-enriched food crops, fertilizing soil with selenium is often considered as the most effective, safest and fastest strategy. However, the Se concentration in food crops obtained from Se fertilized soils was found to be influenced by various soil parameters, such as soil pH and redox potential (Eh), organic matter, Fe-oxides, sulphates, mode of occurrence, soil weathering, physiography, climate, and (an)oxic conditions (Aubert and Pinta, 1977). Moreover, it depends on the form of Se used to fertilize the soils (Gissel-Nielsen et al., 1984). Although several researchers previously investigated soil factors affecting Se uptake by different food crops in greenhouse experiments, much less attention was given to accumulation and longer-term fate of the fertilizer in the soil, and field studies. Moreover, it is not yet clear to what extent the overall nutritional quality of the crops, such as their content of other essential micronutrients, is affected by the fertilization.

It should also be mentioned that the biological significance of Se is not only dependent on the total amount of Se consumed but it also depends on the speciation of Se in the food crops (Tamas et al., 2010), i.e. the form in which Se occurs, which in turn depends on the type of food crop and factors affecting availability of different Se species in the soil. This speciation determines its chemical fate, bioavailability, biological role and toxicity in the human body. For example, the anti-carcinogenic role of some organic Se forms (e.g., MeSeCys, γ-glut-cyst) was shown to be higher compared to other organic and inorganic Se forms (Finley and Davis, 2001, Finley et al., 2000, Ip et al., 2000). Moreover, the bioavailability of Se in fortified food crops can also be affected by its bioaccessibility, i.e. fraction of the compound that after ingestion is mobilized into the gut fluids or chyme and is available for assimilation (Rossi et al., 1996, Ruby et al., 1996). This bioaccessibility usually depends on the speciation as well as characteristics of the food matrix itself (Thomson, 2004). Although several of studies have previously focussed on agronomic strategies for Se biofortification, only few focussed on factors affecting speciation of Se in the crops and its bioaccessibility (Kápolna et al., 2007; Jaiswal et al., 2012).

1.2 Objectives

This study aimed to contribute to the development of an effective Se biofortification strategies. Leek and kenaf were used as study crops. Leek, which belongs to the *Allium* family was chosen as it has not yet been studied before in Se biofortification studies although *Allium* crops were previously identified to have a high potential for Se accumulation and conversion to Se species that may be beneficial for human health, such as *γ-glut-cyst*, SeCys and MeSeCys. Kenaf, which belongs to *Malvaceae* family, was chosen as it can be used as food as well as feed ingredient and has proven to have potential for Se accumulation although its Se speciation and physiological response to Se fertilization have not yet been studied before (Banuelos et al., 1997a; Lopez et al., 2006; Kubmarawa et al., 2009).

In particular, we aimed to:

- assess the impact of Se fertilizer type and dose on Se uptake by leek and its speciation in leek grown on a sandy loam soil (chapter 3)
- assess in a field experiment how soil properties may affect uptake of Se by leek from non-fertilized soils and soils fertilized with selenite fertilizer (chapter 4)
- compare the response of different genetic leek varieties to different types of Se fertilizers (chapter 5)
- assess accumulation and speciation of Se in kenaf and its physiological response to Se fertilization (chapter 6)
- assess how contents of other micronutrients in kenaf may be affected by Se fertilization (chapter 7)
- assess how liming and supply of organic amendments, such as compost, pig and cow manure, may affect availability of Se in a sandy loam soil, and how this evolves in the first months after application of the Se fertilizer (chapter 8)
- assess the bioaccessibility of Se from the Se-enriched food crops (in vitro) and compare it with commercially available food supplements, meanwhile assessing also the role of microorganisms in the release and biotransformation of Se in the gastrointestinal tract (chapter 9).

The work concludes with a general discussion in which technical, economic and practical feasibility of using Se-enriched leek and kenaf in biofortification programmes is assessed (chapter 10).

Chapter 2. Literature Review

2.1 Physical and chemical properties

The atomic number of Se is 34 and its atomic mass is 78.96. It belongs to Group 6 *chalcogens* (Group VIa) of the periodic table and is considered as a non-metal element. The six stable isotopes of Se are ^{74}Se (0.87%), ^{76}Se (9.02%), ^{77}Se (7.58%), ^{78}Se (23.52%), ^{80}Se (49.82%), and ^{82}Se (9.19%) (Hoffmann and King, 1997). Selenium replaces sulphur in sulphide minerals such as pyrite, chalcopyrite, pyrrhotite and sphalerite being a chalcophile (sulphur-loving) element. In the periodic table, the common valence electron configuration of s^2p^4 causes parallel valances and equivalent bonding structures among the group elements. Due to the electron configuration, Se exists in three common oxidation states of 6, 4, and -2, identical to sulphur. Selenium is a member of the sulphur group of nonmetallic elements and is similar to this sulphur in terms of its forms and compounds. As observed from the periodic table, the Se atom is larger than S with a radius of 0.5 Å compared to 0.37 Å for S. The inorganic Se forms mainly exist as elemental selenium (Se), selenide (Se^{2-}), and in the +4 and +6 oxidation state. In the +4 oxidation state it can occur as selenium dioxide (SeO_2), selenite (SeO_3^{2-}) or selenious acid (H_2SeO_3) and in the +6 oxidation state it occurs in the form of selenic acid (H_2SeO_4) or selenate (SeO_4^{2-}) salts. The fact that Se exists in different oxidation numbers allows it to become biologically active as it participates in electron donor and acceptor reactions (Shrift, 1973).

2.2 Se sources and geochemistry

Selenium in soils mainly originates from the weathering of Se-containing rocks, volcanic activity and dust arising from coal combustion (Weiss et al., 1971). In unweathered rocks and mineral ores, the occurrence of Se is nearly always connected with presence of sulphur minerals. The abundance of Se in the lithosphere relative to sulphur is on average 1 to 6000. When compared to tellurium, Se is approximately 50 times more abundant. However, sulphur and Se slightly differ in their oxidation. During weathering, sulphide is oxidized to sulphate whereas selenide stops with the formation of selenite. A very high oxidation potential is

needed to form selenate. Under a humid climate, a large part of the sulphate can leach into rivers and oceans. However, sulphate in sea water may occur in the sediments as sulphates of the alkaline earth metals, or be transformed into heavy metal sulphides or elemental sulphur. Selenites often stay readily available in sea water, although they may also be absorbed and co-precipitated with the iron and manganese hydroxide in sediments and with organic material, where they can further be reduced to elemental selenium or selenide (Bisbjerg, 1972, Weiss et al., 1971).

2.3 Selenium toxicity and deficiency

Selenium was initially believed to be a toxic element when toxicity symptoms were observed in horses grazing on seleniferous soils. In these horses, hair loss from mane and tail, sloughing of hooves, joint erosion and lameness hair loss were observed which is termed as the Alkali disease (Toole et al., 1995). In the 13th century such toxic symptoms in animals feeding on plants with high Se concentrations were observed by Marco Polo, an explorer. Subsequently, Se toxicity in humans was reported in China in regions where soil Se concentrations are higher than 40 mg kg^{-1}. First deficiency symptoms, such as cardiomyopathy, were also reported in China in 1935, and referred to as the Keshan disease (Cheng, 2002). In 1941, a study was conducted in which chickens were fed a diet containing of corn, barley and wheat grown on seleniferous soils. The diets contained 0, 2, 5, 8 or 10 mg Se kg^{-1}. The observed chickens fed with 2 mg Se kg^{-1} showed significant and rapid growth, while a declined growth was seen in chickens fed with 10 mg Se kg^{-1} (Poley et al., 1941). A decade later, in 1957, the nutritional importance of Se was claimed by Schwarz and Foltz . Subsequently, the importance of Se for animals and humans was further highlighted in several studies (Schwarz et al., 1957, Chen et al., 1980, Gupta and Gupta, 2010).

2.4 Soil Se levels and their availability to plants

Selenium is unevenly distributed in the soil. Most soils are relatively low in Se, with concentrations varying in a normal range of 0.01 to 2.00 mg Se kg^{-1} (average: 0.4 mg Se kg^{-1})

(Fordyce 2005). However, also soils with more than 1200 mg Se kg^{-1} occur. These are termed seleniferous soils and are widespread in the Great Plains of the USA, Canada, South America, China and Russia (Birte, 1972). The highest Se contents are found most frequently in phosphates, uranium ore, fossil coal and oil, and in shale with a high content of organic matter (Fleming, 1980). Processes influencing Se cycling are volcanic activity, weathering of rocks, sea spray and volatilization–recycling induced by biota. Besides release from the native substrate, rainfall plays an important role in determining the selenium content of a soil (Fleming, 1980). In regions with less than 500 mm of rain, the soil formed from rocks with a high Se content contains potentially toxic Se concentrations. When the substrate is low in Se, the soil forming on it will have a low Se concentration regardless of climate.

In soil solution with high redox potentials, selenate (SeO_4^{2-}) is the most abundant species (pe+pH>15). In the medium redox range (pe+pH=7.5–15), selenite species are prevailing, while selenide species are stable only at low redox state (pe+pH<7.5) (Elrashidi et al., 1987). Selenite is more stable under lower redox than higher redox potentials, and selenate entering drainage systems is readily reduced to selenite if there is a fall in pe/Eh. At lower pH, selenite is strongly absorbed by hydrous secondary iron oxides and possibly to a lesser extent by clays and organic matter (Dhillon, 2009, Elrashidi et al., 1987, Masscheleyn et al., 1990). If soils are rich in Ca and Mg, $CaSeO_4$ and $MgSeO_4$ both contribute to the total Se concentration at higher redox potentials, whereas KHSe, NH_4HSe and MnSe are the major contributors at lower redox potentials (Elrashidi et al., 1987). Plants absorb Se from the soil solution primarily as selenate and to a much lesser extent as selenite (Geering et al., 1968). Selenate is more readily available to plants and is stable in higher pH soils while selenite, which is dominant in acidic soils, is bound to sesquioxides, decreasing its availability for uptake (Geering et al., 1968). The order of availability of inorganic Se forms to plants is SeO_4^{2-} > $HSeO_3^-$ > SeO_3^{2-}> Se^0 (Mayland et al., 1991). In humid regions and acid soils, the prevailing form is selenite, which is firmly adsorbed on sesquioxides and clay minerals. This form of Se is thus not readily available to plants. On the other hand, selenate, which occurs mainly under well-aerated conditions in alkaline soils of semiarid regions, does not form insoluble salts and thus is readily available to plants (Cary et al., 1967). However, leaching of available Se forms may lead to reduced Se availability to plants.

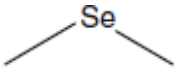
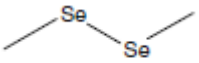
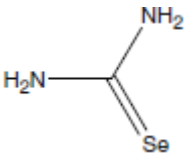
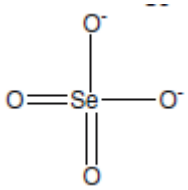
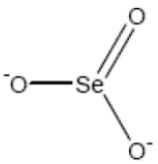
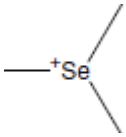
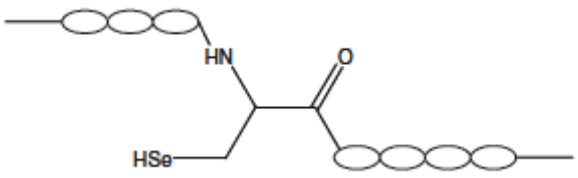
2.5 Agronomic Biofortification

The potential for using Se-enriched fertilizers to increase crop Se concentrations and dietary intake has been demonstrated previously in Finland, the UK and Australia (Adams et al., 2002, Rayman, 2002, Arthur, 2003, Broadley et al., 2006). Most studies focused on pastures to increase dietary Se uptake (Gissel-Nielsen, 1998, Gupta and Gupta, 2002). Due to low dietary Se intake and its potential health consequences in Finland, widespread use of Se in combination with other fertilizers was initiated by the Finnish Ministry of Agriculture and Forestry in 1983 (Ylaranta, 1984, Varo et al., 1988, Eurola et al., 1989, Eurola et al., 1991, Aro et al., 1995, Rayman, 2002, Eurola et al., 2004). With an application dose of 10 mg Se kg^{-1} fertilizer, the Se concentration of wheat bread was increased 10-fold from 0.03 to 0.35 mg Se kg^{-1} DW (Eurola et al., 1991, Eurola, 2005, Aro et al., 1995). In Australia, a Se application ranging from 4 to 120 g Se ha^{-1}, sprayed onto the soil at the time of sowing or applied after flowering, leads to 133-fold and 20-fold increase of Se concentrations in wheat, respectively (Lyons *et al.,* 2005). In the United Kingdom, application of Na_2SeO_4 solution as a single, high volume drench significantly increased Se concentration in wheat grain and straw for all four sites which were examined; in this study, the Se concentration increased by 0.0167 mg kg^{-1} DW for straw and 0.026 mg kg^{-1} DW for grain for each g Se ha^{-1} applied (Broadley et al., 2010). In Malawi, application of Na_2SeO_4 at a rate of 5 g Se ha^{-1} and 10 g Se ha^{-1} leads to increases of 12.6- 15.7% and 6.5-10.8%, respectively in maize (Chilimba et al., 2012). In the various studies, the percentage of applied Se recovered in the crops varies, sometimes being below 10% due to the fact that applied selenate is converted to selenite, which is easily adsorbed by iron oxides and hydroxides in acidic soils (Cary et al., 1967, Geering et al., 1968, Christensen et al., 1989, Balistrieri and Chao, 1990). In an early study conducted by Davies and Watkinson (1966), the recovery of applied Se by the plants varied from only 1 to 2 % in case of selenite application, while 65% was found to be adsorbed to soil colloids while the remaining 30% was unaccounted. In a later study, conducted by Curtin et al. (2006), it was reported that the recovery in the crop differs with Se application rate, timing and method of application, and crop yield (Curtin et al., 2006).

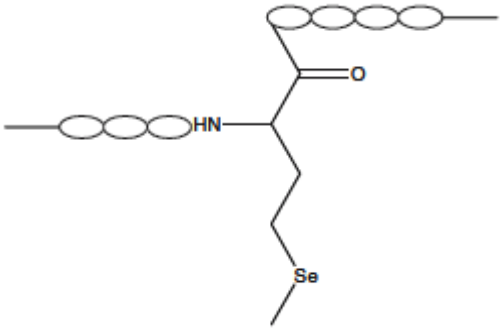
2.6 Selenium speciation in plants

Selenium also exists in different forms in different plant species. Both inorganic and organic Se species occur in the plants (Table 1). In plants, selenate is taken up and distributed by means of sulphate-proton co-transporters (Smith et al., 1995), whereas, selenite uptake is an active process likely mediated, at least partly, by phosphate transporters (Li et al., 2008). In general, plants accumulate high amounts of Se from soil-selenate due to its active transport through sulphate transport mechanism. Moreover, selenate binds weaker to soil particles and tends to have a higher bioavailability (Zhang and Sparks, 1990). Although the uptake of selenate is higher, the formation of organic species from selenate is lower because the reduction of selenate to selenite is a rate-limiting step in the Se assimilation pathway. Thus, most plants supplied with selenate accumulate predominantly selenate while plants supplied with selenite accumulate organic Se (Souza. et al., 1998).

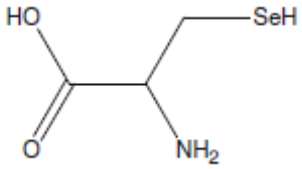
Table 1. Selenium compounds and their structural formulae

Name	Structural formulae
Dimethylselenide	
Dimethydiselenide	
Selenourea	
Selenate (selenic acid)	
Selenite (selenous acid)	
Trimethylselenonium ion	
Selenoprotein	

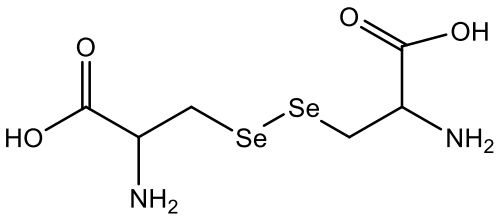
Se-containing protein



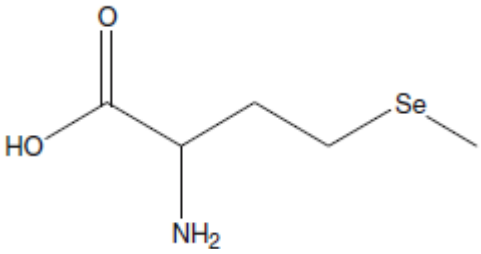
Se-cysteine (SeCys)



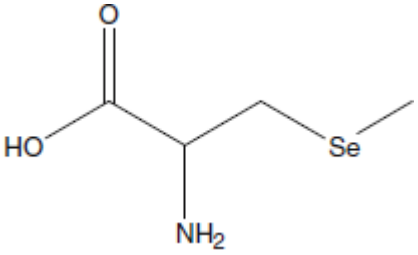
Se-cystine ($SeCys_2$)



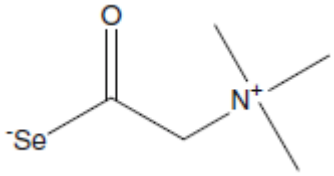
Selenomethionine



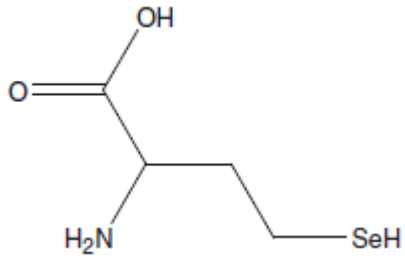
Se-methylselenocysteine (MeSeCys)



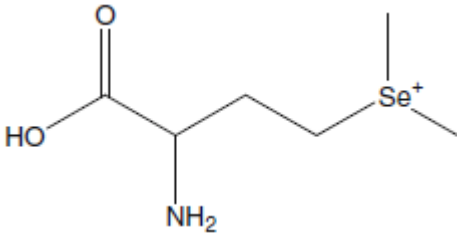
Selenobetaine



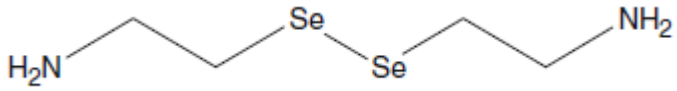
Se-homocysteine



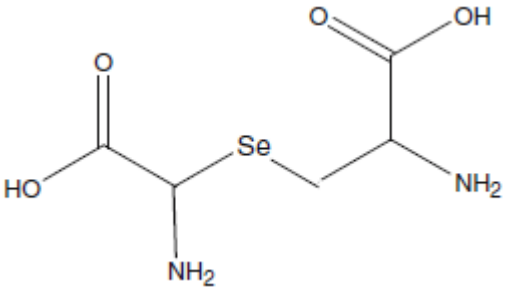
Se-methylSemethionine (MeSeMet)



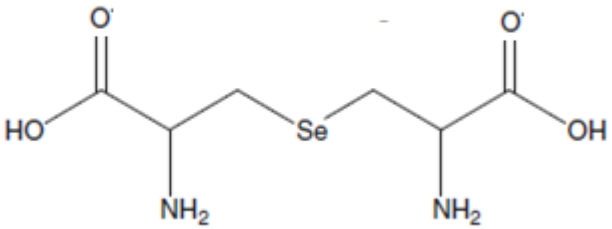
Se-cystamine



Se-cystathione

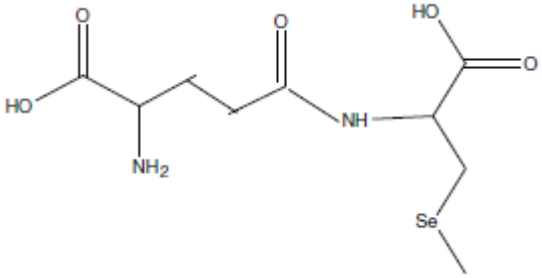


Se-lanthionine

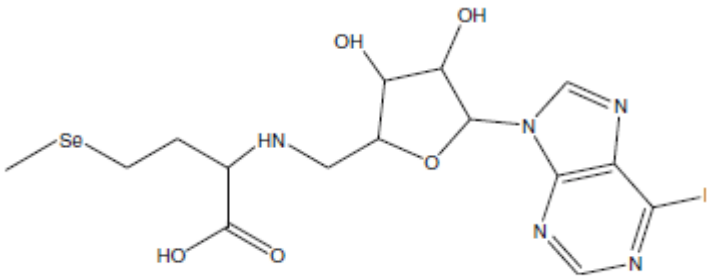


γ-Glutamyl-Se-methylselenocysteine

(γ-glut-cyst)



Se-adenosyl-homocysteine



The conversion of selenate to selenite involves the consecutive action of two enzymes. ATP sulphurylase (APS) couples selenate to ATP, forming adenosine phosphoselenate (APSe) (Wilson and Bandurski, 1958). This is subsequently reduced to selenite by APS reductase (APR). Further reduction of selenite to selenide is mediated by sulfite reductase, in analogy with sulfite reduction. However, it has also been suggested that nonenzymatic reduction by reduced glutathione (GSH) may play a significant role in selenite reduction (Anderson, 1993, Terry et al., 2000). After, selenide has been formed, it can be coupled to O-acetylserine (OAS) to form SeCys, by means of OAS thiol lyase (also called cysteine synthase). OAS is synthesized by the enzyme serine acetyl transferase and functions as a signal molecule that upregulates the activity of sulphate transporters and sulphate assimilation enzymes (Figure 1a).

The major organic Se compounds are selenomethionine (SeMet), selenocystine ($SeCys_2$), and Se-methylselenocysteine. More than 25 Se-containing proteins (selenoproteins) are now known. It has been postulated that SeCys is metabolized to SeMet by cystathionine-g-synthase (CgS), which couples SeCys to O-phosphohomoserine to form Se-cystathionine. A second enzyme, cystathionine-β-lyase, converts Se-cystathionine into Se-homocysteine. Finally, Se-homocysteine is converted to SeMet via the action of Met synthase. The other organic species such as MeSeCys and γ-glut-cyst are formed from SeMet which is further metabolized to Se–adenosyl–Se–Met and MeSeMet. The formation of MeSeCys in plants occurs through two pathways, via the SeMet pathway and the SeCys pathway (Figure 1a and 1c).

Volatilization of Se from plant is recognised as a detoxification process (Wilber, 1980). Detoxification can occur via two different pathways as indicated in Figure 1b and c (Lewis et al., 1974). Selenium hyper-accumulating plants show a higher detoxification ability compared to non-accumulating plants, with the formation of MeSeCys via the action of

SeCys methyltransferase being a major process in these hyper accumulators (Lyi et al., 2005). As MeSeCys is not incorporated into proteins like SeCys does, the detoxification mechanism in hyper accumulators leads to higher formation of DMDSe (Figure 1c).

In general, selenomethionine (SeMet) is identified in many plant species as the main organic Se species. However, in accumulating plants of the *Allium* and *Brassica* family, MeSeCys and γ-glut-cyst were identified as the dominant Se species. Inorganic Se species were also identified in many plants and their degree of occurrence differs between the species. In the past decade, many studies focused on achieving organic Se species in plants as these species are considered to be more beneficial for humans (see section 2.9).

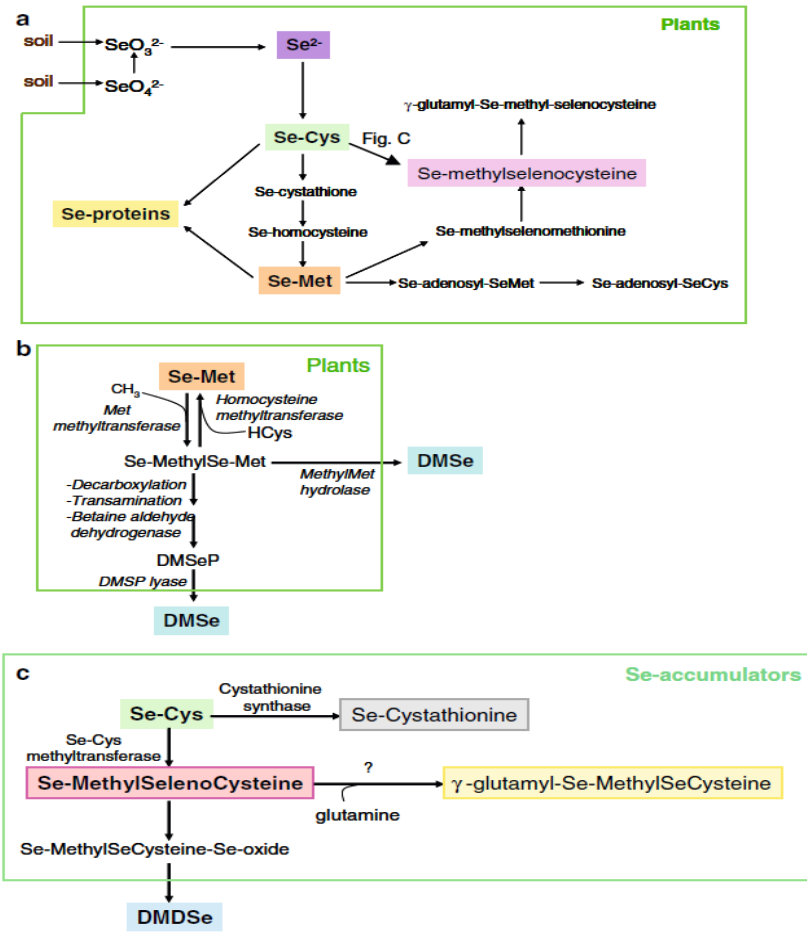


Figure 1. Schematic representation of the metabolization of Se in plants (a), of the volatilization of Se in plants (b) and of the metabolization of Se in Se accumulating plants (c) (Dumont et al., 2006b)

2.7 Role of Se in human health

The essentiality of Se for humans and livestock was previously illustrated in various studies. A total of 25 selenoproteins have been identified in humans, including iodothyronine deiodinases, thioredoxin reductases, glutathione peroxidases, and a range of other selenoproteins (e.g. SelP, SelM, SelT) (Brown et al., 2001, Fairweather et al., 2011, Rayman 2002). Studies in farm animals indicate that Se deficiency affects both cell-mediated and hormonal components of the immune response (Arthur, 2003, Hoffmann and Berry, 2008), whereas in humans, limited data suggest that when intake of Se is sub-optimal, Se supplements can improve immune response (Hoffmann et al., 2008). In case of low serum Se in humans, when consuming Se less than 40 μg Se d^{-1} which is associated with low levels of natural killer cells, health disorders, including cardiovascular disorders, occur (Fairweather-Tait et al., 2011). In such situations, Se supplementation (200 μg Se d^{-1}) increased T-lymphocyte-driven tumor lysis, lymphocyte proliferation and enhanced immune response. The Keshan disease (a cardiomyopathy) and Kashin-Beck disease (an osteoarthropathy) were reported in case of extremely low Se (<20 μg Se d^{-1}) intake levels (Fairweather-Tait et al., 2011). Several studies indicate that low dietary Se intake can be linked to pancreatitis, asthma, inflammatory response syndrome, impacts on immune system functioning, lower response to viral infection, lower female and male fertility, and abnormal thyroid functioning (Rayman, 2000, Rayman and Rayman, 2002). Correlation studies relating low Se status with functioning of the immune system pointed towards a role in spreading of HIV/AIDS (Cirelli et al., 1991, Look et al., 1997).

The protective role of 15 selenoenzymes and their characteristic biological function is shown in Table 2. Among them, four glutathione peroxidases (GPx) and three forms of thioredoxin reductases have important roles in regenerating antioxidant systems and maintaining the intracellular redox state, and three forms of iodothyronine deiodinases are involved in the production of active thyroid hormone. The active site of the potent GPx contains SeCys residues. It is known that GPx activity and expression have been used in many human studies as biomarkers for selenium status. However, other selenoproteins (e.g., selenoprotein P and thioredoxin reductase) also have been shown to possess antioxidant properties in defense action against peroxynitrite, by reduction of this potent oxidizing and nitrating species to nitrite (Arteel and Sies, 2001).

Table 2. Known selenoproteins involved in metabolic processes (adapted from Rayman, 2000)

Selenoprotein	Function
Glutathione peroxidases (GPx1, GPx2, GPx3, GPx4)	Antioxidant enzymes: remove hydrogen peroxide, and lipid and phospholipid hydroperoxides (thereby maintaining membrane integrity, modulating eicosanoid synthesis, modifying inflammation and likelihood of propagation of further oxidative damage to biomolecules such as lipids, lipoproteins, and DNA) .
(Sperm) mitochondrial capsule selenoprotein	Form of glutathione peroxidase (GPx4): shields developing sperm cells from oxidative damage and later polymerises into structural protein required for stability/motility of mature sperm.
Iodothyronine deiodinases (three isoforms)	Production and regulation of level of active thyroid hormone, T3, from thyroxine, T4.
Thioredoxin reductases (probably three isoforms)	Reduction of nucleotides in DNA synthesis; regeneration of antioxidant systems; maintenance of intracellular redox state, critical for cell viability and proliferation; regulation of gene expression by redox control of binding of transcription factors to DNA.
Selenophosphate synthetase, SPS2	Required for biosynthesis of selenophosphate, the precursor of SeCys, and therefore for selenoprotein synthesis.
Selenoprotein P	Found in plasma and associated with endothelial cells. Appears to protect endothelial cells against damage from peroxynitrite.
Selenoprotein W	Needed for muscle function.
Prostate epithelial selenoprotein (15kDa)	Found in epithelial cells of ventral prostate. Seems to have redox function (resembles GPx4), perhaps protecting secretory cells against development of carcinoma.
DNA-bound spermatid selenoprotein (34 kDa)	Glutathione peroxidase-like activity. Found in stomach and in nuclei of spermatozoa. May protect developing sperm.
18 kDa selenoprotein	Important selenoprotein, found in kidney and large number of other tissues. Preserved in selenium deficiency.

The various mechanisms that may play a role in the anti-carcinogenic functioning of Se are illustrated in Figure 2. They include regulation of the cell cycle, apoptosis, and antioxidant effects, which are due to the action of selenoproteins (in particular, GPx1, GPx4, Sep15, SEPP1, and TXNRD1), modulation of angiogenesis and the extracellular matrix, histone deacetylase inhibition, carcinogen detoxification, induction of GSTs, alteration of DNA damage and repair mechanisms, and also immune system modulation (Jackson et al., 2008,

Lu and Jiang 2005, Selenius et al., 2009). Clinical studies on Se supplementation strongly supported the role of Se in reducing incidence of cancer in recent years. For instance, the Nutritional Prevention of Cancer (or NPC) trial carried out by Clark and co-workers in the USA using a supplementation in the form of selenized yeast at 90 µg Se d^{-1}, showed 50% lower total cancer mortality ($p<0.002$) and 37% lower total cancer incidence ($p < 0.001$) with 63% fewer cancers of the prostate, 58% fewer cancers of the colon, and 46% fewer cancers of the lung (Clark et al., 1996). In support of these results, Se supplementation with 200 µg Se d^{-1} resulted in a 60% decrease in prostate cancer. A significant decrease in other cancer incidences was also reported with a significant decrease in esophageal cancer prevalence and also reduced total mortality and gastric cancer mortality. In contrast to these studies, the Selenium and Vitamin E Cancer Prevention Trial (SELECT), the largest ever prostate cancer prevention trial, showed that Se or Vitamin E taken alone or in combination did not prevent prostate cancer in a population of relatively healthy men over an average period of five years (Klein et al., 2011). However, in this study pure L-selenomethionine was used instead of selenized yeast, which contains a much wider variety of Se species. The effect of selenium on cancer is assumed to depend on the dose of Se, as well as its speciation and bioavailability, and it may be affected by metabolism and genotype (Figure 2).

The role of Se in reducing toxicity caused by other metals, e.g. (methyl) mercury found in seafood (Chen et al., 2006), was also reported. This is attributed to the formation of inert metal selenide complexes. A Se-induced reduction in prooxidant and genotoxic effects of arsenic has been demonstrated in humans suffering from arsenic-related skin lesions (Gailer et al., 2000). Moreover, Se was reported to reduce oxidative stress induced by cadmium in various animal tissues (Zwolak and Zaporowska, 2012).

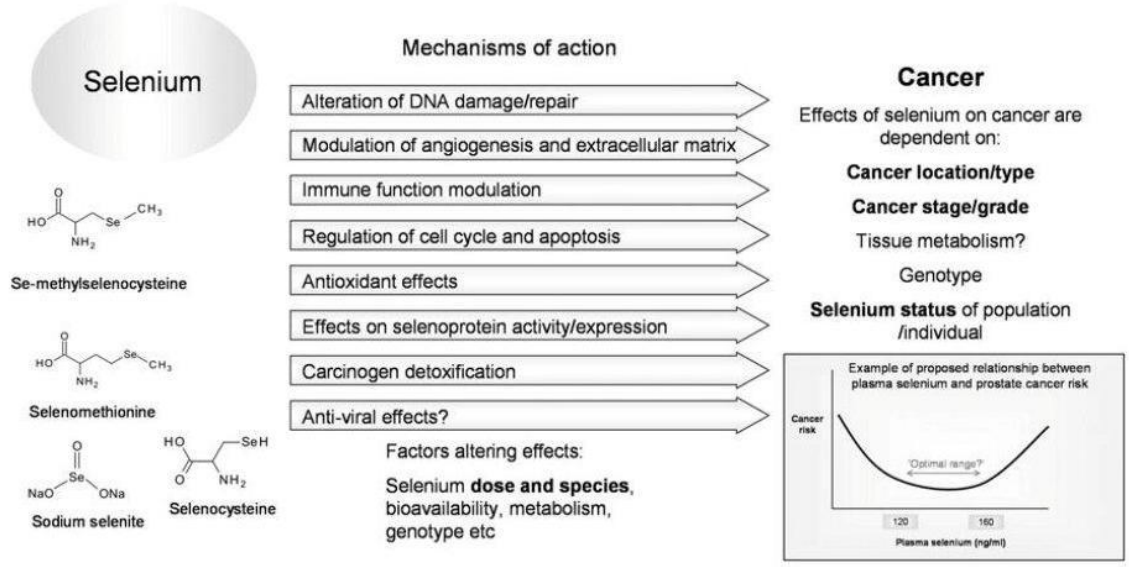


Figure 2. Mechanisms that may play a role in the anti-carcinogenic functioning of Se (Fairweather-Tait et al., 2011)

2.8 Selenium intake and daily required allowance

The daily intake of Se varies among different countries across the world (Figure 3). The Se intake in most European countries is significantly lower when compared to Japan and the USA. The amount of Se available in the soil for plant uptake is clearly related to the intake of Se by humans living in an area. It varies considerably between regions and countries. Selenium intake below 11 μg d^{-1} is often associated with serious health effects and intake below 20 μg Se d^{-1} has been observed to induce deficiency symptoms (Fairweather et al., 2011). In Australia, Bangladesh, Canada, Finland, Greece, Russia, United Kingdom, USA, Venezuela and Germany intake ranges from 29 to 500 μg d^{-1} (Reilly, 1998). The mean intake in Finland increased from 30 to 113 μg Se d^{-1} between 1984 and 1986 due to the national supplementation programme (Eurola et al., 2003). The intake and status of Se in New Zealand also increased when Australian wheat containing higher levels of Se was imported (Thomson and Robinson, 1980, 1996, Watkinson, 1981).

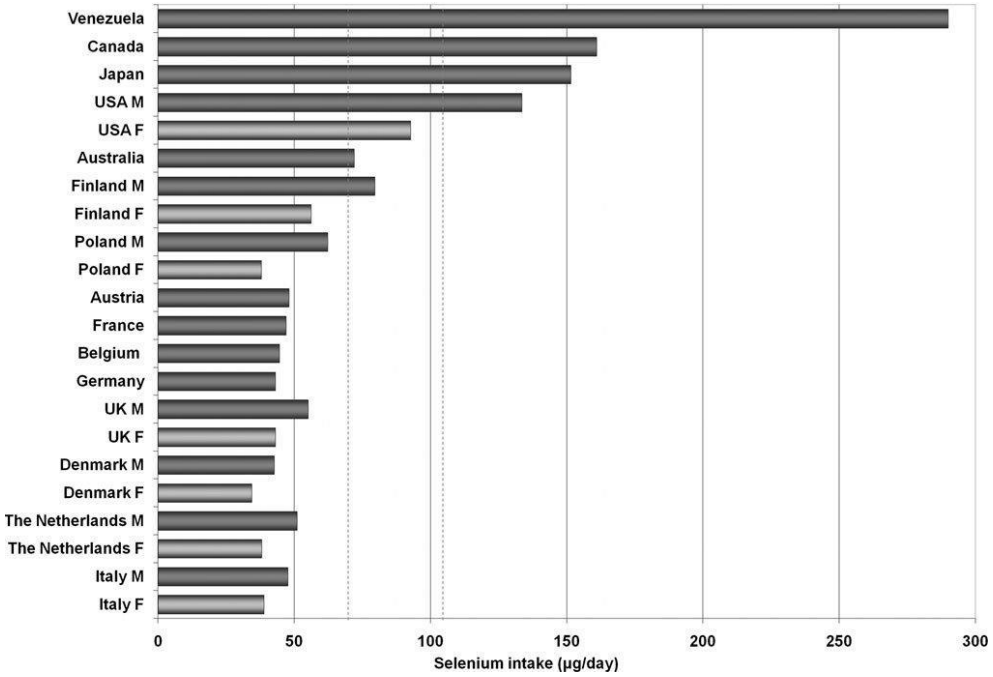


Figure 3. Global variation in selenium intake. Data shown here were compiled from Se intake data (adapted from (Fairweather-Tait et al., 2011). Data are presented for the intakes for males (M) and females (F) separately where available, with the latter shown with a lighter bar

There are also regions with very high Se intake like in India where an estimated intake of 475 µg d^{-1} for women and 632 µg d^{-1} for men was reported. In these regions, more than 80% of the Se is taken in through consumption of cereals locally grown in soils rich in Se (Hira et al., 2004). In many parts of the world such as Africa and many parts of Asia and Latin America, no data on Se intake are available yet.

There is no general, international recommended dietary Se intake because the recommended dose varies with age, sex and source of dietary Se (Thomson, 2004). The recommended Se intake in the USA, Canada and Europe was set at 55 µg d^{-1} (Thomson, 2004), a value intended to achieve and maintain the maximum plasma GPx activity. However, as there is growing evidence that additional beneficial effects, such as cancer prevention, may be provided when dietary Se intake exceeds the normal nutritional range, it may be inappropriate to rely solely on GPx activity to define optimal Se intake (Rayman, 2002). Recently, it was reported that consumption of Se in amounts up of 3–5 times the recommended dietary allowance of 70 µg d^{-1} for men and 55 µg d^{-1} for women (National Research Council 1989)

may prevent certain cancers including colon cancer. Plasma Se levels exceeding 120 μg L^{-1} may be a useful target value for minimizing cancer risk (Combs, 2001). To obtain these plasma Se concentrations, it is recommended that the dietary intake should be at least 1.5 μg Se kg^{-1} body weight d^{-1}, which is equivalent to 90 and 120 μg d^{-1} for people weighing 60 or 80 kg, respectively. The lowest Se plasma levels in Europe are found in Eastern Europe and several parts of Poland, Slovenia and Turkey (Kljai and Runje, 2001, Micetic-Turk et al., 2000, Ochoka et al., 2000, Vrca et al., 2004). The plasma Se concentration in the Belgian population was reported to be 84.3±9.4 μg L^{-1} in year 2004 and 79.8±4.4 in 2007 (Cauwenbergh et al., 2007, Cauwenbergh et al., 2004).

In Belgium, Robberecht et al. (1994) reported the daily Se intake to range between 28 and 61 μg Se d^{-1}, with an average of 45 μg Se d^{-1}. A recent study conducted by Waegeneers et al. (2013) assessed Se intake by the Belgian population. The estimated average Se daily intake was 60 μg Se d^{-1} (Waegeneers et al., 2013). Although the latter study used much more recent data, it was based on analysis of non-prepared food samples collected on the market, whereas data used by Robberecht et al. (1994) referred to cooked food which is ready to consume.

2.9 Selenium speciation and human health

Selenium speciation is gaining attention in human health due to the different role the different species may play. The metabolic pathway of Se in the body is presented in Figure 4. Organic Se was found to increase blood Se concentrations more than inorganic Se species (Slavik, 2008). In general, methionine from proteins competes with SeMet, predominantly seen in tissue proteins such as skeletal muscle, erythrocytes and plasma. Selenomethionine is more effective in increasing apparent selenium status because it is non-specifically incorporated into protein, thus acting as a reservoir of Se. Although inorganic Se leads to formation of selenoproteins, they cannot be stored for later use (Alfthan et al., 1991). Clinical data indicated that organic and inorganic Se species result in a similar Gpx activity in plasma. However, in two studies it was concluded that, upon supplementation with SeMet and seleni(a)te over a period of time, Se concentrations of plasma reached a plateau in the subjects supplemented with inorganic Se forms, while it was still rising in SeMet supplemented subjects (Levander et al., 1983, Thomson et al., 1982). This suggests that long

term effects of SeMet are larger than those of mineral Se. In some of the studies, Se-enriched yeast containing mainly SeMet was used as Se source. Se-methylselenocysteine (MeSeCys), another organic species, is a naturally occurring seleno-amino acid that is synthesized by plants of the *Allium* and *Brassica* family, such as garlic and broccoli, and also by Se-enriched yeast at lower levels. Unlike SeMet, which is incorporated into proteins substituting methionine, MeSeCys is not incorporated into proteins and shows a behavior similar to inorganic Se species. The formed MeSeCys in plants can also convert to γ-glut-cyst (Dumont et al., 2006b). It is fully available for the synthesis of Se-containing enzymes, such as Gpx (Zeng et al., 2008). Anticarcinogenic effects observed in rat trials have been attributed to this species, and other species mainly occurring in *Allium* and *Brassica* crops, such as γ-glut-cyst and MeSeCys (Ip et al., 2000, Ip and Lisk, 1995).

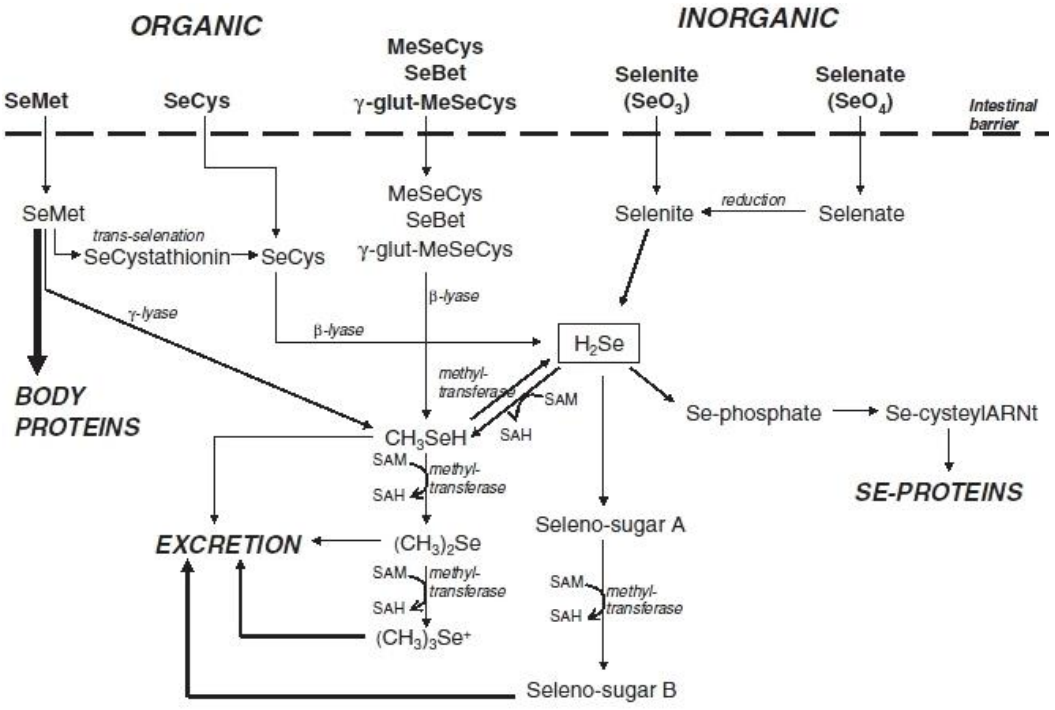


Figure 4. Proposed schematic representation of Se metabolism in humans (adapted fromSuzuki et al., 2006a, Suzuki et al., 2006b, Suzuki et al., 2008). CH_3SeH: methylselenol; $(CH_3)_2Se$: dimethylselenide; $(CH_3)_3Se^+$: trimethylselenonium; γ-glut-methylselenocysteine: gamma glutamyl methylselenocysteine; GSH: glutathione; H_2Se: hydrogen selenide; MeSeCys: Se-methylselenocysteine; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; SeBet: selenobetaine; SeCys: selenocysteine; SeMet: selenomethionine

2.10 Importance of *Allium* species in Se biofortification

The genus *Allium* includes 600 to 750 species, making it one of the largest plant genera. *Allium* species are consumed by indigenous population across the world. Mainly *Allium sativum* (garlic), *Allium cepa* (onion), *Allium schoenoparsum* (chives), *Allium ampeloprasum* (great-headed or elephant garlic), *Allium tuberosum* (Chinese or garlic chives), *Allium fistulosm* (Japanese bunching onion), *Allium tricoccum* (ramp), *Allium ursinum* (bear's garlic or ramson/rank), and *Allium ascalonicum* (shallot) are widely grown species. Among all vegetables in diet, *Allium* species particularly contain high concentrations of sulphur analogues such as cysteine (Eric, 2010). The latter gives a lot of potential to these crops to accumulate large amounts of organic Se species (Table 3) due to the fact that Se uptake and metabolism follows the sulphur pathways. The ancient Indian Ayurvedic medical treatise called Charaka-Samhita already assigned beneficial health effects to *Allium* species, such as garlic and onion.

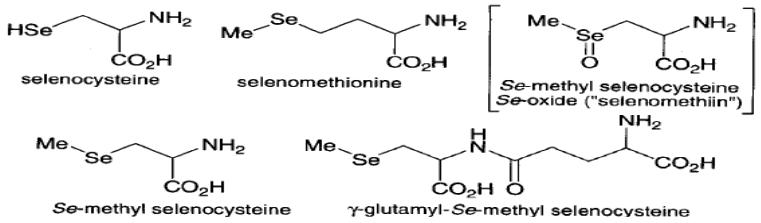


Figure 5. Major organoselenium compunds occurring in *Allium* plants (Eric, 2010)

Various sulphur and Se analogues were reported to occur in the *Allium* species, some of which were reported to be anti-carcinogenic (Figure 5) (Eric, 2010). A cysteine analogue in which Se replaces sulphur leads to formation of SeCys, called the 21st amino acid which is considered essential for ribosome-directed protein synthesis. However, $SeCys_2$, an oxidised form of SeCys is often reported to be the dominant Se species. This might be due to speciation transformations occurring during the extraction prior to analysis or oxidation reactions occurring under biotic and abiotic where glutathione activity is predominant (Arteel et al., 2001). Selenocystine may also exist as a free amino acid in certain organisms when

they grown in Se rich conditions, and the possibility still exists that $SeCys_2$ may be incorporated in proteins (Huber and Criddle, 1967). Moreover, MeSeCys and γ-glut-cyst, to which anticarcinogenic effects were assigned in rat trials, are often present in high concentrations (Finley et al., 2000, Ip et al., 1995). The major organic Se species reported in *Allium* crops are reported in Table 3. The total concentration was found to range between 100 and 1355 mg Se kg^{-1} DW when fertilizing the plants with selenate or selenite salts (Ip et al., 1995).

Table 3. Prevalence of selenium species in Se-enriched *Allium* species[I]

Plant	Se addition	Total Se[b] (µg/g)	Selenium species (%)[c]					
			Se(IV)	Se(IV)	SeMet	$SeCys_2$	MeSeCys	γ-Glu-MeSeCys
Garlic (*Allium sativum*)[II,III,IV]	Na_2SeO_4+mycc-orhiza (50 mg kg^{-1}, 4 weeks)	969	Enzymatic/water extraction					
			-	-/9[d]	2/1[d]	-	3/5[d]	64/62[d]
		296	Enzymatic/water extraction					
			-	2[d]	13[d]	0.5[d]	3[d]	73[d]/85[d]
	$BaSeO_{3+}$ $BaSeO_4$ (500 mg m^{-3} of each, 8 months)	96	Water extraction					
			-	-	15.5[e]	6.0[d]	28.8[e]	49.7[e]
Onion (*Allium cepa*) [V]			$HClO_4$-ethanol extraction					
	Na_2SeO_3	154	-	-	0.3	0.5[f]	4.0	-
	Na_2SeO_4 (15 mg kg^{-1}, 8 days)	601	-	-	0.2	0.1[f]	1.9	-
Green onion (*Allium fistulosum*) [VI]	Na_2SeO_3 (15 mg kg^{-1}, 4 months)	30.3	Enzymatic extraction/HCl hydrolysis					
			+/-	-/-	+/-	+/-	+/-	+/-
Ramp (*Allium tricocum*) [VII]	Na_2SeO_4 (30 mg L^{-1})	252	-	42	-	-	35	1.4
Shallot (*Allium ascalonicum*) [VII]	$BaSeO_{3+}$ $BaSeO_4$ (500 mg m^{-3} of each, 8 months)	226.8	Water extraction					
			-	28	-	-	5.4	66
			Enzymatic extraction					
Garlic [IX]		68	-	1	18	0.5	2.5	68
		235	-	1.5	17	0.5	3	70
		1355	-	4	13	-	60	8
Ramp		48	-	1	21	-	34	3
		524	-	22	5	-	44	1.5
Onion		96	-	10	5	1	1	63

		140	-	33	10	-	5	35
Chives (*Allium schoenpprasum*) [x]			$HClO_4$-ethanol extraction/enzymatic extraction					
	Na_2SeO_3	222	-/3	21/5	-/5	40/42	28/36	-
	Na_2SeO_4	613	-/-	81/51	-/-	5/2	3/20	-
	SeMet (10 mg L^{-1}, 14 d-1)	265	-/1	5/-	-/3	35/37	46/48	-

[a]+ detected but not quantified; [b]Based on dry weight; [c]Relative to total Se in the sample; [d]Relative to total chromatographed selenium; [e]Relative to total Se in the extract; [f]Se-cysteine. (I. Pyrzynska, 2009; II. Larsen et al., 2006; III. Ip et al., 2000; IV. Dumont, E. et al., 2006; V. Wróbel et al., 2004; VI. Shah et al., 2004; VII. Whanger et al., 2000; VIII. Ogra et al., 2005; IX. Kotrebai et al., 2000; X. Kápolna et al., 2007b).

Chapter 3. **Selenium uptake and speciation in leek (*Allium ampeloprasum* var. *porrum*) as affected by Se fertilizer type and dose**

This Chapter has been redrafted from:

Lavu R. V., Du Laing G.,Van de Wiele T., Pratti V. L., Willekens K., Vandecasteele B., Tack F. 2012. Fertilizing soil with selenium fertilizers: impact on concentration, speciation, and bioaccessibility of selenium in leek (*Allium ampeloprasum*). Journal of Agricultural and Food Chemistry. 60:10930-5.

3.1 Abstract

The effect of fertilizing soil with sodium selenite (Na_2SeO_3), sodium selenate (Na_2SeO_4) and barium selenate ($BaSeO_4$) on Selenium uptake and speciation in leek (*Allium ampeloprasum* var. *porrum*) was studied in a greenhouse experiment. A sandy loam soil with baseline Se concentration of 0.2 mg kg^{-1} was enriched with 0.2, 1.3, 2.6 and 3.8 mg Se kg^{-1} as Na_2SeO_3, Na_2SeO_4, and $BaSeO_4$ in different treatments. Leek was grown for 3 months on these soils, harvested and analysed for Se contents and speciation. Identified selenium species in the leek were selenite, selenate, MeSeCys, SeMet, $SeCys_2$ and γ-glut-cyst. When the soil was amended with Na_2SeO_4 or $BaSeO_4$, about half of the Se in the leek was found to be inorganic (58 % and 48%, respectively). When Na_2SeO_3 was applied, only 38 % was inorganic. Although applying selenate results in a higher total Se accumulation in the crop, applying sodium selenite seems to be better to enhance particular organic Se species, which were previously described for other *Allium* species to exhibit potential anticarcinogenic properties. However, the lower plant uptake when using Na_2SeO_3 as fertilizer results in a higher risk for Se accumulation in the soil on longer term. Therefore, more research is needed to assess the factors affecting plant uptake and fate of selenite in the soil.

3.2 Introduction

Selenium is an essential trace element for humans and animals. It can occur in different inorganic and organic forms (= species), which may have different effects on mobility, availability and toxicity (Tamas et al., 2010). The most important source of Se is the diet. Selenium uptake depends on the eating habits of the individual. Vegetables were found to provide more than 85 % of the average daily human dietary Se intake (Cassens, 1997). Selenium supplementation is often needed due to a lack of Se in the soils and crops grown on these soils, resulting in Se deficiency in humans and animals. Biofortified food crops such as Se fortified *Brassica* (broccoli, rapeseed, cabbage) and *Allium* (onion, garlic, chives, ramps) species were suggested especially as these plants are capable of accumulating higher amounts of Se during cultivation and transforming Se into appropriate chemical forms having potential

positive effects on human health. These forms include MeSeCys and γ-glut-cyst which are known to be more effective inhibitors of tumor formation (Benoit and Ceustermans, 1994, Ip et al., 2000). The Se uptake by crops and Se species formed during crop growth depend on the Se form and its concentration available in the soil, as well as soil conditions (Gissel-Nielsen, 1971, Johnsson, 1991, Robberecht et al., 1982). Several studies described that *Allium* family species are capable of transforming inorganic forms applied to the soil into organic forms (Dumont et al., 2006b, Ip et al., 2000, Larsen et al., 2006, Wróbel et al., 2004). Amongst the *Allium* species, focus was laid on studying chives, onions and garlic. Not much attention was paid to leek (*Allium ampeloprasum* var. *porrum*) yet, even though it is one of the economically most important vegetable crops in Europe (Benoit et al., 1994). Leek is a good source of vitamin A, vitamin B6, vitamin C, vitamin K, dietary fiber, folate, calcium, iron and magnesium, and low in saturated fat, sodium and cholesterol. It is intensively cultivated in Indonesia, Turkey, France, Belgium, China and Poland. Daily use of leek in the diet has been shown to have beneficial effects on the body, particularly on circulatory system (Liu et al., 2006).

In the present study, the effect of Se fertilizer type and dose on Se accumulation and speciation in leek was studied. Leek plants were grown on soil fertilized by Na_2SeO_4, Na_2SeO_3 and $BaSeO_4$. The plants were harvested and their Se contents and speciation were determined.

3.3 Materials and methods

3.3.1 Experimental setup

For the cultivation of the Se-enriched leek, commercially available Leek (*Allium ampeloprasum* var. *porrum*) plantlets of Harston variety were purchased. Recipients were filled with 25 kilograms of soil, which had a sandy-loamy texture and contained Se (0.23 mg kg^{-1}), Al (4683 mg kg^{-1}), Cd (0.24 mg kg^{-1}), Cr (10.4 mg kg^{-1}), Cu (12.4 mg kg^{-1}), Fe (6817 mg kg^{-1}), Mn (262 mg kg^{-1}), Ni (5.0 mg kg^{-1}), Pb (65.1 mg kg^{-1}), Zn (33.3 mg kg^{-1}), K (65.1 mg kg^{-1}), Mg (115 mg kg^{-1}), Ca (1001 mg kg^{-1}), Na (22.1 mg kg^{-1}), P (214 mg kg^{-1}), and organic carbon (1.09 %). Its pH was 6.15.

The required amount of Se was added to each soil and the soil was completely homogenized in mixing rotator. In each recipient, eight plantlets were planted in two rows and watered with deionized water. The treatments consisted of applying three sources of selenium, i.e. Na_2SeO_4, Na_2SeO_3 and $BaSeO_4$ at four levels of selenium, i.e. 0.2, 1.3, 2.6 and 3.8 mg Se kg^{-1} soil. The experiment was replicated four times. To prevent pests, pyrethra pur (Eco-style, Belgium) was sprayed twice over a period of 3 months. After three months, plants were harvested and washed gently with tapwater to remove surface contaminants. Washing with tap water was followed by rinsing with deionized water. The weight of each plant was recorded and the entire plant was manually cut into pieces. The samples were shock-frozen immediately with liquid nitrogen after transferring them into polyethylene boxes. They were stored at -80°C. Finally, they were freezedried by a lyophiliser (Heto power dry, Belgium) and ground to a fine powder in a mechanical grinder (MF 10 IKA, Werke Germany) to pass through a 1 mm sieve.

3.3.2 Reagents and standards

Sodium selenite (Na_2SeO_3), sodium selenate (Na_2SeO_4), barium selenate ($BaSeO_4$), SeMet, $SeCys_2$ and MeSeCys were purchased from Sigma Aldrich (St. Louis, MO, USA), and γ-glut-cyst and γ-glut-meth were purchased from pharmaSe (Austin, Texas, USA). For chromatographic purposes, citric acid was obtained from Sigma Aldrich (St. Louis, MO, USA), heptafluorobutryic acid was obtained from Fluka and ammonium hydroxide was obtained from J.T.Baker (Deventer, The Netherlands). For sample preparation procedures, protease XIV, lipase and Tris-HCl was purchased from Sigma, while concentrated HNO_3 and H_2O_2 were purchased from Chemlab (Zedelgem, Belgium). MilliQ® (MQ) water from Water Systems Ltd. (Brussels, Belgium) was used throughout the experiment. Chromatographic standards and other solutions were prepared freshly every day.

3.3.3 Selenium analysis

3.3.3.1 Sample preparation for total selenium analysis in soil

Conventional aqua regia digestion was performed in 250-mL glass beakers covered with watch glasses. A well-mixed sample of 1.000 g was digested in 10 mL of aqua regia (3:1 HCl:HNO_3) on a hotplate for 3 h at 105°C. After evaporation to near dryness, the sample was diluted with 2% nitric acid and transferred into a 100-mL volumetric flask after filtering through Whatman no. 42 paper. The filtrate was diluted to 100 mL with deionized water, and analysed with ICP-MS (see 3.3.3.4).

3.3.3.2 Sample preparation for total selenium analysis in plants

For the determination of total Se in the leek samples, 0.2 g sample was placed into a centrifuge tube followed by addition of 2.5 mL concentrated HNO_3 and 2.5 mL 30% H_2O_2. After 16 h, the tubes were capped and placed in a microwave oven (Mars, North Carolina, USA) (Williams et al., 2007). In a first step, the temperature was raised to 55 °C in 10 min at 600 Watt and 100% power. Afterwards, the temperature was raised to 75 °C in 10 min. Finally, it was maintained at 100 °C for 30 min. The clear digests were diluted to 50.0 mL with deionized water for analysis with ICP-MS (see 3.3.3.4). For validation of the procedure, the certified reference plant material BCR-CRM 402 (white clover, 6.7 ± 0.27 mg Se kg^{-1}) was digested using the same procedure. Three replicates of BCR-CRM 402 were analysed with each sample batch.

3.3.3.3 Sample preparation for Se speciation analysis in plants

In order to extract the protein-bound Se species in all samples, an enzymatic extraction was performed. Plant sample (0.2 g) and 80 mg of the enzyme Protease XIV were dissolved in 5

mL of water. This mixture was shaken in a 10 mL centrifuge tube for 24 h at 37 °C (Mazej et al., 2008) using a shaker fitted incubator chamber (Sartorius, Goettingen, Germany) and centrifuged (Sigma 2-16PK centrifuge, Germany) for 30 min at 3000 g. The supernatant was separated from the residue and filtered through a 0.45 µm syringe-type PVDF membrane filter. Supernatant and residue were stored at -20°C until they were analysed for total Se and Se speciation using ICP-MS and HPLC-ICP-MS, respectively (Table 4). In addition, an enzymatic digestion that also targets the lipid-bound fraction was included. A 5 mL of Tris–HCL buffer adjusted to pH 7.5 was added to 0.2 g of sample in a 50 mL centrifuge tube, followed by addition of 20 mg protease XIV and 10 mg lipase VII. This mixture was further processed as mentioned above.

3.3.3.4 Analysis

An Inductively Coupled Plasma Mass Spectrometer (ICP-MS, PerkinElmer DRC-e, Sunnyvale, CA, USA) was used for total Se and speciation analysis as an element-specific detector. The ICP-MS was fitted with a Babington nebulizer and a Scott double pass spray chamber. Among the measured Se isotopes, ^{80}Se was choosen for the calculations. The interference of $^{40}Ar_2^+$ on mass 80 was removed successfully using CH_4 as reaction gas. Results were calculated using external calibration.

For speciation analysis, the ICP-MS was coupled as detector to a liquid chromatographic system (Series 200 HPLC, PerkinElmer, Sunnyvale, CA, USA). It consisted of a P680 HPLC pump and an ASI-100 automated sample injector. A Hamilton PRP-X100 anion exchange column and Altima C_8 column (250 mm × 4.6 mm I.D., 5 µm, 120 Å) were used as stationary phase. Both columns were equipped with a guard column containing the same stationary phase material. The different species were quantified using data obtained from the anion exchange column. However, the reversed phase column was also used to quantify the oxidised form of SeMet (SeMet oxide), which cannot be distinguished from $SeCys_2$ on the Hamilton PRP-X100 column. If SeMet oxide was found to be present, its concentration was subtracted from the concentration of SeCys calculated using the Hamilton PRP-X100 column. HPLC-ICP-MS conditions are presented in Table 4.

Table 4. Optimized instrumental parameters for ICP-MS

ICP-MS parameters:	
Power	1250 W
Plasma Ar flow	15 L min^{-1}
Isotopes monitored (mass)	76,77,78, 80, 82
Reaction gas and flow rate	CH_4, 0.9 mL min^{-1}
Dwell time for each isotope	0.1 s
Chromatographic conditions:	
Anion exchange (isocratic elution)	
Column	PRP-X100 (250mm × 4.6 mm, 5 µm)
Mobile phase	10 mM citric acid, 5 % (v/v) methanol, pH 5.0
Flow rate	1.0 mL min^{-1}
Injecton volume	25 µl
Reversed phase (isocratic elution)	
Column	Alltech Altima C8 (250mm × 4.6 mm, 5 µm)
Mobile phase	0.15 % (v/v) Hepta flurobutyric acid, 5% (v/v), methanol
Flow rate	1.0 mL min^{-1}
Injection volume	25 µl

3.3.4 Statistical analysis

The significance of effects was evaluated using ANOVA with 0.05 as significance level. In addition, differences in Se concentrations with the control plant were evaluated using an LSD (Least Significant Difference) test. Regression analysis was conducted to identify correlations (linear/quadratic) between the tested parameters and applied Se doses. Statistical analysis was conducted with SPSS (version 21).

3.4 Results

3.4.1 Effect of Selenium fertilizers on biomass production

The application of the different Se fertilizers at different doses did not result in significant differences in biomass production ($p \geq 0.05$) (Figure 6). Accordingly, no relationship was observed between the applied Se dose and biomass production of the leek.

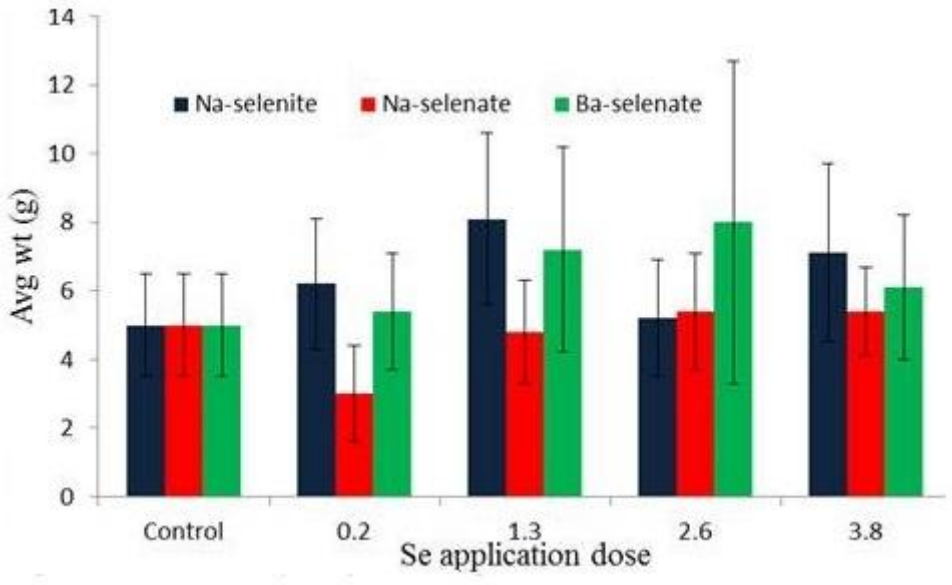


Figure 6. Biomass production of leek (g DW) as function of Se fertilizer type and dose (mean±standard deviation, n=3)

3.4.2 Total selenium concentrations in the plants

The plants differed significantly ($p < 0.05$) in their total Se concentration when they were exposed to Na_2SeO_4, Na_2SeO_3 and $BaSeO_4$ supplied to the soil (Table 5). A significant linear relationship was observed for both selenate-based fertilizers but not for the selenite-based fertilizers between Se application dose and plant Se concetrations (mg Se kg^{-1}).

Na_2SeO_3: (plant Se) = -8.14 + 22.97*(soil Se); r^2=0.47

Na_2SeO_4: (plant Se) = -316.66+233.31*(soil Se); r^2=0.76

$BaSeO_4$: (plant Se) = -128.80+93.10*(soil Se); r^2=0.77

The capability of the plants to accumulate Se was very high when they were grown on soil fertilized with Na_2SeO_4 and to a lesser extent when grown on soil fertilized with $BaSeO_4$. In these cases, Se concentrations reached 982±159 and 288±143 mg kg^{-1}, respectively, when applying a dose of 3.8 mg kg^{-1} soil. When the plants were grown on soil treated with Na_2SeO_3 only 103±33 mg kg^{-1} accumulated in the plants even at the highest dose (3.8 mg kg^{-1}). Thus, application of Na_2SeO_3 results in the lowest Se accumulation in the leek (Table 5).

Table 5. Effect of Se fertilizer type and dose on Se concentration (mg kg^{-1}) in leek plants (mean±standard deviation, n=3)

Se application dose	Fertilizer type		
	Na_2SeO_3**(%)	Na_2SeO_4**(%)	$BaSeO_4$**(%)
control treatment: 6.6±0.04			
0.2 mg kg^{-1} soil	24.1±5.2 (9.6)	102±24(39)	61.4±40(16)
1.3 mg kg^{-1} soil	49.7±63*(5.3)	313±29(38)	63.2±42(8.9)
2.6 mg kg^{-1} soil	71.2±13*(4.5)	582±72*(48)	255±61*(16)
3.8 mg kg^{-1} soil	103±33*(4.6)	982±159*(36)	288±143*(15)

*indicates statistically significant difference at $p \leq 0.05$ compared to the control leek (LSD); ** indicates ANOVA overall significance ($p \leq 0.05$). Total percentage Se uptake from applied Se dose in leek was presented between brackets.

3.4.3 Selenium speciation in the plants

Recoveries of total Se measured in the protease and protease:lipase enzymatic extracts were found to be 87.8 ± 5.4% and 79.8 ± 7.7% respectively, relative to total Se found in the HNO_3/H_2O_2 extracts. The obtained enzymatic extracts were measured using chromatographic methods which are optimized to quantify seven Se species (Figure 7 and 8). The sum of identified species ranges between 55 and 76% of the total Se present in the leek from two different enzymatic extraction methods (protease and protease:lipase). In the plants grown on soils treated with Na_2SeO_4 55.4 % of total Se in the plant was found to be protease extractable selenate and the most abundant protease-extractable organic species was SeMet (12.3 %), followed by MeSeCys (4.3 %) (Figure 9). When using $BaSeO_4$ as fertilizer, the main protease extractable species was selenate (47.8 %), followed by SeMet (13.9 %) and MeSeCys (3.7 %). When the soil was treated with Na_2SeO_3 the main protease extractable Se species in the plants were selenate (21.6%), SeMet (16.7 %) and MeSeCys (6.8 %) (Figure 9). Using enzymatic method (protease:lipase) showed a higher chromatographic recovery (10-15%). However, trends in relative amounts of inorganic and organic species in leek were similar when fertilized with different Se forms (data not shown). The chromatograms of enzymatic extracts of plants treated with Na_2SeO_4, Na_2SeO_4 and $BaSeO_4$ have two or three unknown peaks, the largest unknown representing approximately 2–3% of the total Se in the leek. A similar number of unknown peaks was identified on both anion and reversed phase columns. In all treatments, selenate, SeMet and MeSeCys were dominant species.

3.5 Discussion

Depending upon the oxidation-reduction potential of the soil, Se occurs in the soil mainly as selenate, selenite and organic forms (Mazzafera, 1998). Selenium plant uptake differs with the Se form available in the soil. In general, selenite is less bioavailable to plants in comparison to selenate because the former is more strongly adsorbed by iron oxides and/or hydroxides and the latter is more water-soluble (Banuelos et al., 2005). It was previously also identified that selenate is the predominant species when wheat plants were treated with selenate, whereas in selenite-treated plants the selenite was readily converted to other forms,

including SeMet and other organic forms (Li et al., 2008). The fact that total Se was higher in leek when the soil was treated with selenate forms illustrates that the uptake of selenate follows sulphur pathways from soil to plant (Liang et al., 1999). However, in case of selenite, translocation in treated leek was lower, possibly due to the uptake by plant roots being not metabolically dependent (Arvy, 1993).

In leek, Se is accumulated from selenate fertilizer to a larger extent compared to when selenite fertilizer is used. This indicates that more residual Se is left in soils after selenite application. The fate of this residual Se in soils is still unclear. To our knowledge no long-term studies, quantitatively reporting on the cycling of Se supplied through Se fertilizers and its impact on crop quality and the environment, exist yet. However, some studies already reported the trend of Se uptake by crops directly after the first harvest. For instance, in a pot study using different ryegrass cultivars (sodium selenite, 30 and 60 g ha^{-1}), Se uptake from the residual Se left in the soil was found to be similar in the next crop **(**Cartes et al., 2011**).** Moreover, also in laboratory experiments, it was also proven that residual selenate is adsorbed less to soil surfaces and leaches faster than selenite (Alemi et al., 1989). Moreover, some studies have also indicated that most of the applied selenate is converted to selenite, which is easily adsorbed by iron oxides and hydroxides in acidic soils (Cary et al., 1967, Geering et al., 1968, Christensen et al., 1989, Balistrieri and Chao, 1990).

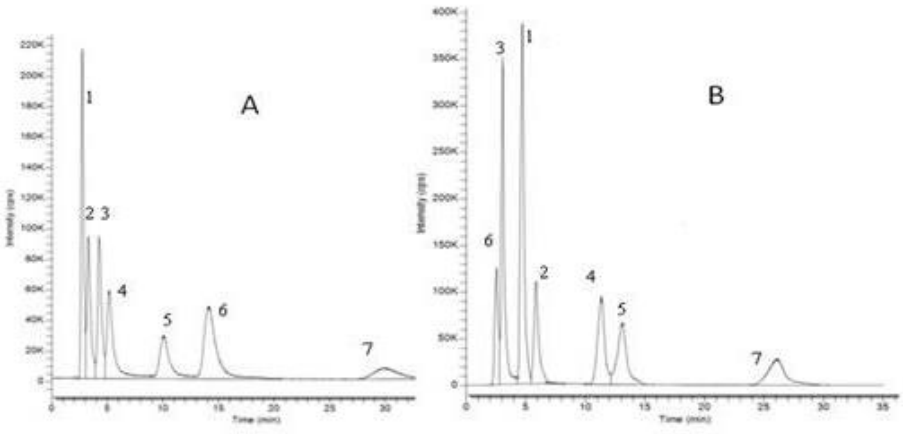


Figure 7. HPLC–ICP MS analysis of a mixture of 7 selenium species using (A) Anion exchange, (B) ion pairing reversed phase separation. 1. Se-cystine, 2. Se-methylselenocysteine, 3. selenite, 4. Se- methionine, 5. γ-glutamyl methyl selenocysteine, 6. selenate, 7. γ-glutamyl selenomethionine

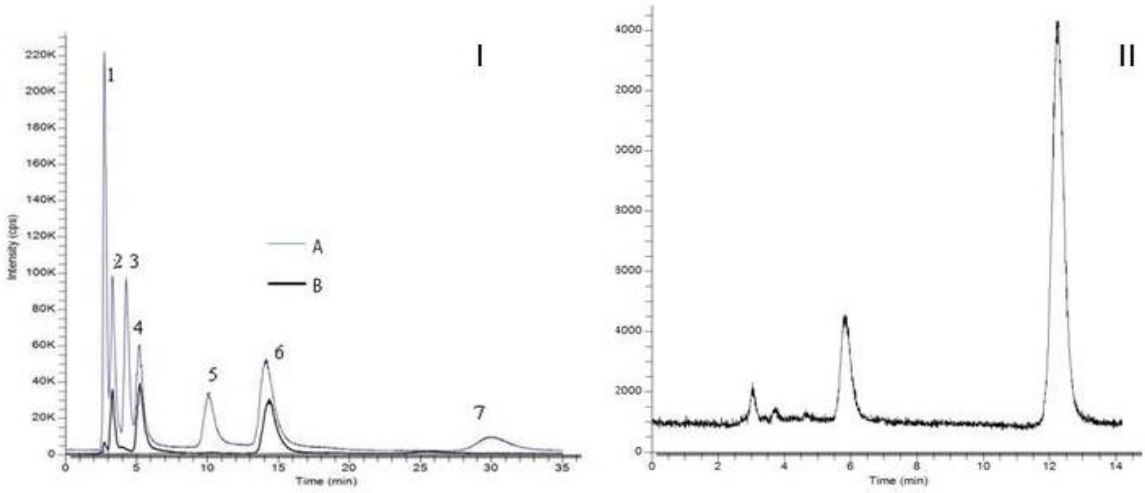


Figure 8. (I) Overlay of chromatograms of standard containing 7 Se-species anion exchange column (A) Reference standards mixture 1. Se-cystine, 2. Se-methylselenocysteine, 3. selenite, 4. Se-methionine, 5. γ-glutamyl methyl selenocysteine, 6. Selenate 7. γ-glutamyl selenomethionine (analyses conducted by HPLC-ICP-MS) (B) enzymatic (protease) extract of Se-enriched leek fertilized by Na_2SeO_3. (II). HPLC–ICP MS analysis of non-treated leek

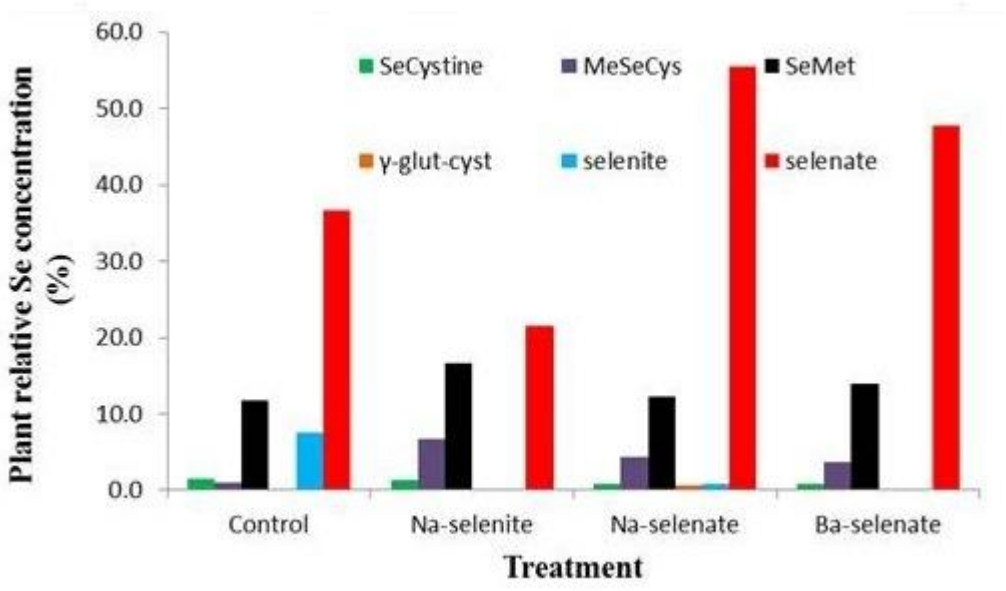


Figure 9. Sum of Se species in leek plants extracted using protease enzymatic extraction method grown on soils fertilized with Na_2SeO_3, Na_2SeO_4 and $BaSeO_4$ (3.8 mg Se kg^{-1} soil) and a non-fertilized control soil (expressed as % of total Se)

Finally, it is also already known that Se uptake from the soil depends on soil conditions, such as pH and organic matter, and addition of P and S inputs through manure and fertilizers, which may also change between different cropping seasons and affect the availability of the residual Se. This complicates assessment of Se cycling and the role of residual Se in longer-term field experiments.

Among the vegetables, *Allium* species draw particular attention due to their potential for Se accumulation and transformation into bioactive species (Larsen et al., 2006, Wróbel et al., 2004). In the present study, fertilization of soil with Se also resulted in accumulation of Se in leek, which also belongs to the *Allium* family. The extent of accumulation also significantly varied with the applied dose and Se form used in the fertilization, with the use of Na_2SeO_4 as fertilizer resulting in a 10-fold higher concentration in comparison to the use of Na_2SeO_3. Similar differences in Se accumulation after applying selenate versus selenite were previously also reported in literature for other *Allium* species, such as chives (Kapolna and Fodor, 2007, Kápolna and Fodor, 2007a). These differences previously seemed to be less pronounced when working in hydroponic solution, which emphasizes the role of soil in retaining selenite (Srivastava et al., 2005). In our study, the use of Na_2SeO_4 resulted in a higher Se concentration in the plants compared to when $BaSeO_4$ was applied at a similar Se dose. This supports findings of previous studies, in which $BaSeO_4$ was mentioned to act as a slower-releasing Se salt in comparison to Na_2SeO_4 (Whelan and Barrow, 1994). Amongst *Allium* species reported till now, higher Se concentrations were previously reported for garlic, but these higher concentrations were also reached at higher soil Se concentrations (Larsen et al., 2006). The prevalence of inorganic species in leek is similar to its prevalence in chives, which was reported to be 21% (when applying Na_2SeO_3) and 51% (when applying Na_2SeO_4) of total recovered Se (using the same extraction method). Higher extraction efficiency of Se from *Allium* species was reported in studies using protease enzyme.The majority of Se in these species is associated with proteins which could lead to higher extraction efficiency (70-100%) from total Se (Table 3). Similarly, in leek, a combination of protease and protease:lipase enzyme extracts about 85% of total Se (Kápolna and Fodor, 2007a). The results of current study achieve a similar range of extraction efficiency to that of earlier reported studies on *Allium* species. However, use of alternative enzymes might improve extraction efficiency of total Se present in leek.

In samples, Se species chromatographic recovery was approximately 10-15% higher in Tris-HCl enzymatic extracts than in the extract obtained by water enzymatic extracts. This result

suggests that pH which is more stable in Tris buffer extracts increases the chromatographic sensitivity. In leek, the highest amount of inorganic species was observed in the plants treated with Na_2SeO_4. Under these conditions, only little transformation to organic species occurred, which was previously also reported for various other food crops (de Souza et al., 2000). In contrast, applying selenite resulted in a higher fraction of organic Se species. However, it results in lower plant uptake, so also a higher risk for Se accumulation in the soil on longer term. Selenite concentrations were found to be very little to below detection limits, also in plants grown on Na_2SeO_3 treated soils. A higher prevalence of selenite was previously reported for garlic grown on a selenate-enriched medium and chives grown on a selenite-enriched medium (Kápolna et al., 2007b). Both MeSeCys and SeMet were the major organic species in all three treatments. MeSeCys was found in higher concentrations when the soils were treated with Na_2SeO_3. This species was previously reported to exhibit potential anticarcinogenic properties (Ip et al., 1995).

3.6 Conclusion

Leek accumulates Se and responds to Se fertilization as other *Allium* plant species do. Therefore, Se-enriched leek can also be considered as a food crop that may induce beneficial health effects. Total Se accumulation in the plants is higher when Na_2SeO_4 is supplied as fertilizer but this result in little transformation to organic species. When aiming to increase the fraction of organic Se species in leek, Na_2SeO_3 seems to be the best fertilizer. However, the lower plant uptake when using Na_2SeO_3 as fertilizer results in a higher risk for Se accumulation in the soil on longer term. Therefore, more research is needed to assess the factors affecting plant uptake and fate of selenite in the soil.

Chapter 4. Potential of selenite as fertilizer to biofortify leek (*Allium ampeloprasum* var. *porrum*): a field study

4.1 Abstract

The aim of this study was to investigate how yield and uptake of elements (Se, Pb, Al, Zn, Cu, Fe and Mn) in leek plants are affected by use of selenite as fertilizer under field conditions, and how the uptake is affected by soil properties. Leek was cultivated in 26 different fields across Flanders (Belgium). Soil samples were collected prior to cultivation of the leek to assess soil properties and metal concentrations. In each study field, a subplot of 50×50 m^2 was chosen. In each subplot, half of the plot was considered as control and on the other half, Se fertilizer was applied. Fully grown leek plants were harvested after four months, and elemental concentrations were analysed in the control as well as Se fertilized plants. No significant effect of Se fertilization on yield was observed. The Se concentration in leek significantly increased with Se fertilization. The percentage of applied Se taken up by the leek plants ranged from 0.2 to 4.8 %. Among all metals analysed, a significant increase in Se concentrations was observed upon Se fertilization, whereas no significant effect was observed for the other metals. Soils grouping based on soil characteristics and other soil parameters (using PCA) shown significant differences in Se uptake between groups.

4.2 Introduction

Most of the essential nutrients are obtained through the human diet (Parr et al., 2006). Soils play a major role in providing these essential nutrients to the food chain by delivering nutrients to the food crops. Even though some nutrients are present in adequate amounts in soils, they can be poorly available to plants due to soil conditions affecting their availability, such as organic matter content, clay, pH etc. (Gissel-Nielsen, 1971, Johnsson 1991). A too low uptake of some essential nutrients through food crops may result in adverse health effects such as cardiovascular diseases, inflammatory bowel diseases and anaemia (Prasad, 2009). Biofortification, i.e. fortification of food crops with essential nutrients (Hotz and McClafferty, 2007) as well as nutritional supplements were proposed as alternatives to

overcome deficiencies of essential nutrients (Hotz et al., 2007) and to induce positive health effects in humans and animals (Welch et al., 1999).

Among other nutrients, selenium (Se) is an essential micronutrient, known for its antioxidant and potential anticarcinogenic properties by enhancing the glutathione peroxidase activity (Brown and Arthur, 2001). However, the geographical distribution of Se in soils is uneven. A majority of countries has Se deficient soils leading to Se deficiency in the population. Therefore, use of Se fertilizers to obtain Se-fortified crops was recommended in various countries and the Se status in Se deficient regions was improved by adding Se fertilizers to soil on which crops were grown (Brown et al., 2001). Use of inorganic Se forms as Se fertilizers could aim at obtaining organic Se forms in the food crops (Pyrzynska, 2009). Some food crops, including *Allium* and *Brassica* species, do not only accumulate considerable amounts of Se but also convert inorganic Se forms into organic forms such as MeSeCys and γ-glut-cyst, which were reported to have anticarcinogenic properties (Dumont et al., 2006a, Larsen et al., 2006, Wróbel et al., 2004). Supply of selenite was found to result in a higher relative occurrence of these organic species in the crop compared to the supply of selenate (Kapolna et al., 2007). The accumulation of Se in food crops is not only affected by plant parameters but also influenced by soil properties, trace metals and Se forms available in the soil (Gissel-Nielsen, 1971, Johnsson, 1991, Landberg and Greger, 1994). In addition, soil parameters such as pH, organic matter, and Fe-oxide contents were found to affect the mobility of Se in soil (Johnsson, 1991).

Although the response of different *Allium* and *Brassica* crops to Se fertilization was previously studied under laboratory and greenhouse conditions, there is a lack of field studies focusing on the effects of Se fertilization on these crops, and there is a need to define relationships between Se supply, soil properties and plant uptake of Se, and other toxic or essential trace elements. This can only be done by studying trace and other element uptake by crops grown on several field plots differing in soil physicochemical properties.

Therefore, this study aimed to: (1) examine the variability of biomass growth, Se and trace element uptake by leek crops grown on different field plots across the Flanders region (Belgium), (2) study the effect of fertilizing the soils with selenite on biomass growth, uptake of Se and other elements by the leek crops, and (3) study the effect of soil properties on Se uptake by the crops.

4.3 Materials and Methods

4.3.1 Experimental setup

Twenty-six fields were selected across the Flanders region in Belgium (Figure 10) during the year 2009. In each field 1.5 m wide beds were raised using a tractor to improve soil drainage. On sandy soils flat beds were used, whereas ridge beds were installed on other soils (Figure 11). Each bed accommodates four plant rows, 40 cm apart, with spacing of about 20 cm between the plants in different rows. The Se fertilization plot was located in a representative subpart of each field, on an area of approximately 50 x 50 m^2. From this 50 x 50 m^2, half was considered as control and the other half was used to apply Se fertilizer. Leek plantlets were planted manually in 10 to 15 cm deep holes. In each plot, one of two commercially available varieties (Harston or Poulton) was planted. After planting, each plantlet from the Se fertilizer plot was fertilized with 0.5 mg Se as sodium selenite supplied through a 60 mL solution with a disposable syringe. Control plants were supplied with 60 mL deionized water. In the autumn, leek was harvested manually from the Se fertilized and non-fertilized plot over a length of 3 meters. The whole plants, including the root system, were harvested and washed thoroughly before being weighed for yield determination. From each of the Se-fertilized plots and non-fertilized plots, three plants were dried in an oven at 45 ºC. The three plants were ground together and considered as one composite sample for further analysis.

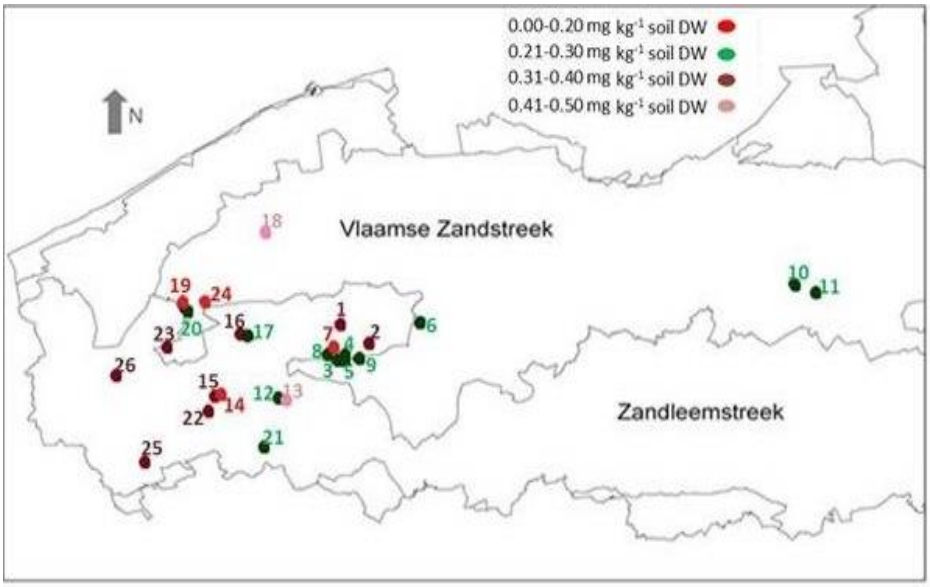


Figure 10. Selenium in soils at the studied field sites in Flanders, Belgium

4.3.2 Soil sampling

Soil samples were collected from the Se-fertilized plots on each site. The samples were analysed for various soil properties and total metal concentrations. Soil sampling took place in spring (April-May) before fertilization and soil tillage. In each plot, 12 soil cores were augered in a grid pattern to a depth of 30 cm, and bulked into one composite sample. The soil samples were air-dried, ground with a hammer-cross beater mill and stored in air-tight polythene bags until analysis.

4.3.3 Soil and plant analysis

Various soil parameters such as pH-KCl, organic matter (OM), cation exchange capacity (CEC) and electrical conductivity (EC) were determined according to Van Ranst et al. (1999). Elemental concentrations were analysed in the soil samples after aqua regia extraction (Van Ranst et al., 1999), whereas plant samples were digested using a digestion procedure with HNO_3 in open microwave vessels described by Lavu et al. (2012). In the plant

and soil extracts, Se, Cd and Pb were determined using ICP-MS (PerkinElmer DRC-E, Waltham, MA, USA). The other metal contents (Cr, Cu, Mn, Ni, Pb, Zn, Al, Fe, Ca, and Mg) in the plant extracts and soil extracts obtained through aqua regia extraction were determined using ICP-OES (Varian Vista MPX, Palo Alto, CA, USA). In addition, soil extractable P (ammonium lactate extracts) and S ($CaCl_2$) were determined using ICP-MS.

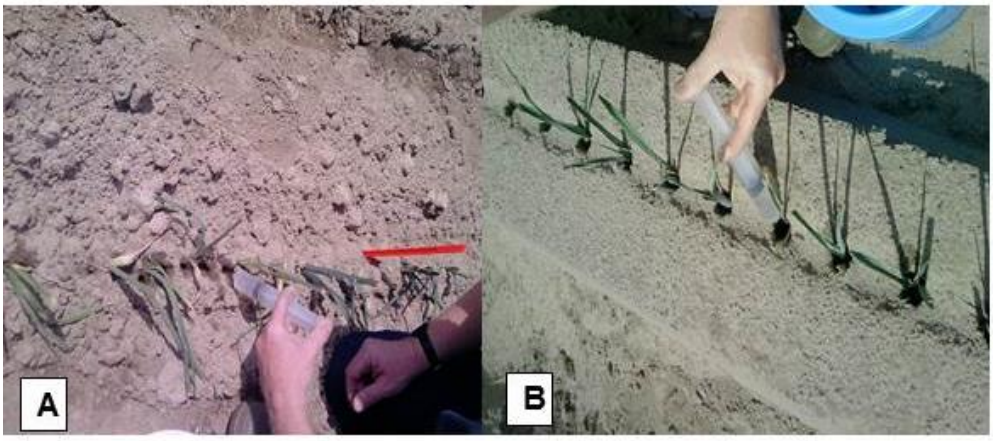


Figure 11. Selenium fertilization conducted for the two different types of leek cultivation (1A. Flat type, 1B. Ridge type)

4.3.4 Statistical analysis

The differences in metal uptake by leek between the non-fertilized group and the Se-fertilized group were analysed with a paired-sample t-test ($p < 0.01$). A correlation matrix (Pearson's correlation) was constructed to assess relations between metal concentrations and metal uptake in the plants, soil parameters (pH, OC, EC, CEC, sand, clay) and elements in the soils (Cr, Cu, Mn, Ni, Pb, Zn, Al, Fe, Ca, Mg, and extractable P and S). Subsequently, a stepwise multiple regression analysis was conducted with the aid of SPSS 21.0 software package. Data distributions were skewed and transformed prior to analysis using either a log10 or quadratic function to improve normality. The categorical variables as ''dummy'' variables 0 or 1 were assigned to each plant variety and cultivation type. For example, to test whether plant variety influenced plant metal uptake, all Harston variety samples were given the indicator ''H = 1'', while all Poulton variety samples were assigned ''P = 0''. This was done to assess the

influence of plant variety and cultivation type on the relationship between soil parameters and accumulation of other essential and non-essential nutrient accumulation. In addition, using principal component analysis (PCA), two sets of groups were created based on soil properties, and differences in Se uptake between the soil groups were evaluated using ANOVA. A first grouping was conducted based on soil characteristics (sand, clay, Mn, Ca, Mg, Fe, OC, pH and HWC) and a second grouping was conducted based on parameters which were previously reported to affect Se uptake by plants (count of aerobic Bacteria , EC, CEC, Se, OC, pH, extractable S and P).

4.4 Results and Discussion

4.4.1 Soil Se and other element concentrations

Soil Se concentrations ranged between 0.16 and 0.46 mg Se kg^{-1} across Flanders (Appendix 1). In general, total soil Se contents of 0.10 to 0.60 mg kg^{-1} are considered to reflect Se deficient soils, i.e. soils which induce Se deficiency in humans living in the region due to the lower Se contents in the food crops grown on the soils (Rayman, 2000). Selenium deficient soils were previously reported to occur in e.g. New Zealand, Denmark and the Atlantic Region of Canada (Gupta et al., 2010). The soils under study can thus also be considered as Se deficient. This motivates the need for Se fertilization, which is already in practice in various other countries such as New Zealand, Finland and to a limited extent in China, the United States, and Canada (Gupta et al., 2010). The majority of our study fields contained soil Se concentrations ranging between 0.21 and 0.30 mg Se kg^{-1} DW (46%). Only two fields contain more than 0.41 mg Se kg^{-1} DW. Other trace metals and nutrients were also analysed (Table 6 and Appendix 1 & 2). Soil properties, which were previously reported to affect Se uptake by food crops, are presented in Table 7.

Table 6. Range of essential and non-essential element concentrations (mg kg^{-1}) in the studied soils before Se application

Element	mg kg^{-1}
Cr	9.1 – 31.3
Cu	11.1 – 54.6
Cd	< 0.5
Mn	100 – 428
Ni	4.1 – 12.4
Pb	9.3 – 149
Zn	31.3 – 82.5
Al	5328 – 13075
Fe	4679 – 19905
Ca	5483 – 1068
Mg	428 – 2265
Se	0.2 – 0.5

4.4.2 Response of leek to selenite fertilizer

An increase in plant Se concentrations following soil Se application was reported in various studies (Broadley et al., 2006, Chilimba. et al., 2012, Ip et al., 1995). Also in our study, Se applied to the soils in the form of selenite in an aqueous solution was found to increase Se concentrations in leek. Selenium concentrations in non-fertilized plants ranged from 0.04 to 0.17 mg Se kg^{-1}, whereas they varied between 0.08 and 0.68 mg Se kg^{-1} in plants grown on Se-fertilized soils (Table 8 and 9). The Se uptake in the field experiment, 0.3-4.9% from the applied dose, was lower compared to the pot experiment (4.5-9.6%), although the biomass production in the pot experiments was lower. In the pot experiment, Se fertilizers were thoroughly mixed with soil and the plants were grown in pots that were closed at the bottom. This may induce a higher Se availability in the root zone due to lower Se leaching (Brenda

and Robert, 1997). In the field study, Se was added near to the root zone and Se have have leached from this zone, reducing the effectiveness of Se uptake.

Table 7. Soil properties, leek planting system and leek variety used on the selected field plots

Field No.	pH-KCl	OC (%)	EC (mS cm^{-1})	CEC (cmol/kg)	Planting system	Leek variety*
1	5.4	0.7	0.1	7.5	Ridge	H
2	6.3	0.9	0.2	7.2	Ridge	P
3	5.5	0.7	0.1	6.4	Ridge	H
4	5.1	0.6	0.2	5.7	Ridge	H
5	5.6	0.8	0.2	6.7	Flat	P
6	7.2	1.2	0.3	8.8	Flat	P
7	6.0	1.1	0.2	7.9	Ridge	H
8	5.9	1.1	0.2	7.1	Ridge	P
9	5.3	0.6	0.1	8.7	Ridge	H
10	6.9	2.5	0.2	11.5	Flat	H
11	5.3	1.6	0.1	6.2	Flat	H
12	6.6	0.9	0.3	8.4	Flat	H
13	7.1	1.1	0.3	8.3	Flat	P
14	6.4	1.4	0.5	12.7	Flat	P
15	6.0	1.4	0.6	12.0	Flat	P
16	7.1	1.3	0.4	9.9	Ridge	H
17	5.6	2.0	0.3	11.4	Ridge	H
18	4.8	1.7	0.3	11.2	Flat	H
19	5.5	1.5	0.3	8.0	Ridge	P
20	5.5	0.9	0.2	6.8	Ridge	H
21	6.4	1.0	0.6	9.0	Flat	P
22	6.9	1.7	0.3	13.4	Flat	P
23	6.1	0.8	0.3	9.9	Ridge	H
24	5.8	1.1	0.2	7.2	Flat	P
25	4.8	2.3	0.3	15.8	Flat	H
26	6.6	1.8	0.4	10.4	Flat	H

* Leek variety: H: Harston; P: Poulton

Table 8. Elemental concentrations in plants grown on non-Se-fertilized soil (mg kg^{-1} DW)

Field No.	Cu	Cd	Mn	Pb	Zn	Fe	Al	Se
1	5.5	0.11	27	0.52	27	123	88	0.08
2	5.5	0.09	25	0.33	30	154	100	0.17
3	6.8	0.28	55	0.46	38	222	136	0.08
4	7.4	0.13	20	0.41	35	120	59	0.11
5	7.1	0.09	48	1.50	31	544	484	0.20
6	6.9	0.08	31	0.77	31	532	298	0.06
7	5.9	0.11	30	0.58	41	278	198	0.11
8	6.1	0.07	17	0.38	38	97	64	0.09
9	7.2	0.17	20	0.40	32	167	109	0.07
10	4.8	0.14	27	1.48	36	92	43	0.17
11	5.6	0.23	33	0.53	65	70	26	0.04
12	6.3	0.09	23	0.35	30	113	66	0.10
13	5.3	0.10	23	0.56	22	225	164	<0.01
14	5.4	0.14	32	0.73	33	375	200	0.04
15	6.1	0.06	26	0.53	34	231	149	0.04
16	6.5	0.07	17	0.37	28	209	116	<0.01
17	5.9	0.04	19	0.61	24	299	191	0.09
18	4.9	0.21	38	0.40	44	263	144	0.04
19	6.1	0.08	20	0.41	34	189	127	0.05
20	5.1	0.07	23	0.51	26	222	183	0.08
21	5.0	0.19	26	0.86	28	186	165	0.04
22	5.2	0.11	23	0.85	27	340	177	0.16
23	4.9	0.10	15	0.22	20	109	100	0.07
24	5.8	0.07	17	0.30	32	100	59	0.05
25	6.7	0.17	19	0.73	26	654	177	0.09
26	6.5	0.09	11	0.37	34	257	227	0.14

Table 9. Elemental concentrations in leek grown on Se-fertilized soil (mg kg^{-1} DW of Se also the percentage of Se applied to the soil taken up by crop is reprensted between brackets.

Field No.	Cu	Cd	Mn	Pb	Zn	Fe	Al	Se (% taken up by the crop)
1	5.6	0.10	28	0.44	34	198	152	0.68 (2.1)
2	6.7	0.17	32	0.55	40	232	182	0.40 (1.7)
3	9.7	0.23	47	0.23	37	109	78	0.31 (4.2)
4	7.2	0.14	25	0.37	37	108	63	0.68 (1.3)
5	8.1	0.12	42	0.51	39	233	201	0.34 (1.5)
6	5.7	0.06	26	0.6	28	503	317	0.32 (1.8)
7	5.8	0.11	32	0.42	28	144	113	0.36 (3.5)
8	5.5	0.05	19	0.4	36	105	80	0.42 (1.5)
9	6.9	0.11	18	0.37	25	189	131	0.27 (0.6)
10	4.8	0.13	33	3.08	32	323	197	0.24 (0.2)
11	6.3	0.44	54	0.66	95	100	58	0.08 (4.5)
12	4.9	0.07	18	0.24	23	110	81	0.66 (1.5)
13	5.0	0.09	22	0.44	19	143	142	0.14 (1.6)
14	5.4	0.13	28	1.28	39	588	393	0.21 (0.5)
15	4.1	0.09	31	0.98	30	559	380	0.10 (1.7)
16	6.8	0.09	18	0.4	31	205	139	0.24 (1.9)
17	6.2	0.04	19	0.41	26	211	149	0.44 (0.4)
18	4.5	0.16	35	0.52	49	225	137	0.12 (1.4)
19	6.1	0.17	22	0.45	48	296	208	0.24 (1.0)
20	5.3	0.23	23	0.37	29	127	98	0.24 (4.9)
21	5.6	0.12	25	0.63	27	187	179	0.44 (2.0)
22	5.6	0.08	32	0.3	31	998	598	0.44 (1.6)
23	5.0	0.16	15	0.43	21	104	89	0.34 (2.3)
24	5.0	0.11	23	2.3	29	120	85	0.24 (1.3)
25	7.0	0.09	23	0.22	23	376	371	0.33 (0.3)
26	6.6	0.07	11	0.36	24	325	373	0.19 (2.1)

4.4.3 Differences in Se uptake by leek as affected by soil characteristics

Based on soil characteristics, soils were divided into different groups (Figure 12). A first grouping was conducted based on soil characteristics (sand, clay, Mn, Ca, Mg, Fe, OC, pH and HWC) and a second grouping was conducted based on parameters which were previously reported to affect Se uptake by plants (count of aerobic Bacteria, EC, CEC, Se, OC, pH, extractable S and P).

The PCA based on soil characteristics resulted in four soil groups (Figure 12.I). Similarly, the PCA based on parameters which were previously reported to affect Se uptake by plants resulted in three groups (Figure 12.II). Differences in Se uptake between these soil groups were evaluated. No significant differences in Se uptake by leek plants fertilized with Se were observed between the groups. However, in non-fertilized leek, significant differences in Se uptake were found between group A and group C when grouping is based on general soil characteristics (Figure 13.I). This was also the case when grouping was based on soil parameters expected to affect Se uptake (Figure 13.II) ($p \leq 0.05$).

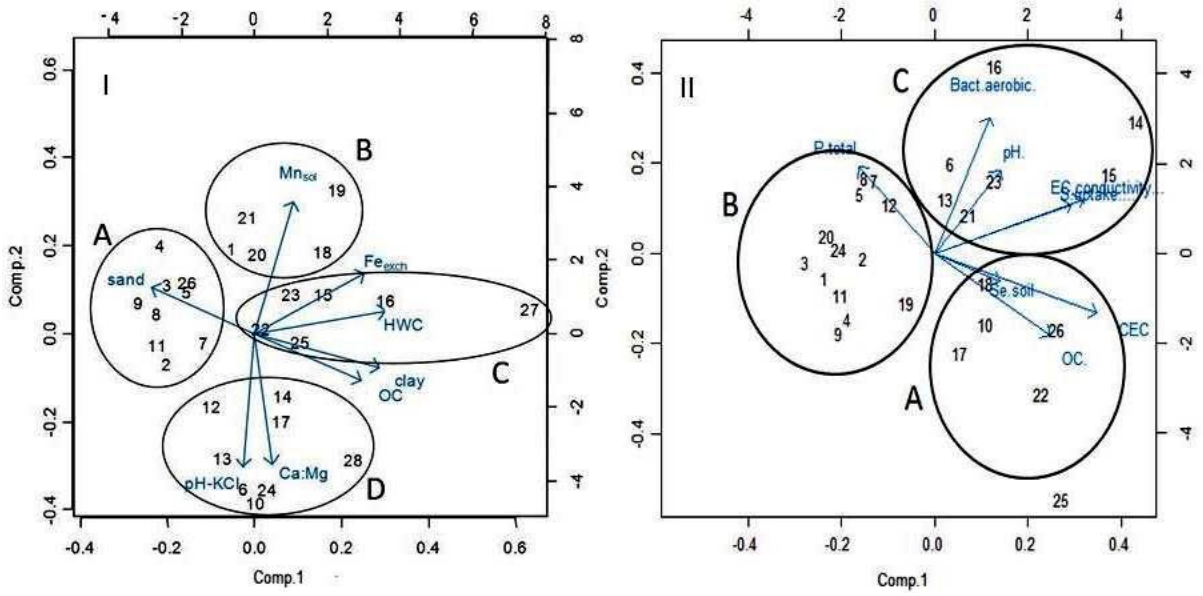


Figure 12: Grouping of study plots using principal component analysis. I: Based on soil characteristics. II: Based on other soil parameters

Leek plants grown in plots with high sand content (Figure 12I - group A) accumulate higher Se contents compared to soils having more clay and organic carbon (Figure 12I -group C). Although the sand content of group C and D is significantly different from group A, Se

uptake in plants grown on soils from group D is not significantly different from plants grown on soils from group A. The higher pH in soils of group D may have facilitated the accumulation of Se in plants grown on soils of this group. The results clearly illustrate that soils with higher organic carbon content decrease Se uptake by plants (Borowska and Koper, 2011). When soils are grouped based on parameters that are supposed to affect Se uptake, a clear difference in Se uptake was seen between group A and group C (Figure 12.II, Figure 13) ($p \leq 0.05$). Even though soils from group A contain more OC, the leek plants may have accumulated more Se due to slightly higher Se contents in soils of this group. In addition, soils from this group contain lower available P content compared to soils from group B and C. Earlier studies reported a decreasing Se uptake by plants with increasing P content in soils due to competition between P and Se for plant uptake (Hopper and Parker,1999; Broyer et al., 1972). On the other hand, phosphate is also considered to lead to desorption of selenite ions bound to minerals in the soil, as it is bound more strongly to trivalent iron and aluminium compared to selenite (Liu Q et al., 2004; Nakamaru et al., 2006). However, in our study, lower extractable phosphorus in soils of group A tends to be related to higher Se uptake by the plants, which may indicate presence of other Se forms or the dominant role of competition between Se and P for plant uptake. Count of aerobic Bacteria is significantly higher in group C compared to group A, which is an indicative measurement of prevailing aerobic conditions in these soils. Aerobic conditions are supposed to induce selenate formation, leading to a higher Se uptake by the plant, which is however not in agreement with our observations.

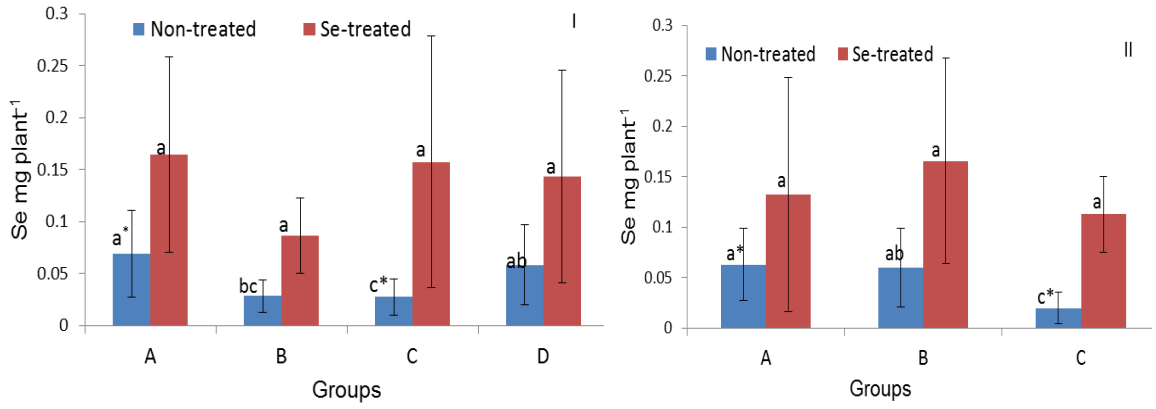


Figure 13. Se uptake by leek plants grown on soils grouped by PCA (Figure 12). I: Groups based on soil characteristics. II: Groups based on other soil parameters considered to affect Se uptake; a*, c* indicates statistical differences between two groups LSD ($p \leq 0.05$).

4.4.4 Accumulation of elements in leek plants and factors influencing their uptake and concentrations

Selenium uptake was not significantly different between the two different plant varieties, i.e., Harston and Poulton ($p>0.05$). The differences in uptake and concentrations of metals between non-fertilized leek and Se-fertilized leek were significant ($p \leq 0.05$) based on the paired-sample t-test. However, uptake and concentrations of other trace metals were not significantly different between fertilized and non-fertilized plants. No significant differences in crop yield (on dry matter basis) were found between fertilized and non-fertilized leek (($p>0.05$). The observed differences in yield between a Se fertilized and the adjacent non-Se fertilized plot were considered to be non-existing. The uptake of Se and other metals (expressed as mg m^{-2}) was calculated considering equal yield values for fertilized and non-fertilized plots. Therefore, the metal uptake from a non-fertilized plot on a certain field was calculated based on the dry matter yield of the respective Se fertilized plot.

The results of step-wise multiple regression analysis for the Se concentrations and uptake by the plant are shown in Table 10. Out of the various soil parameters simultaneously entered into the model, only OC was significant in influencing plant Se. It explained 42% of the concentrations and uptake of Se in Se-fertilized leek plants, with the correlation being negative (Table 10). Selenite is very well bound to soil organic carbon which could have decreased Se uptake by the plants (Gibson et al., 2012). In addition, stepwise linear multiple regression was applied to find the dominant soil parameters influencing uptake of other elements and their concentrations in the plants. Total metal concentrations in soil were found to be the main factor, being correlated positively with metals in the plants in most cases. Interestingly, most of the cationic metals show positive correlations with other elements in the plants, such as Al, Ca, Ni and Mg (Table 10). Such relations were reported earlier already in other studies. For example, Mg increases with increasing Fe concentrations in soybeans, and Ca is affected by Al and other polyvalent cations (Clarkson and Sanderson, 1971, Lingle et al., 1963). The extractable P in soils shows a negative correlation with Al concentrations in the plants and a positive correlation with the uptake and concentration of Zn. In addition, soil pH and CEC correlated negatively with Zn in the plants and with Pb uptake. However, no

significant differences in metals between non-fertilized plants and Se-fertilized plants were observed based on the independent samples t-test, except for Se and Al. The parameters influencing the metal uptake are different between non-fertilized plants and Se-fertilized plants (Table 10).

Table 10. Results of stepwise linear multiple regression analysis

Element	Regression equation		% R^2	
	Non-treated (NT)	Se-treated (ST)	NT	ST
Regression equation based on uptake of metals by leek (mg m^{-2}):				
Se	NA	Se (p) = 0.243-0.078 OC	NA	42.0
Cu	NA	NA	NA	NA
Mn	NA	NA		
Pb	Pb (p) = -0.150+0.110 soil Ni-0.003 $(CEC)^2$	*Pb (p) = -0.013+0.012 Pb(s)	65.7	69.6
Zn	* Zn (p) = 14.202+15.711 P uptake	* Zn (p) = 14.294+35.715 $(P\ uptake)^2$	58.0	68.3
Fe	Fe (p) = -15.780+0.169 Mg (s)+0.001 Al (s)	Fe (p) = -119.953+0.232 Mg (s)	72.7	73.5
Al	Al (p) = 71.844+0.005 Ca (s)	Al (p) = -71.811+0.157 Mg (s)	51.6	74.5
Regression equation based on concentration of metals in leek (mg kg^{-1} DW):				
Se	NA	Se (p) = 0.354-0.442 log OC	NA	41.9
Cu	NA	NA	NA	NA
Mn	Mn (p) = 0.166+0.003 Al (s)	NA	46.0	NA
Pb	Pb (p) = -0.016+0.002 Mn (s)+0.068 $(OC)^2$	*Pb (p) = 0.059+0.018 Pb (s)	71.7	77.6
Zn	Zn (p) = 72.069+30.604 P uptake-59.903 log pH	Zn (p) = 112.232+46.959 P uptake-114.758 log pH	63.7	62.4
Fe	Fe (p) = -33.184+0.184 Mg (s)+0.002 Al (s)	Fe (p) = -181.993+0.363 Mg (s)	75.1	75.2
Al	Al (p) = 155.384-424.498 $(P\ uptake)^2$+0.006 Ca (s)	*Al (p) = -95.291+0.202 Mg (s)-222.161 log OC	61.6	83.6

*Variety of leek (Harston and Poulton) shows a significant influence and is included in the regression equation, NA: $P > 0.05$, p: plant, s: soil

Elemental concentrations in plants grown on Se fertilized and non-fertilized soils are summarized in Table 8 and 9. Some studies reported that Se application in soils could also lead to a decrease or increase in other trace metals (Feroci et al., 2005, He et al., 2004, Landberg et al., 1994). In our study, the Pb concentration of leek grown on the majority of the study fields was higher than 0.3 mg kg^{-1} DW in non-fertilized and Se-fertilized leek which is the general limit for vegetables (Codex alimentarius commission (FAO/WHO)., 2001) (Table 8). In three Se fertilized plots, the leek contains 1-3 mg kg^{-1} DW Pb. The obtained results do not confirm findings of He et al. (2004), who reported for Chinese cabbage and lettuce that fertilization with 1 mg Se kg^{-1} selenite leads to a decrease in Pb concentration. In addition, Al in leek plants was higher in the majority of the study fields when plants were grown on Se-fertilized plots compared to plants grown on non-fertilized plots. An earlier study reported that Se fertilization leads to a decrease in Al concentrations in *Stylosanthes humilis*. However, the Se form used in this study was selenate (0.1 µM). The results of current study show that Se application may increase Al concentrations in plants when selenite is used as fertilizer at 0.5 mg Se per plant. For the other metals (except Se), concentrations in the plants did not differ significantly between Se fertilized and non-fertilized plots.

4.5 Conclusion

The soils in Flanders (Belgium) can be considered as Se deficient, leading to Se deficiency in humans, and hence there may be a need for Se fertilization. Such fertilization increases Se concentrations in leek when selenite is used in an aqueous solution at a dose of 0.5 mg per plant. The two leek varieties used in the study fields show no significant difference in Se uptake. Moreover, no differences in dry biomass yield were observed between plants grown on different Se fertilized soils. Among the metals tested, only Se concentrations in the plants showed a significant response to Se fertilization. In leek significant difference in Se uptake was observed based on soil characteristics and soil parameters. A clear indication of decreasing Se uptake with soil organic carbon and phosphorous content was observed. On the other hand, Se uptake increased with soil pH and sand content in non-fertilized leek; such effects were less pronounced in Se fertilized leek.

Chapter 5. Selenium accumulation in leek (*Allium ampeloprasum* var. *porrum*): role of genetic variation

5.1 Abstract

Twenty leek cultivars were tested under field conditions for their ability to accumulate Se from the soil after application of liquid fertilizers containing Se as selenate or as selenite. The Se doses were 0.5 and 2 mg Se per plant in the first year and 4 mg Se per plant in the second year. Fully grown leek plants were harvested and dry weight contents and Se concentrations were analysed to evaluate whether type or dose of Se fertilizer influences the biomass production and Se uptake by the leek, and how this is affected by genetic variation. In the current study, the main focus was on the consumable part of leek, i.e. the white belowground part. Leek fertilized with selenate was found to accumulate higher Se concentrations in its consumable part. This was the case for all leek cultivars tested. At rates of 4 mg Se per plant, up to 51% of the Se added as selenate was taken up by the plant, whereas only up to 4% was taken up when Se was added as selenite. The biomass was lower in the majority of leek cultivars that were fertilized with selenate compared to these fertilized with selenite. When using selenite fertilizers, the Se concentration in the leek increases 4-5 times, whereas it increases 10-15 times when using selenate fertilizers. Significant differences in Se uptake were observed between the cultivars. Six cultivars were selected as superior cultivars, accumulating more than 40% of the Se applied as selenate.

5.2 Introduction

Increasing attention is paid to Selenium (Se) in human and animal health as it is an essential component of several proteins such as the antioxidant enzyme glutathione peroxidase (GSH-Px) as well as other Se-containing enzymes, including iodothyronine deiodinases, thioredoxin reductase and selenoprotein W (Birringer et al., 2002, Pallud et al., 1997). However, Se is also toxic at higher doses and the concentration range between its requirement and its toxicity is relatively narrow. Selenium deficiency in diet leads to a decline in blood plasma concentrations, which was previously reported to occur in areas of Australia, China, Finland, New Zealand, North America and Sweden (Gissel-Nielsen et al., 1984, Gupta and Gupta,

2000, Hartikainen, 2005b). To overcome Se deficiencies in entire human populations, growth of food crops on Se fertilized soils was proposed in various countries, such as the UK, Finland and Malawi (Broadley et al., 2006, Chilimba, Allan et al., 2012, Eurola et al., 2003). In this context, it should be noted that Se is not considered as an essential element for plants, and at higher Se doses, a decrease in plant growth was previously reported (Kopsell and Randle, 1997, Sharmaet al., 2010). Among the vegetables, some authors previously focused on species of the *Allium* and *Brassica* families (Dumont et al., 2006a, Ip et al., 2000, Larsen et al., 2006, Wróbel et al., 2004), as the Se accumulation potential of these families is higher compared to other vegetables. Several *Allium* vegetables have already been investigated for accumulation of Se (total content) and its individual species (Pyrzynska, 2009) and the Se uptake was reported to vary among the different species. However, leek (*Allium schoenoprasum*) has not been extensively studied before. Moreover, little attention was paid to the effects of genetic variability on Se accumulation, i.e. differences between the cultivars.

Leek is used as a vegetable in many parts of America, Asia and Europe. It is rich in flavor (Eric et al., 1992, Mondy et al., 2002) and has higher antioxidant capacity than tomato, cauliflower and cucumber, but less than spinach, broccoli and red cabbage (Bernaert et al., 2012, Fattorusso et al., 2001, Paganga et al., 1999). The usage of leek in sausages enhances the quality during storage (Madentzidou et al., 2012). Its richness in soluble plant fibers helps to reduce adherence of diarrhoea-associated pathogens to intestinal epithelial cells (Simpson et al., 2012). Leek also contains significant levels of lutein, β-carotene, vitamin C and vitamin E (Hart and Scott, 1995, Proteggente et al., 2002). Various leek varieties are commercially available; among them, F1 hybrids are gaining popularity due to their higher yields and improved uniformity compared with open-pollinated cultivars. A recent study on 30 different leek cultivars showed differences in the antioxidant capacity between the cultivars, which may be related to variability in Se uptake between these different cultivars (Bernaert et al., 2012). In the present study, 20 different leek cultivars were selected and the Se uptake in their consumable parts was evaluated. Therefore, a liquid Se fertilizer was added to the rhizosphere of the plantlets. Two different Se doses of 0.5 and 2.0 mg Se per plant (selenite) were tested in the first year and, two different Se forms (selenate and selenite) at 4.0 mg Se per plant were evaluated in the second year.

5.3 Materials and methods

5.3.1 Experimental setup and sample collection

Twenty seven leek (*Allium ampeloprasum* var. *porrum*) cultivars were studied (Table 11). Leek seeds were obtained from the collection of the Institute for Agricultural and Fisheries Research (ILVO, Merelbeke, Belgium). The seeds of the leek cultivars were sown in a greenhouse in March and the plantlets were transferred to the experimental field plots of ILVO (Merelbeke, Belgium) between May and June. This was done in the year 2010 and 2011. Properties of two soil samples collected in both years are presented in Table 12.

The plantlets were planted into holes made in the soil. This was repeated two times in separate rows (Figure 14) with 15 plants. In the year 2010, three plants were fertilized with 0.5 mg Se per plant applied as selenite, three plants were fertilized with 2.0 mg Se per plant applied as selenite and the remaining plants were left non-fertilized (control). Similarly, in the year 2011, three plants were fertilized with 4 mg Se per plant applied as selenite, three plants were fertilized with 4 mg Se per plant applied as selenate and the remaining plants were left non-fertilized (control). The solution containing the Se (60 mL) was added manually into each hole by using a surgical syringe one week after the plantlets were sown (Figure 15). Each cultivar (n=3) was harvested manually when the optimal harvest period was reached. After harvest, the plants were gently washed with tap water, followed by rinsing with deionized water to remove surface contaminants. The weight of each plant and the weight of the consumable part (white part) were recorded. The consumable part was separated and manually cut into pieces. Samples were stored at -80 ºC (New Brunswick, Rotselaar, Belgium) prior to freeze-drying during five days (CD-Energie, Eke, Belgium) and subsequently ground to fine powder in a mechanical grinder (Fritsch, Rotterdam, The Netherlands) to pass through a 1 mm sieve.

Figure 14. Experimental field plot containing different leek cultivars

5.3.2 Sample preparation for total Se and S determination in leek

All replicates of all cultivars were analysed for total Se and S contents. The methodology used for determination of total Se contents was similar to the methodology used for wheat plants which is described in section 8.3.

5.3.3 Statistical analysis

Statistical analysis was conducted in SPSS version 21.0. The significance of differences between the two Se fertilizer types and two doses of selenite was evaluated using factorial ANOVA with 0.05 as significance level. Pearson correlation coefficients were determined to correlate Se contents in the leek cultivars with biomass and S contents of the cultivars. For Pearson correlation analysis and to highlight cultivars with lowest and highest Se accumulation, only the 20 cultivars which were common in both study years (2010 and 2011) were considered.

Table 11. Overview of the analysed leek cultivars

ID	Commercial name	Type	Category	Origin
1	Albana	Summer	open pollinated	Nunhems
2	Miracle F1	Summer	F1 hybrid	Enza
3	Zeus F1	Summer	F1 hybrid	S&G
4	Striker F1	Summer	F1 hybrid	Bejo
5	Breugel F1	Autumn	F1 hybrid	Rijkzwaan
6	Tadorna	Autumn	old cultivar	Enza
7	Alcazar	Autumn	open pollinated	Rijkzwaan
8	Belton F1	Autumn	F1 hybrid	Nunhems
9	Pretan F1	Autumn	F1 hybrid	Nickerson-Zwaan
10	VLimberg R	Winter	breeder selection	Sint Katelijne Waver
11	Coolidge F1	Winter	F1 hybrid	Hortiplan
12	Artico	Winter	old cultivar	IPK
13	Farinto	Winter	open pollinated	Nunhems
14	Arkansas	Winter	open pollinated	Royal Sluis
15	Gavia	Winter	open pollinated	Enza
16	Toledo	Winter	old cultivar	Thompson & Morgan
17	Uytterhoe E	Winter	breeder selection	Onze Lieve Vrouw Waver
18	Engels P	Winter	breeder selection	Putte
19	Harston F1	Winter	F1 hybrid	Nunhems
20	Fahrenheit F1	Winter	F1 hybrid	Royal Sluis
21	Varna	Summer	open pollinated	Royal Sluis
22	Nelli	Summer	open pollinated	Svalöf Weibull
23	Nebraska	Autumn	old cultivar	Royal Sluis
24	Buelens Willy	Winter	breeder selection	Onze Lieve Vrouw Waver
25	Electra	Autumn	open pollinated	Clause
26	Poribleu	Autumn	open pollinated	Nickerson-Zwaan
27	Vervloet M	Winter	breeder selection	Sint Katelijne Waver

Figure 15. Application of liquid Se fertilizer

Table 12. Properties of soil samples collected on the experimental fields in 2010 and 2011

soil parameter	study year 2010	study year 2011
pH (KCl)	4.7	5.4
OC (%)	0.9	0.9
P (mg 100 g^{-1})	16.0	21.8
K (mg 100 g^{-1})	5.3	11.4
Mg (mg 100 g^{-1})	9.9	14.3
Ca (mg 100 g^{-1})	67	85.0
Na (mg 100 g^{-1})	4.0	<1.9
Se (mg 100 g^{-1})	0.03	0.03

5.4 Results and discussion

5.4.1 Biomass production as affected by fertilizer type and dose

The biomass of plants fertilized with 0.5 and 2.0 mg selenite-Se ranges between 26 and 85 g, and between 41 and 100 g DW per plant, respectively (Figure 16). The analysis of variance (ANOVA) for the effects of cultivars and fertilizer on biomass production and Se uptake is presented in Table 13. In study year 2010, only the factor "cultivar" significantly affected biomass while the interaction of fertilizer with cultivars had no significant effect on Se uptake and biomass (Table 13). Notably, when selenite was used at lower doses, differences in biomass production were not statistically significant between cultivars (0.5 mg Se per plant), whereas at higher dose (2 mg Se per plant) differences in biomass production between cultivars were statistically significant. It was reported earlier that Se fertilization significantly decreased the dry matter of shoots and roots of *Brassica* species (Banuelos et al., 1997a, Sharmaet al., 2010). Similarly, in crops like maize, wheat, sunflower and rice, which were considered as non-accumulators of Se, the DM yield was found to decrease with increasing fertilizer dose (Prasad, 2009, Singh and Singh, 1978). In the next study year (2011), the biomass of plants fertilized with 4.0 mg Se as selenate and selenite ranged from 16 to 87 g, and from 23 to 113 g DW per plant, respectively (Figure 17). Both cultivars and fertilizer forms significantly affected biomass. There was also a significant interaction between leek cultivars and Se fertilizer for biomass yield (Table 13). This interaction indicates that leek cultivars responded differently when different Se forms were used as fertilizers. In chapter 3, when soil was fertilized with 3.8 mg Se kg^{-1}, biomass production was slightly influenced. Earlier studies also reported that biomass production is influenced by Se fertilizers (Hartikainen, 2005b, Malik et al., 2011, Pilon-Smits et al., 2009, Yao et al., 2009). In various studies, it was observed that fertilization with selenate seems to result in lower plant biomass compared to selenite (Hopper and Parker 1999; XimenezEmbun et al., 2004; Sharma et al., 2010). When selenate is the dominant Se form in the soil, the active uptake of Se by the plants via the sulphur pathway could lead to higher accumulation and conversion to organic Se species. The replacement of amino acids in plant proteins by their Se analogues (selenocysteine and selenomethionine) is considered to be the underlying cause of selenium toxicity. The majority of plants in the *Allium* family, including leek, contains high levels of sulphur compared to other vegetables (Cerella et al., 2011).

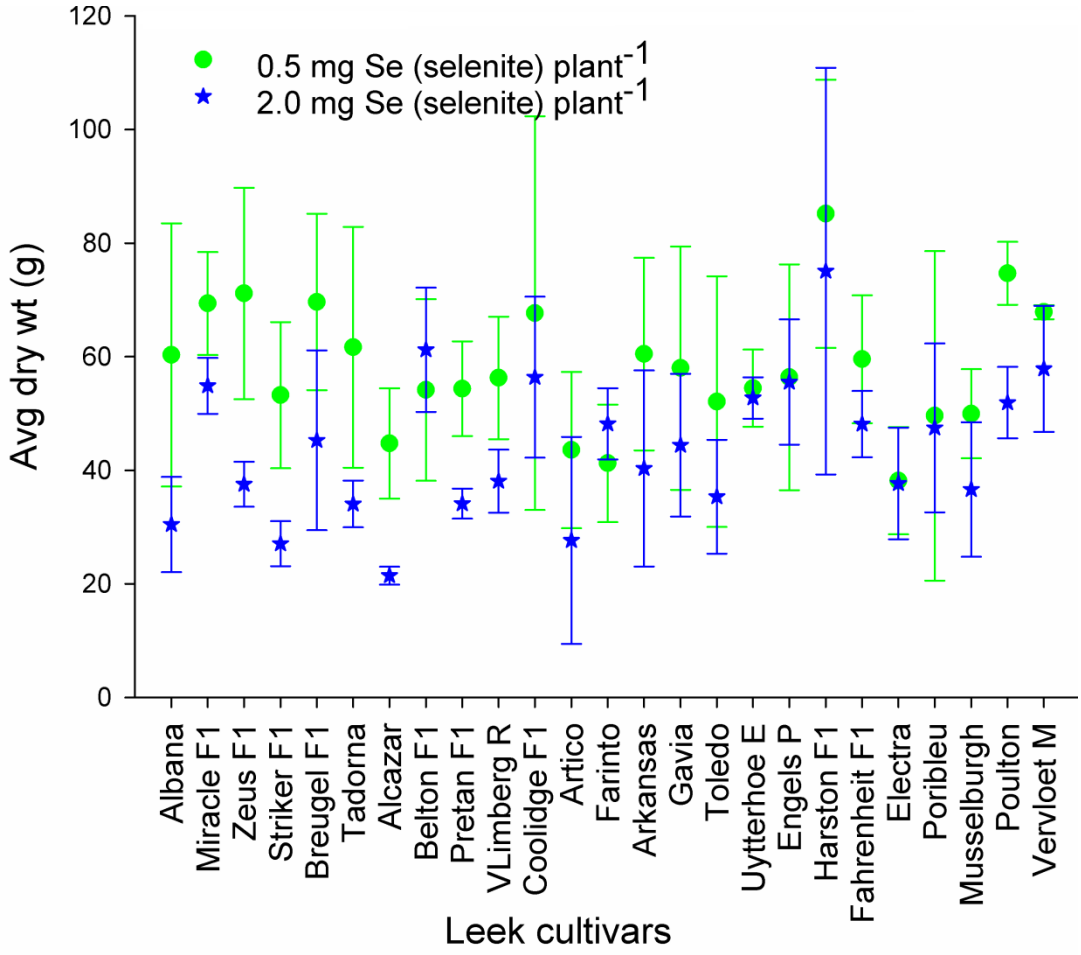


Figure 16. Dry matter yields of whole leek plants grown on soil fertilized with two different doses of Se applied as selenite (Na_2SeO_3) at 0.5 and 2.0 mg Se per plant

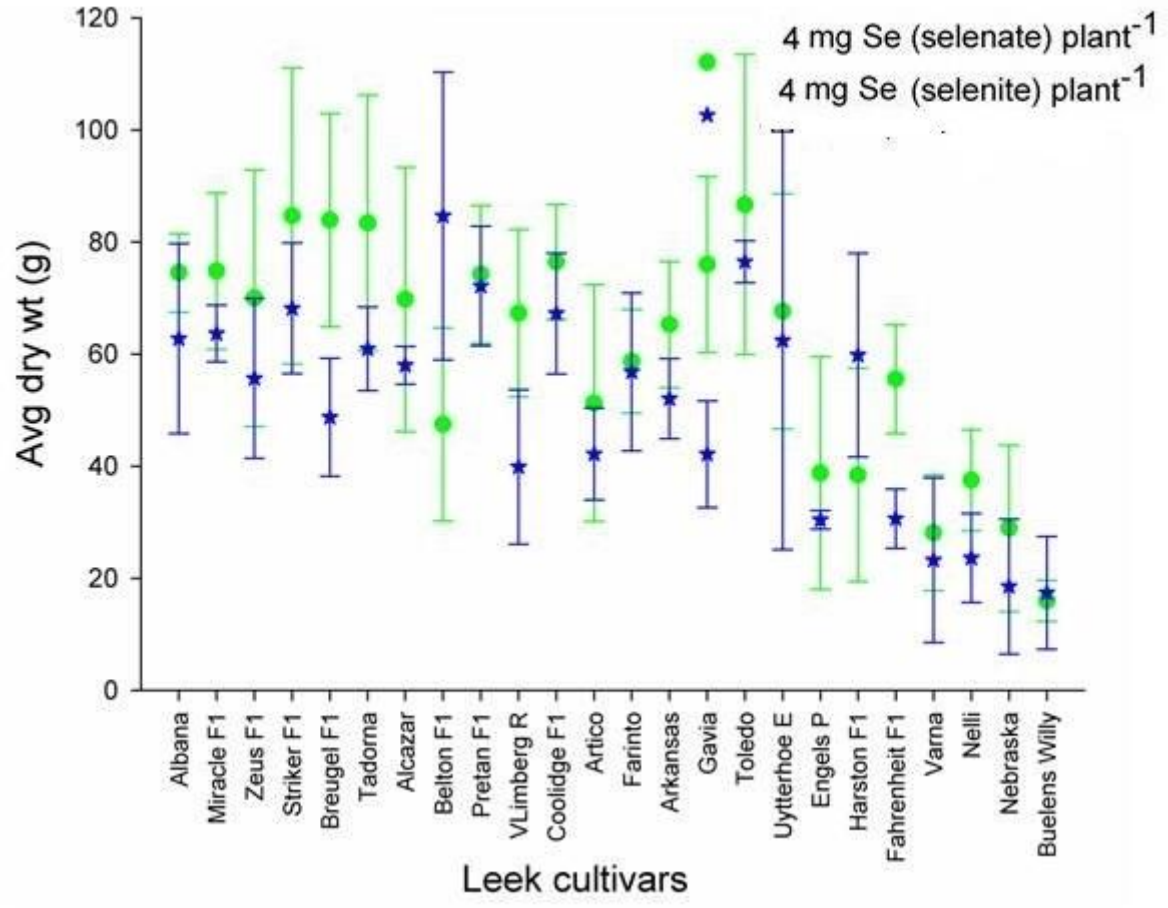


Figure 17. Dry matter yields of whole leek plants grown on soil fertilized with two types of Se fertilizer, selenate (Na_2SeO_4) and selenite (Na_2SeO_3), at 4.0 mg Se per plant

5.4.2 Effect of fertilizer type and dose on Se uptake in various leek cultivars

Previously no studies have reported differences between leek cultivars in terms of Se accumulation when subjected to Se fertilization at various Se doses and Se forms. In the present study, we observed that supply of different Se species influences Se uptake in various leek cultivars. Fertilizer doses significantly affected Se uptake while cultivars and the interaction of fertilizer with cultivars had no significant effect on Se uptake when 0.5 and 2 mg selenite-Se per plant were used as fertilizer in study year 2010 (Table 13). In next study year (2011), cultivars and fertilizer form (selenate and selenite) significantly affected Se uptake. In that study, there was also a significant interaction between leek cultivars and Se treatment for Se uptake (Table 13). This interaction indicates that leek cultivars responded

differently when two Se forms were used. These differences in Se accumulation between both fertilizer types were previously also observed in the greenhouse experiment with leek (see chapter 3) and for other food crops (Kapolna et al., 2007, Larsen et al., 2006). Concentrations of Se in non-fertilized leek (control) were presented for selected cultivars in figure 18. Concentrations of Se in these control plants were similar to control leek data obtained in the field survey presented in chapter 4. Among the 20 leek cultivars, some cultivars show significant changes in Se uptake when increasing the selenite-Se dose from 0.5 mg Se per plant to 2.0 mg Se per plant ($p \leq 0.05$) (Figure 19). The Se uptake by plants fertilized with 0.5 and 2.0 mg selenite-Se per plant ranged from 3.0±0.5 to 10.4±6.1 and from 9.3±0.8 to 68±72 µg per plant, respectively. When cultivars were fertilized with 4 mg Se per plant as selenite and selenate in the subsequent year, significant differences in Se uptake were observed for all cultivars (Figure 20). Leek fertilized with selenate fertilizer shows a higher uptake for these cultivars.

No differences between cultivars were observed when the plants were grown on 4 mg Se per plant supplied as selenite, whereas significant differences between cultivars were observed when plants were grown on 4 mg Se per plant supplied as selenate (Table 13). The fact that selenate fertilization results in larger differences between cultivars may be related to selenate being translocated to the aerial organs in plants whereas selenite was reported to be accumulated mainly in the roots (Arvy, 1993). Moreover, its uptake rate is independent of external concentrations (White et al., 2004). On the other hand, selenate readily competes with sulphate for uptake by plants and is probably assimilated by the sulphur transport pathway in chloroplasts (Ellis and Salt, 2003).

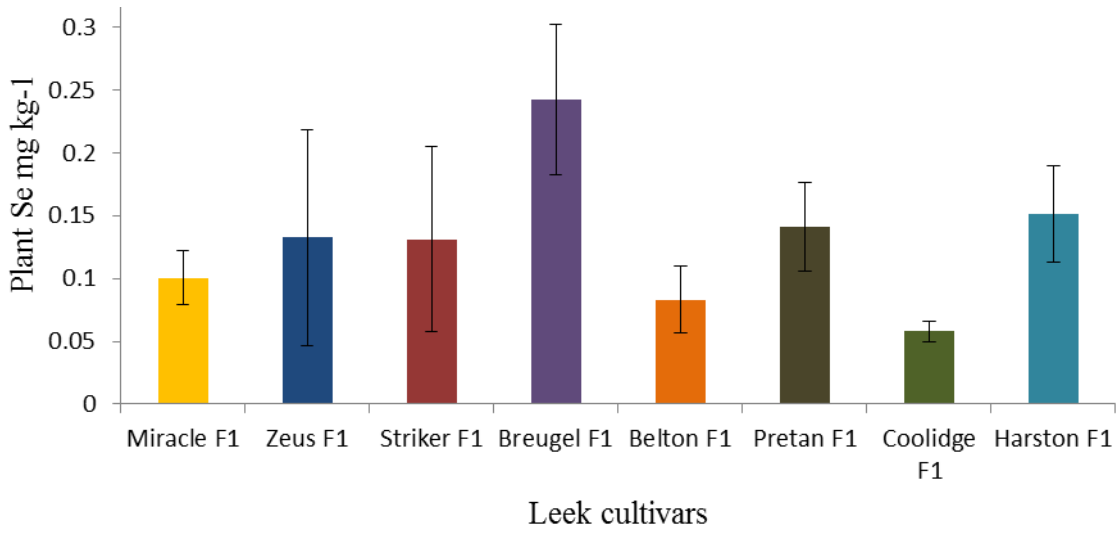


Figure 18. Selenium concentration in non-fertilized (control) plants of various leek cultivars grown in study year 2011

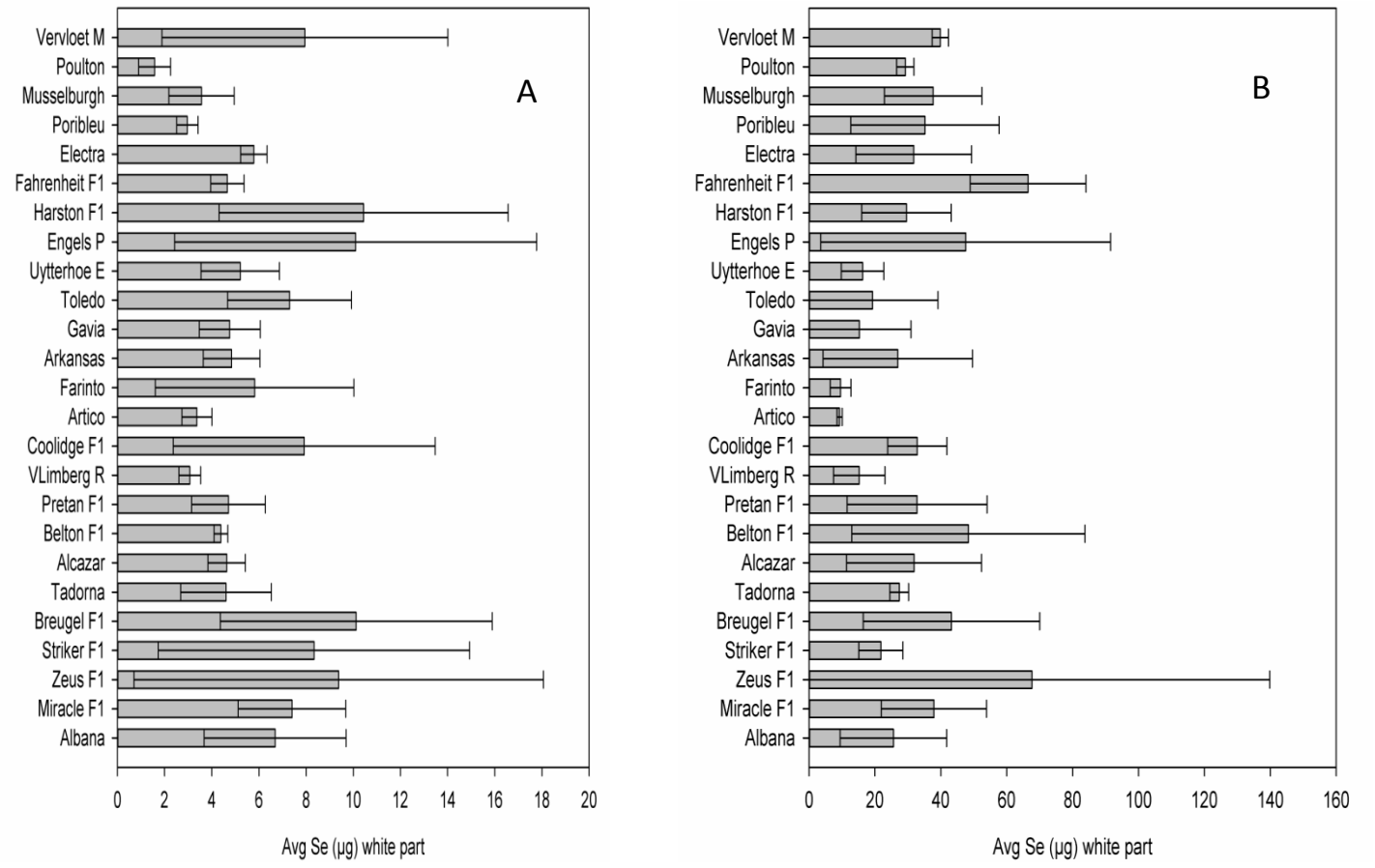


Figure 19. Average Se content (µg) in consumable white part (DW) of leek grown on two different doses of Se supplied as selenite (Na_2SeO_3) at (A) 0.5 mg Se per plant (B) 2.0 mg Se per plant

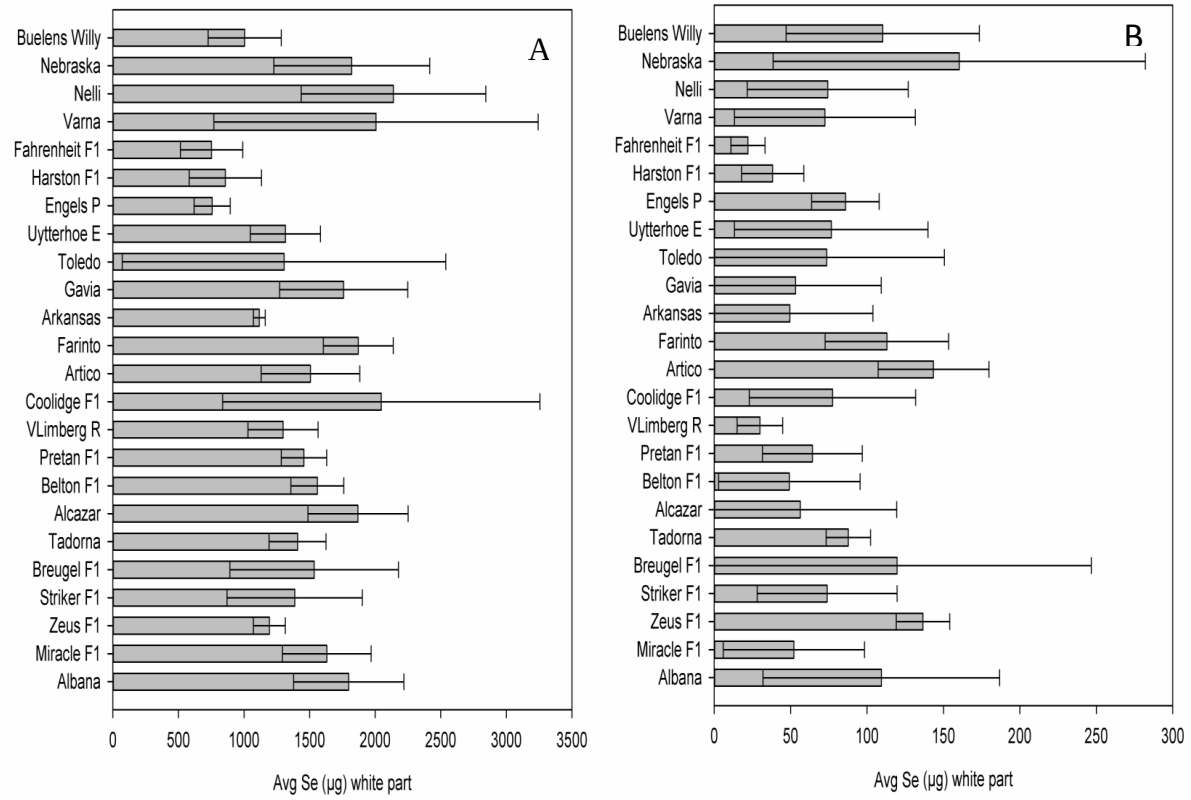


Figure 20. Average Se content (µg) in consumable white part (DW) of leek, grown on soils fertilized with two types of Se fertilizer at (A) 4 mg Se per plant (selenate) and (B) 4 mg Se per plant (selenite)

5.4.3 Effect of Se application on leek sulphur content in various cultivars

Selenium and sulphur (S) uptake in the leek were found to be significantly positively correlated (P <0.05) when leek was grown on 4 mg selenate-Se per plant (Table 14 and Appendix 3). The high S uptake in the plants grown on soils spiked with selenate corresponds with a high plant Se uptake. In the first study year, S uptake of the plants fertilized with 0.5 and 2.0 mg selenite-Se per plant ranged from 42±7 to 129±9 and from 50±9 to 169±68 µg per plant, respectively. In the second study year, the S uptake of the plants fertilized with 4.0 mg Se per plant as selenate and selenite ranged from 86±5 to 304±115 and from 76±4 to 200±52 µg per plant, respectively. When Se was supplied as selenite at a dose of 4 mg Se per plant, Se and S showed a positive correlation. The ability of Se to enhance S uptake and accumulation in some cultivars was unexpected since Se and S

are competitive and absorbed into the plant by the same carrier (Kopsell et al., 1997). Significant differences in contents of organic sulphur compounds were previously observed between leek cultivars (Bernaert et al., 2012a). We now also observed differences in total S between the cultivars. In general, *Allium* species contain more S compared to other vegetables, and it was previously hypothesized that plants which are prone to the highest disease incidence contain more sulphur analouges (Cerella et al., 2011; Coleysmith 1986).

Table 13. ANOVA (p-values) for effects of cultivars and fertilizer on biomass production and Se uptake in both studies (2010 and 2011). Treatments are considered significant at $p \leq 0.05$

	Study-I (2010) 0.5 & 2 mg selenite-Se plant^{-1}		Study-II (2011) 4 mg selenite-Se & selenate-Se plant^{-1}	
	Biomass	Se uptake	Biomass	Se uptake
Cultivars	0.00*	0.07	0.00*	0.00*
Fertilizer	0.82	0.00*	0.07	0.00*
Cultivars*fertilizer	0.43	0.28	0.05*	0.01*

Table 14. Linear correlation coefficients (r) for correlation of Se uptake (µg Se per plant) with biomass (g per plant) and S uptake (µg S per plant) for 20 leek cultivars in all fertilizer treatments

Regression Variables	Study-I (2010)		Study-II (2011)		Study-I & II (2010 & 2011)
	0.5 mg selenite-Se plant^{-1}	2 mg selenite-Se plant^{-1}	4 mg selenite-Se plant^{-1}	4 mg selenate-Se plant^{-1}	
Biomass (g per plant)	0.178 (0.122)	0.357 (0.453)	0.096 (0.688)	0.498* (0.025)	0.102 (0.369)
S (µg per plant)	0.048(0.901)	-0.012(0.938)	0.028 (0.864)	0.592** (0.000)	0.259* (0.020)

* and ** indicate statistical significance at the probability level of $p < 0.05$, and $p < 0.01$, respectively. Probability values are indicated between brackets

5.4.4 Total Selenium transferred from soil to consumable part

The major focus of the current study was to assess how Se uptake may differ between different cultivars aimed at optimizing Se biofortification strategies. Accordingly, the prime focus is given to the consumable white part of leek (shoot). However, biomass production data were presented for the whole plant. Selenium uptake by the leek was calculated using Se concentrations measured in the white part as an estimation of Se concentrations in the whole plant. These data are presented in Table 15.

Table 15. Average percentage (%) uptake of Se in leek calculated using Se concentrations measured in the white part as an estimation of Se concentrations in the whole plant, expressed as percentage of the amount added to the soil in the form of Se fertilizer

		Fertilizer type and dose			
sample ID	commercial name	0.5 mg Se per plant (Selenite)	2.0 mg Se per plant (Selenite)	4.0 mg Se per plant (Selenite)	4.0 mg Se per plant (Selenate)
1	Albana	1.3	1.3	2.7	45.0
2	Miracle F1	1.5	1.9	1.3	40.8
3	Zeus F1	1.9	3.4	3.4	29.8
4	Striker F1	1.7	1.1	1.8	34.7
5	Breugel F1	2.0	2.2	3.0	38.4
6	Tadorna	0.9	1.4	2.2	35.2
7	Alcazar	0.9	1.6	1.4	46.7
8	Belton F1	0.9	2.4	1.2	39.0
9	Pretan F1	0.9	1.6	1.6	36.4
10	VLimberg R	0.6	0.8	0.7	32.4
11	Coolidge F1	1.6	1.6	1.9	51.2
12	Artico	0.7	0.5	3.6	37.7
13	Farinto	1.2	0.5	2.8	46.8
14	Arkansas	1.0	1.3	1.2	27.9
15	Gavia	1.0	0.8	1.3	44.0
16	Toledo	1.5	1.0	1.8	32.6
17	Uytterhoe E	1.0	0.8	1.9	32.9
18	Engels P	2.0	2.4	2.1	18.9
19	Harston F1	2.1	1.5	1.0	21.4
20	Fahrenheit F1	0.9	3.3	0.6	18.8

The Se content in the white part varied between 0.75 and 2.00 mg per plant when plants were grown on soils fertilized with selenate. When grown on soils fertilized with selenite at a dose of 4 mg Se per plant, the Se content varied between 0.02 and 0.14 mg per plant. At a dose of 0.5 and 2 mg selenite-Se per plant, contents between 0.004-0.011 and 0.009-0.07 mg per plant were measured, respectively. It is evident that 95% of the Se remained in the soil when leek was fertilized with selenite whereas less than 50% of the Se remained in the soil when it was fertilized with selenate. For some cultivars, reproducibility of Se uptake within the cultivars seems lower. This could be due to uneven Se availability to the plants through application of the liquid Se fertilizer in the root zone. The amount of Se taken up from the applied Se dose of 0.5 mg Se per plant in form of selenite by the various leek cultivars in the current experiment (0.6-2.1%) is lower compared to the amount taken up by leek (0.3-4.9%) grown on various fields throughout Flanders region upon application of the same fertilizer type and dose (chapter 4). Notably, the pH of the soil of the current experiment in study year 2010 was lower (pH 4.7) compared to the soils of the field survey, described in table 7 (pH 4.8-7.1), and a lower pH may result in a lower Se uptake (Mayland et al., 1991).

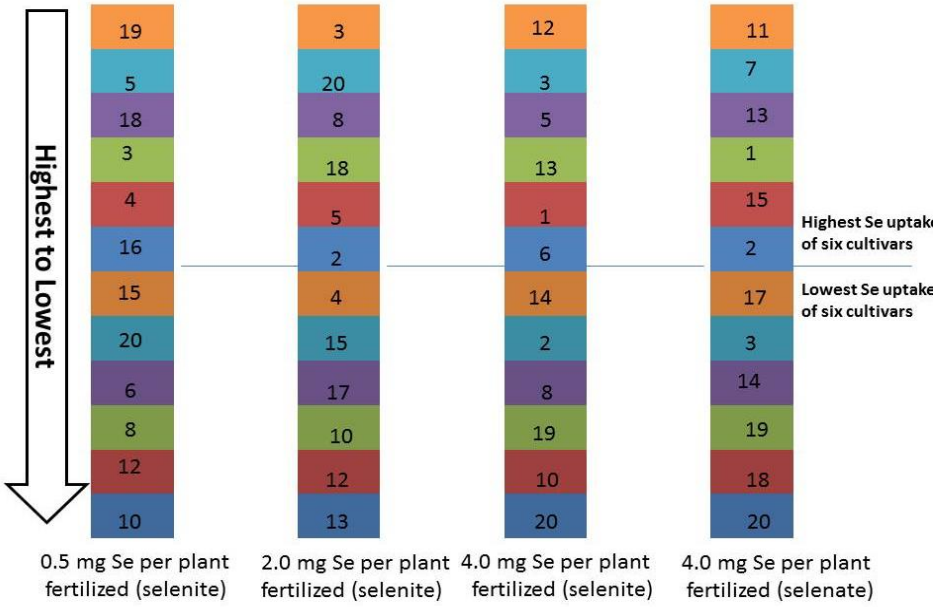


Figure 21. Six cultivars with the highest and lowest Se uptake for the different fertilization treatments. Serial numbers given to the cultivars are explained in Table 11

The response of cultivars on Se fertilization differs between Se fertilization doses and Se forms. In the first study year, among the six cultivars which recorded the highest Se uptake, three cultivars were common for both selenite fertilization doses (0.5 and 2.0 mg Se per plant), i.e. Breugel F1, Engels P and Zeus F1. Among the six cultivars exhibiting the lowest Se uptake, three other cultivars were common (VLimberg R, Artico and Gavia) (Figure 21). Similarly, in the second study year, among the six cultivars which recorded highest Se uptake, two cultivars were common for both fertilizer types (selenate and selenite at a dose of 4 mg Se per plant), i.e. Farinto and Albana. The cultivars exhibiting the lowest Se uptake, three other cultivars recorded the lowest Se uptake (Fahrenheit F1, Harston F1 and Arkansas) (Figure 21). Irrespective of the fertilization dose, VLimberg R and Fahrenheit F1 cultivars recorded the lowest Se uptake in the majority of the cases. Among all cultivars, six cultivars showed a Se uptake exceeding 40% of the applied Se dose (Albana, Miracle F, Alcazar, Coolidge F1, Farinto and Gavia)

5.5 Conclusion

The Se accumulation in leek is not only affected by Se fertilizer type and dose, but also differs between different leek cultivars. Among 20 different cultivars, six cultivars showed higher Se uptake when fertilized with selenate. Differences between cultivars were affected by Se dose and form tested. However, there is no common cultivars which can be ranked as having the highest Se uptake for each Se dose and fertilizer type. The higher amount of Se in the consumable part of leek when plants are grown at 4 mg Se per plant supplied as selenate could eventually lead to a decrease in production of dry biomass. All varieties respond to Se application and show a dose-dependent response in Se uptake. As in the previous chapter, it can be concluded that use of selenite (Na_2SeO_3) as fertilizer results in a higher risk for Se accumulation in the soil on longer term.

Chapter 6. Use of selenium fertilizers for production of Se-enriched Kenaf (*Hibiscus cannabinus*): effect on Se concentration and plant productivity

This Chapter has been redrafted from:

Rama V. S. L., De Schepper V., Steppe K., Tack F., Du Laing G. 2013. Use of selenium fertilizers for production of Se-enriched Kenaf (*Hibiscus cannabinus*): Effect on Se concentration and plant productivity. Journal of Plant Nutrition and Soil Science. 176: 634–639.

6.1 Abstract

Due to selenium (Se) deficiency, Se fortification of food and feed is applied in many countries. Therefore, this potential use of Se-enriched kenaf was investigated based on its Se accumulation, its potential to transform accumulated Se to other Se species and effect of Se accumulation on its growth. Kenaf was grown with different levels of two Se fertilizers (selenite and selenate) at concentrations ranging from 0 to 4 mg Se kg^{-1} soil. Total Se concentrations in the plants grown on selenate-treated soil amounted to 1019±136 mg Se kg^{-1} dry weight and were much higher compared to plants grown on selenite-treated soil. Identified Se species were selenite, selenate, SeMet and $SeCys_2$. Biomass yield, net photosynthesis and chlorophyll content index of the plants decreased when plants were grown on soils treated with high doses of selenate.

6.2 Introduction

Selenium (Se) at low levels is essential for humans and mammals and its deficiency is still a cause of concern in many countries. Therefore, Se fortification of food and feed has increased in the past decade. Countries with low Se status have introduced Se-fortified food and feed crops as well as Se-enriched food and feed supplements in their policies to increase the Se status of livestock and human population (Aro et al., 1995). Several studies have proven the possibility of overcoming Se deficiency by enriching yeast with Se and by applying Se fertilizers when growing forage, wheat and maize (Broadley et al., 2006, Chilimba et al., 2012, Filley et al., 2007, Kahakachchi et al., 2004). Although Se is needed for biological processes, it can be toxic when present in too high concentrations. In addition, the form in which Se is present, i.e. its speciation, largely influences its toxicity. Because for Se the range between optimal required levels and toxic effects is narrow and depends on its speciation, it is important to control concentrations in crops carefully and to evaluate how Se fertilization affects the concentration and speciation in the crops. The Se form and dose applied to the soil, as well as some soil parameters, were reported to be important in this context (Gissel-Nielsen, 1971, Johnsson, 1991, Robberecht et al., 1982).

Selenium is not a very abundant element and is present at average levels of 1 mg Se kg^{-1} in the soil. Deficient soils contain less than 0.4 mg Se kg^{-1}, whereas seleniferous soils may contain concentration up to 100 mg Se kg^{-1} soil. On seleniferous soils most plant species contain 1-10 mg Se kg^{-1} dry weight (DW), but the so-called hyperaccumulators (e.g. from the genera Stanleya and Astragalus) accumulated 1000-15000 mg Se kg^{-1} DW even from low external soil Se concentrations (Broadley et al., 2006, Pilon-Smits et al., 2009). The potential of several plant species to accumulate Se still needs to be evaluated. Attention should go to not only total concentrations in the plants, but also to their speciation. Kenaf (*Hibiscus cannabinus*) was previously classified as a Se indicator and identified as a Se-accumulating plant (Banuelos et al., 1996). This means that concentrations between 100 mg Se kg^{-1} DW, i.e. the upper limit of non-accumulators, and 1000 mg Se kg^{-1} DW, i.e. the lower limit of hyperaccumulators can be expected. It is a crop of interest to both humans and animals due to its usage as food and feed (Kubmarawa, 2009, Lopez et al., 2006). Kenaf is intensively cultivated in several countries, such as India, Bangladesh, United States of America, Indonesia, Malaysia, South Africa, Vietnam, Thailand, parts of Africa, and to a small extent in southeast Europe. Since ancient periods, these counties cultivated kenaf as a leafy vegetable used for cooking, cattle grazing and paper production (Adebayo, 2010). It was also recommended as a crop that may be used in phytoremediation, i.e. for the removal of metals from polluted soils (Bada, 2010). Kenaf is particularly interesting as a feed crop due to its soluble protein content which is comparable to the soluble protein content of alfalfa (Phillips et al., 2002). The use of kenaf as a feed crop has been successfully tested on beef cattle and small ruminants, and resulted in high percentage of proteins, non fatty solids and total solids in the milk (Lopez et al., 2006, Xiccato et al., 1998). Recent studies showed that kenaf seed oil enhances apoptosis towards ovarian cancer cells (Yazan et al., 2011). However, not much data on Se accumulation and speciation in kenaf are available. In addition, it is not known whether Se accumulation influences the development and productivity of kenaf plants. Therefore, the current study assessed the effect of fertilizing soil with different doses from two types of Se fertilizers on the Se accumulation and speciation, and their effect on plant performance and development.

6.3 Materials and methods

6.3.1 Experimental setup

Sandy-loamy soil was collected from fields in Merelbeke, Belgium. The soil was air-dried and sieved through a 2 mm sieve. The soil contained 0.23 mg Se kg^{-1}, 1.09% organic carbon and its pH was 6.15. One kg of soil was weighed in each recipient. Two types of Se fertilizer, i.e. sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3) (Sigma Aldrich, St. Louis, MO, USA) were separately tested by adding different Se concentrations 0.5, 1.0, 2.0 and 4.0 mg Se kg^{-1} to the soil, equivalent to 2174, 4348, 8696 and 17391 g per hectare, respectively. The soils were then brought to field capacity with deionised water and allowed to equilibrate for 24h. Thereafter the soils were mixed thoroughly. The moisture content was maintained at 25%. After 10 days, 10 seeds of kenaf were sown in each recipient, from which six plantlets were kept in each recipient by removing the least developed plantlets after five days of seeds sown. The experiment was set up in quadruplicate. The soils were regularly watered with deionized water. After 45 days, leaf characteristics were measured and afterwards the plants were harvested. Soil was washed carefully from the belowground plant parts with tap water, ensuring that root hairs were not disturbed. To further remove surface contaminants, the samples were again washed gently with tap water followed by deionized water. The six plants from each recipient were pooled and considered as one sample, and three independent samples were analysed. The weights of the above and belowground parts of the plants were recorded. The plant material was cut into pieces, transferred to polyethylene boxes, frozen at -20 ºC and lyophilized. Total Se and Se species concentrations were determined in three subsamples. Deionized water (MilliQ water, Water Systems Ltd., Brussels, Belgium) was used throughout the experiment.

6.3.2 Sample preparation for total Selenium content

For determination of total Se in the kenaf samples, the aboveground plant parts of the pooled samples were lyophilized. A subsample of 0.2 g was weighed into a centrifuge tube followed

by addition of 2.5 mL concentrated HNO_3 and 2.5 mL 30% H_2O_2 (Chemlab, Zedelgem, Belgium). After 16 h, the tubes were placed in a microwave oven (Williams et al., 2007). The temperature was raised to 55°C for 10 min at 600 Watt and 100% power. Afterwards, the temperature was raised to 75°C for 10 min. Finally, it was maintained at 100°C for 30 min. The clear digests were diluted to 50 mL with deionized water for further Se determination. For validation of the procedure, the certified reference plant material BCR-CRM 402 (white clover, 6.7±0.27 mg Se kg^{-1}) was digested using the same procedure and the total Se extraction efficiency was found to be 95±3%. Three replicates of BCR-CRM 402 were analysed with the sample batch. Both standard addition and external calibration were used.

6.3.3 Sample preparation for Selenium speciation

For Se speciation analysis, plant samples obtained by fertilizing the soil with Na_2SeO_3 and Na_2SeO_4 at a dose of 0.5 and 4.0 mg Se kg^{-1} soil were used. A 0.2 g plant subsample and 80 mg of the enzyme protease XIV (Sigma Aldrich, St. Louis, MO, USA) were dissolved in 5 mL of water. This mixture was shaken in a 10 mL centrifuge tube for 24 h at 37°C and centrifuged for 30 min at 10,000 g (Mazej et al., 2008). The supernatant was separated from the residue and filtered through a 0.45 µm syringe-type PVDF membrane filter. Supernatant and residue were stored at -20°C until they were analysed for Se speciation. In addition, the total Se content of supernatant was determined to quantify the Se release through the enzymatic digestion. This release was found to range between 75 and 79 %.

6.3.4 Total Selenium and Selenium speciation analysis

An Inductively Coupled Plasma Mass Spectrometer (ICP-MS, PerkinElmer DRC-e, Sunnyvale, CA, USA) was used for Se determination. The ICP-MS was fitted with a Babington nebulizer and a Scott double pass spray chamber. For speciation analysis, the ICP-MS was coupled as detector to a liquid chromatographic system (Series 200 HPLC, Perkin Elmer, Sunnyvale, CA, USA) (HPLC-ICP-MS). The HPLC consisted of a P680 HPLC pump and an ASI-100 automated sample injector. A Hamilton PRP-X100 anion exchange column

and Altima C8 column (250 mm × 4.6 mm I.D., 5 µm, 120 Å) were used as stationary phase. Both columns were equipped with a guard column containing the same stationary phase material. Extraction of Se species was carried out by using a shaker fitted incubator chamber from Sartorius (Goettingen, Germany). Total Se determination was carried out using a microwave digestion apparatus from Mars (North Carolina, USA). HPLC chromatographic standards were sodium selenite (Na_2SeO_3), sodium selenate (Na_2SeO_4), SeMet, $SeCys_2$, MeSeCys and the mobile phase used during HPLC analysis were citric acid, heptafluorobutryic acid ammonium hydroxide and methanol (Sigma Aldrich St. Louis, MO, USA). The mobile phase solutions were prepared freshly prior to analysis. Selenium species were quantified using an external standardization method with reference standards. Linear correlations ($R^2 > 0.99$) between peak areas and analyte concentrations were obtained for these standards.

6.3.5 Physiological leaf characteristics

Before harvesting (after 45 days), one leaf of three plants per treatment was sampled to determine the chlorophyll content index, the photosynthesis rate and the chlorophyll fluorescence parameters. The third fully expanded leaf from the top was measured. Chlorophyll content index was measured using a portable chlorophyll meter system (SPAD 502, Minolta Company, Tokyo, Japan) from 9:30 to 3:00 h. During the same time, net photosynthesis (P_n) at 1000 µmol PAR m^{-2} s^{-1} was determined with a portable infrared gas analyser system (LI-6400, Li-COR, Lincoln, NE, USA). Relative humidity in the leaf chamber was uncontrolled and equal to the humidity in the greenhouse, while the air temperature in the leaf chamber was controlled at 25°C. External air was CO_2 scrubbed and mixed with pure CO_2 to create a standard concentration of 400 µmol mol^{-1}. The air flow rate was set at 200 µmol s^{-1}. Furthermore, the same device (LI-6400, Li-COR, Lincoln, NE, USA) was used as a chlorophyll fluorometer to measure simultaneously with P_n, two light adapted chlorophyll fluorescence parameters: effective quantum yield of PSII (Φ_{PSII}) and PSII operating efficiency (F_v'/F_m').

6.3.6 Biomass growth measurements

At the time of harvesting, plant height and weight were determined for plants of all treatments. Roots and stems with leaves were separated and dried at 55 ºC in an electric drying oven for 48 h (to constant weight) to calculate dry weight percentages.

6.3.7 Statistical analysis

The significance of effects of the two Se fertilizers and their applied doses was evaluated by ANOVA (fixed effects and Duncan's Multiple Range Test). Significance of differences was evaluated at the 0.05 level. Correlation analysis was conducted in order to identify (linear/quadratic) relations between the tested plant parameters and applied Se doses.

6.4 Results

6.4.1 Selenium fertilizers and their effect on Se accumulation and speciation

The Se accumulation in kenaf plants increased significantly and linearly when the dose of selenate and selenite applied to the soil increased (Figure 22A; Table 16). However, the uptake of Se by the plants grown on the selenate-fertilized soil is much higher compared to the uptake by plants grown on the selenite-fertilized soil. The percentage of organic species (relative to the total content) was highest in plants grown on selenite-treated soils (Figure22B). Similar speciation patterns were observed when plants were fertilized with 0.5 mg Se kg^{-1} (data not shown) or 4.0 mg Se kg^{-1} (Figure 22B).

Although relative amounts of organic Se species were lower in plants grown on selenate-treated soils compared to plants grown on selenite-treated soils, absolute amounts were higher. The most abundant organic Se species were SeMet and $SeCys_2$. In both treatments,

selenate was found to be the dominant inorganic species in the plants, whereas selenite was less dominant. Three unknown peaks were identified in kenaf plants grown on selenate-treated soils. However, no unknown peaks were identified in plants grown on selenite-treated soils.

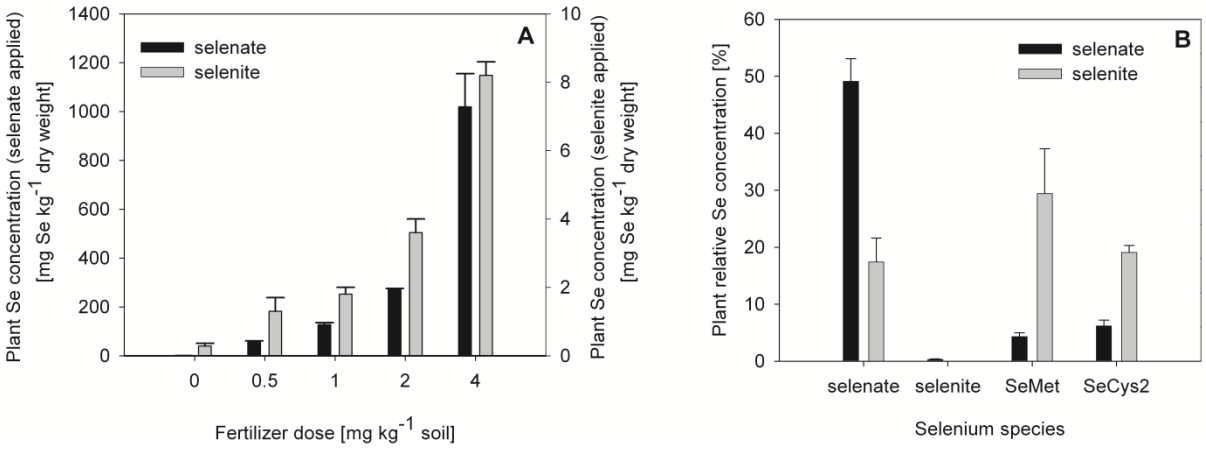


Figure 22. A. Effect of Se fertilizer type (sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3) and dose (0.5 to 4 mg Se kg^{-1} soil) on Se concentration in kenaf plants (mg Se kg^{-1} DW) represented using two different y-axes (left axis for selenate and right axis for selenite); control plants are the same for both treatments. B. Selenium species concentrations in kenaf plants (% of total Se) grown on soils fertilized with selenite and selenate (4 mg Se kg^{-1} soil). Results are expressed as mean ± standard deviation of three independent samples

Table 16. Regression equations predicting plant parameters from applied Se dose for two Se fertilizers tested at various application doses

Parameters	Na_2SeO_4	Na_2SeO_3
P_n	15.08+5.08(soil Se)-1.64(soil Se^2); r^2=0.54	20.33-24.95 (soil Se)+7.63(soil Se^2); r^2=0.94
Φ_{PSII}	0.58-0.23 (soil Se); r^2=0.36	-1.97+8.81 (soil Se); r^2=0.54
F_v'/F_m'	0.35-0.04 (soil Se); r^2=0.47	-1.95+9.86 (soil Se); r^2=0.53
Chlorophyll index	48.88-2.83 (soil Se); r^2=0.42	NS
Total plant weight (g)	NS	NS
Plant above ground weight (g)	NS	NS
Plant height	37.17-4.145 (soil Se); r^2=0.74	NS
Se (mg kg^{-1})	-93.81+261.67 (soil Se); r^2=0.91	0.08+1.98 (soil Se): r^2=0.97
Se (µg pot^{-1})	31.99+288.58 (soil Se); r^2=0.97	0.48+5.07 (soil Se); r^2=0.97

^{NS}Non significant for linear/quadratic ($p \geq 0.05$)

6.4.2 Effect of Selenium fertilizers on plant development

Low doses of both Se fertilizers appear to result in a slight increase in the biomass weight, but a decrease in plant height (Figure 23A). At high doses of selenate fertilizer (4 mg Se kg^{-1} soil), both the plant height and weight decreased significantly, while this was not the case for the selenite fertilizer. The reduction in plant weight was mainly caused by a reduction in aboveground biomass (Figure 23B and 24). However, the regression analysis suggests that applied Se doses have no overall significant effect on biomass yield, although selenate fertilizers seem to decrease plant height (Table 16).

When changing the applied fertilizer dose, net photosynthesis (P_n) (Figure 23C) did not change significantly except for the highest dose of selenate fertilizer (4 mg Se kg^{-1}soil). The

pattern of P_n related to the chlorophyll content index (Figure. 23C) and both measured fluorescence parameters (Figure 23D). The negative effect of the highest selenate dose (4 mg Se kg^{-1} soil) was only significant for the chlorophyll content index and not for the fluorescence parameters. According to these leaf characteristics, selenite fertilizer doses across the full tested range appeared to not influence plant performance or development, whereas selenate

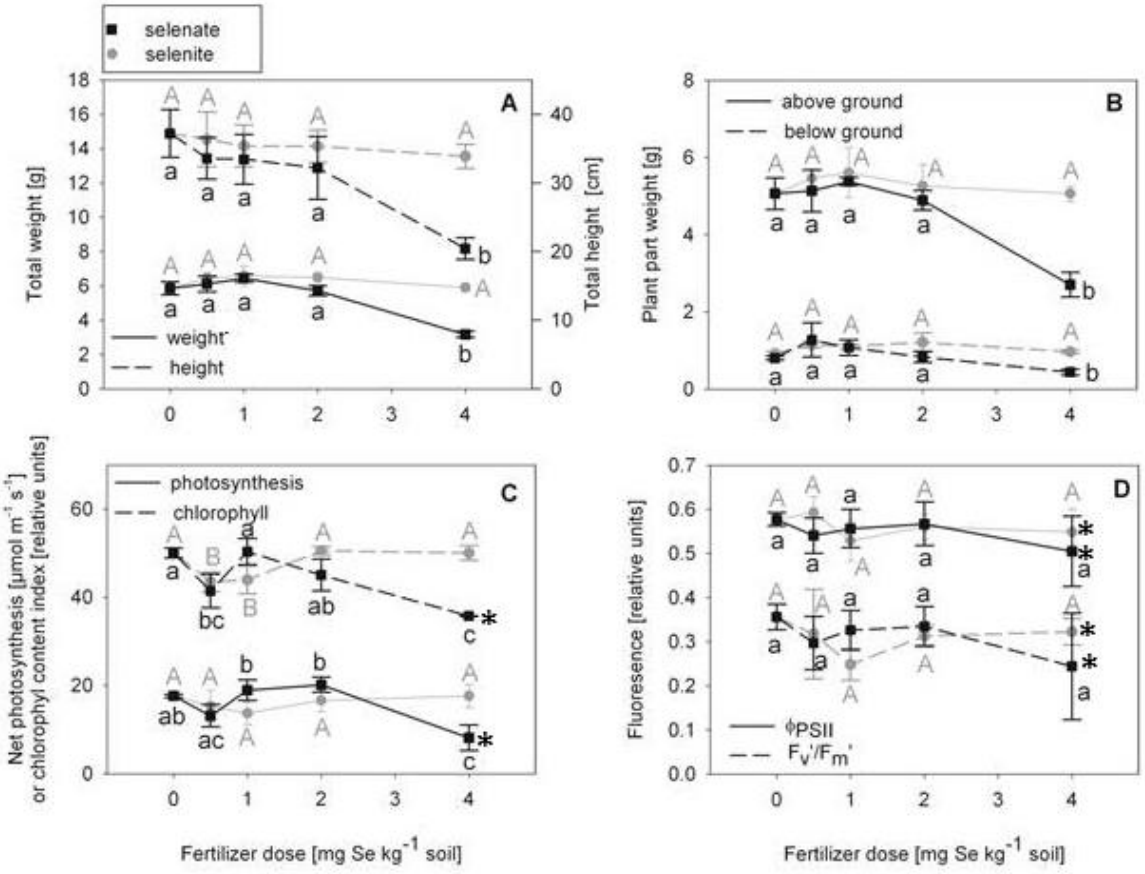


Figure 23. The influence of two selenium (Se) fertilizers, selenate (Na_2SeO_4) and selenite (Na_2SeO_3), and their applied dose (from 0.5 to 4 mg Se kg^{-1} soil) on (A) total plant weight and height, (B) above (stem) and below (root) ground weight, (C) net photosynthesis and chlorophyll content index and (D) fluorescence parameters of kenaf plants. Results are expressed as mean ± standard deviation of four independent samples. Statistical significance of differences between the different doses is denoted for each treatment using alphabets (Duncan's Multiple Range Test). *indicates overall statistical significance of individual parameter ($p \leq 0.05$).

Figure 24**.** Growth of plants as influenced by Se dose when amending soil with selenate

fertilizer doses have to be restricted to 2 mg Se kg^{-1} soil to avoid any effect on growth and performance of the plants (Figure 24). For plants grown on selenate fertilizer, a linear negative association was observed for plant physiological parameters and applied Soil Se dose, whereas for plants grown on selenite fertilizer a positive trend or no association was observed.

6.5 Discussion

The kenaf plants grown on soils treated with selenate seem to accumulate Se at higher concentrations compared to plants of the *Brassica* family, whereas concentrations similar to those observed in plants of the *Brassica* family were found in the kenaf plants grown on the selenite-treated soils (Hopper and Parker, 1999, Sharma et al., 2010). Compared to kenaf, chives (*Allium schoenoprasum*) and dill (*Anethum graveolens* L.) seem to accumulate Se in higher concentrations for both fertilizers (Cankur et al., 2006, Kápolna, et al., 2007). The

percentage of Se removed by kenaf plants grown on selenate amended soils was 32-42%, which is comparable to the percentage removed by wheat plants grown on a similar soil (Broadley et al., 2010). The lower Se uptake in soils treated with selenite is attributed to its lower bioavailability and mobility. On one hand, the higher Se uptake when applying selenate corresponds with the fact that selenate-Se uptake by plants is an active process via sulphate transporters, whereas selenite-Se accumulates through passive diffusion and can be inhibited by phosphate (Sors et al., 2005). On the other hand, selenate also often exhibits a higher mobility and availability in the soil compared to selenite (Broadley et al., 2007). Due to the lower uptake, the amount of Se remaining in the selenite-treated soils can become high, leading to Se accumulation in the soil and an environmental impact on the longer term.

In plants, a Se concentration of more than 5 mg Se kg^{-1} DW inorganic or organic Se is considered to be toxic to feed for cattle pigs (Kim and Mahan, 2001). Therefore, kenaf plants grown on a soil treated with selenate at the doses we tested can be used only as a feed additive and not as a bulk fodder, because the Se concentration level by far exceeded these toxic Se levels (Figure 22A) at all doses tested. However, Se concentrations in kenaf plants grown on soils treated with selenite did not exceed the toxic levels, except for the highest dose of 4 mg Se kg^{-1} soil (Figure 22A).

Inorganic species are considered as less beneficial for animals and humans compared to organic species, because inorganic Se can cause acute Se poisoning (Tiwary et al., 2006). As such, the higher relative abundance of organic species in kenaf plants grown on selenite-treated soils suggests that selenite fortification may be more beneficial compared to selenate treatment without having an influence on plant productivity (Figure 23B). In both treatments, SeMet was the dominant organic Se species, probably because SeMet is randomly incorporated into proteins replacing methionine (Navarro-Alarcon and Cabrera-Vique, 2008). The observed Se speciation for both treatments is in good agreement with results reported for other food crops such as Se-enriched *Allium* species (Pedrero et al., 2007, Wróbel et al., 2004). Even though organic Se compounds are less toxic, when the dominant organic Se form is SeMet, it can be directly incorporated into general proteins instead of other Se compounds which were most effective in reducing tumors. When an adequate amount of SeMet was fed it could lead to accumulation of non-specific pools but at low doses the protective effect of SeMet was effective in raising blood Se concentrations than selenate (Ip, 1988). However, the bioavailability, GSH-Px activities in whole blood and erythrocytes are similar for the two forms (Thomson et al., 1993).

There is no proof of essentiality for Se in plants. However, there have been reports of beneficial effects at low Se doses on plant growth (Hartikainen, 2005b, Malik et al., 2011, Pilon-Smits et al., 2009, Yao et al., 2009). In the kenaf plants, the slight increase in dry biomass for the lowest doses was not statistically significant. In contrast, the physiological toxic effects of high Se doses in kenaf plants were significant. This toxic effect as shown by a decreased biomass and photosynthesis rate. Selenium levels above 300 mg Se kg^{-1} DW in the plant became toxic when selenate doses above 2 mg Se kg^{-1} soil were applied. Many plants show signs of Se toxicity in terms of reduced dry matter yield at high Se plant levels (Banuelos et al., 1997b, Dhillon, 2009, Sharma, et al., 2010). Selenium toxicity is attributed to its similarity to sulphur as Se replaces sulphur in amino acids and can change protein folding, causing reduced growth and deformities (Daniels, 1996, Lemly, 1997, Sors et al., 2005). This sulphur substitution also seemed to occur in the photosynthetic apparatus, inducing a loss in PSII efficiency which could explain the observed growth inhibition (Geoffroy et al., 2007). Both the reduction in chlorophyll content index and fluorescence parameters indicate that the light reactions of photosynthesis are negatively influenced by Se.

Kenaf plants can accumulate Se from Se-soil fertilizers, and as such can be used as a Se-enriched fodder crop or feed additive. However, for use as fodder crop the soil should not contain too much Se in the form of readily available selenate as levels that are toxic to cattle could otherwise be reached. For this purpose, selenate-Se is recommended to be used in doses below the lowest dose tested in our study (0.5 mg Se kg^{-1} soil). Se-selenite fertilizers can also be used at higher doses (until 4.0 mg Se kg^{-1} soil). However, using selenite can lead to higher soil Se accumulation on longer term. Higher Se concentrations in kenaf may be envisaged when kenaf plants would be grown to be used as Se-enriched feed additive instead of fodder crop. In that case, selenate-Se can be applied at doses up to 2 mg kg^{-1}. At higher doses, Se becomes toxic for the plants.

6.6 Conclusion

Our study confirms that kenaf can be considered as a Se indicator plant and might be used for Se supplementation as part of a diet. The extent of Se accumulation strongly depends on the form in which Se is applied as fertilizer to the soil. Using selenate instead of selenite as

fertilizer resulted in a Se uptake that is more than 100 times higher. However, using selenate also resulted in decreased plant productivity at higher doses. The selenium dose that can be applied to the soil to grow Se-enriched kenaf is determined by toxicity of Se to the plant and toxicity of Se to cattle consuming the plant. It depends on the Se form contained in the fertilizer and whether the Se-enriched kenaf would be used as feed additive or fodder crop. Use of selenite may result in a higher appearance of organic species, but also a higher Se accumulation in the soil. For use as bulk fodder crop, selenate as well as selenite can be applied. However, to avoid effects on physiology of the plants the Se dose may not exceed the lowest dose when applying selenate (0.5 mg Se kg^{-1} soil) or the highest dose when applying selenite (4.0 mg Se kg^{-1} soil) tested.

Chapter 7. Influence of soil selenium fertilization on trace element uptake by Kenaf plants (*Hibiscus cannabinus*)

7.1 Abstract

A too low intake of Selenium (Se) by humans and cattle in several world regions has recently resulted in an increased interest in soil Se fertilization to enhance Se concentrations in food and feed crops. The objective of this work was to examine how trace metal concentrations in kenaf (*Hibiscus cannabinus*) plants are affected by soil Se fertilization. Kenaf plants were grown on 0, 0.5, 1.0, 2.0 and 4.0 mg Se kg^{-1} soil, supplied to the soil as selenate (Na_2SeO_4) and selenite (Na_2SeO_3). After 45 days, plants were harvested and the content of some essential (Se, Mn, Zn, Cu and Fe) and toxic (Al, Cd and Pb) metals was measured. The plant tissue concentration of most metals significantly decreased or increased (depending on the metal) when kenaf was grown on soil fertilized with Se. This effect depended on the applied Se dose and form. For instance, when a dose of 2.0 mg Se kg^{-1} soil was applied, the selenite fertilizer resulted in a lower concentration of Al, Cu, Fe, and content of Se in plant tissue compared to when selenate fertilizer was used. When a dose of 4.0 mg Se kg^{-1} soil was applied, both Se-fertilizers significantly increased concentrations of the essential elements Cu, Zn and Se, and significantly decreased the concentration of the toxic Al metal. However, for the majority of the elements, the uptake decreases upon increase of the Se dose irrespective of Se form used in the fertilization. These results emphasize the need to monitor trace metal concentrations when applying Se fertilizer to produce Se-enriched food crops.

7.2 Introduction

Selenium (Se) is essential trace element for humans. It also becomes toxic when certain doses exceed (Bhasin et al., 2012, Greenberg et al., 1986, Greger, 1999). A daily uptake of 55 µg Se per day is considered as an adequate for humans, whereas it becomes toxic when a dose of 300 µg Se d^{-1} is exceeded (Zeng, 2009). The Se uptake from the soil by the plant potentially differs between crops and depends on the available soil Se concentration and forms. Especially the Se form present in the soil highly influences Se uptake by the crop: if a soil contains Se in the form of selenate (Na_2SeO_4) a higher uptake is observed compared to when the soil contains selenite (Na_2SeO_3) (Cartes et al., 2005, Singh, 1991). Due to inadequate soil Se levels in several countries, supplementation strategies, such as application of soil Se-

fertilizers, have become a common practice to obtain Se-enriched crops (Aro et al., 1995, Broadley et al., 2010, Broadley et al., 2006, Eurola et al., 1991, Lyons et al., 2004).

However, there is a risk that concentrations of other essential (Mn, Zn, Cu and Fe) and toxic (Cd, Pb and Al) metals increase or decrease upon fertilization using Se-fertilizers. Elements that are essential for plants play a vital role in plant growth. However, they can also become toxic when they exceed the requirement of the plant (Arnon and Stout, 1939, Rengel and Graham, 1995). There is some evidence that an increased Se content in crops decreases the content of toxic or essential trace elements. For example, Se was found to act as an antagonist on green alga, Chinese cabbage and lettuce (Feroci et al., 2005, Havey et al., 2004, Issa and Adam, 1999).

Kenaf (*Hibiscus cannabinus*) is a crop that is cultivated in many Asian and African countries (Cheng et al., 2004). Its cultivation is very popular due to its various uses. It is used for paper production, biofuel production and as a leafy vegetable in the human and animal diet (Hays, 1989, Kim and Sung, 2007, Webber, 1993). The kenaf plant produces a high vegetative biomass and yield in a short time period. In addition, kenaf plants can grow well in dry environments and can tolerate moderately saline soil conditions (Banuelos et al., 1997a). Hence, the production of Se-enriched kenaf food and feed appears as an attractive option for the use of soils in rather saline and dry areas. The aim of our study was to investigate how the uptake of essential and potentially toxic elements is influenced when kenaf plants are grown on soils fertilized with two different Se forms, selenate (Na_2SeO_4) and selenite (Na_2SeO_3), at five different Se doses.

7.3 Materials and methods

7.3.1 Experimental setup

Kenaf seeds were purchased from a traditional herbal seed supplier in Hyderabad, India and confirmed that kenaf variety which was widely used as food and feed crop from Prof. M.N.V. Prasad at Department of Plant Sciences, Central University, Hyderabad, India. Sandy-loamy soil was collected from Merelbeke (Belgium). One kilogram of soil was weighed into each

recipient. Two types of Se fertilizers, i.e., selenate (Na_2SeO_4) and selenite (Na_2SeO_3) (SigmaAldrich, St. Louis, MO, USA), were separately added to the soil at different doses (0.5, 1.0, 2.0 and 4.0 mg Se kg^{-1}). The soils were then brought to field capacity with deionized water and allowed to equilibrate for 24h. Thereafter, the soils were mixed thoroughly. The moisture content was maintained at 25%. After 10 days, 10 seeds of kenaf were sown in each recipient, from which six plantlets were kept in each recipient by removing the least developed plantlets after five days. The experiment was set up in triplicate. The soils were regularly watered with deionized water, maintaining the moisture content at 25%. After 45 days, the aboveground plant parts were harvested and washed gently, with tap water followed by deionized water. The six plants from each recipient were pooled for elemental analysis on ICP-MS and ICP-OES. Biomass of kenaf plants was measured as described in Chapter 6. Plant tissue concentrations of all elements were expressed on a dry weight basis, and their total uptake per plant was calculated using the respective plant dry weights.

7.3.2 Determination of metal contents in kenaf plants

For determination of metal contents in the kenaf samples, the pooled samples were lyophilized and grounded. The plant samples were digested in a microwave oven using nitric acid (65%) and hydrogen peroxide (37%) (Chemlab, Zedelgem, Belgium) of 2.5 mL for 40 min. In a first step, the temperature was raised to 55 °C in 10 min at 600 Watt and 100% power. Afterwards, the temperature was raised to 75 °C in 10 min. Finally, it was maintained at 100 °C for 30 min. The obtained extracts were filtered and diluted to 50 mL with deionized water. For validation of the procedure, the certified reference plant material BCR-CRM 402 was digested using the same procedure. Three replicates of BCR-CRM 402 were analysed with the sample batch.

An Inductively Coupled Plasma Mass Spectrometer (ICP-MS, PerkinElmer DRC-e, Sunnyvale, CA, USA) fitted with a Babington nebulizer and a Scott double pass spray chamber was used for determination of Se, Cd and Pb (Table 17). An Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) was used to determine Al, Cu, Fe, Mn and Zn (Table 17).

Table 17. Instrumental conditions of ICP-MS and ICP-OES

ICP-MS parameters:	
Isotopes monitored	^{80}Se, ^{111}Cd, ^{208}Pb
Power	1250 W
Plasma flow	15.0 L min^{-1}
Auxiliary flow rate	1.50 L min^{-1}
Reaction gas and flow rate	CH_4, 0.9 mL min^{-1}
Dwell time for each isotope	0.1 s
ICP-OES parameters:	
Elements monitored	Al, Zn, Fe, Cu, Mn
Power	1350 W
Plasma flow	15.0 L min^{-1}
Auxiliary flow rate	1.50 L min^{-1}
Viewing height	10 mm
Replicate read time	5s

7.3.3 Statistical analysis

The significance of effects was evaluated using ANOVA (fixed effects and Duncan's multiple range test) with 0.05 as significance level. In addition, differences between metal uptake and concentrations in treated plants and those in the control plant were evaluated using a LSD test. Statistical analysis was conducted with SAS (version 9.2).

7.4 Results

7.4.1 Effect of Selenium fertilizer on the plant tissue concentration of essential trace metals

The Se concentrations in aboveground parts of plants grown on selenite-fertilized soil were much lower compared to plants grown on selenate-fertilized soil for all Se doses tested (Figure 25A, B). Essential metal concentrations were affected by the dose of Se-fertilizer (Figure 26, 27). A significant decrease in Mn concentration was observed in plants grown on soil fertilized with selenate to 2.0 and 4.0 mg Se kg^{-1} soil, whereas the different doses of selenite had no significant effect (Figure 26A, B). For both Zn and Cu, selenite fertilizers showed a decrease in Zn and Cu concentrations for most applied doses (antagonist effect), whereas the Se-selenate fertilizer had no significant effect unless a strong synergetic effect at 4.0 mg Se kg^{-1} soil (Figure 26C, D and 27C, D). In case of Fe, 1.0 and 2.0 mg Se kg^{-1} soil doses of Se-selenite induced a significant decrease of the Fe content, while the Se-selenate fertilizer significantly decreased the Fe concentrations at doses of 1- 4 mg kg^{-1} Se-selenate (Figure 27A, B). However, a dose of 4 mg kg^{-1} Se-selenate had a significant synergetic effect on the Cu content (Figure 27C, D). In conclusion, if Se enrichment had an effect on trace metals, the effect was antagonistic, except for the highest Se-selenate dose (4.0 mg Se kg^{-1} soil) where an increase of Zn, and Cu content was observed.

7.4.2 Effect of Selenium fertilizer on the plant tissue concentration of toxic metals

None of the Se fertilizers significantly influenced concentrations of Pb (Figure 28E, F), whereas selenate fertilization decreased Cd concentrations upon application of 2.0 mg Se kg^{-1}. However, both fertilizers significantly decreased Al concentrations in the crop. This effect is much stronger when selenite fertilizer is used (Figure 28A, B). When the plants are grown at 4.0 mg Se kg^{-1} soil, a 90% decrease was observed when selenate was applied, whereas only a 29% decrease was seen when selenite was used.

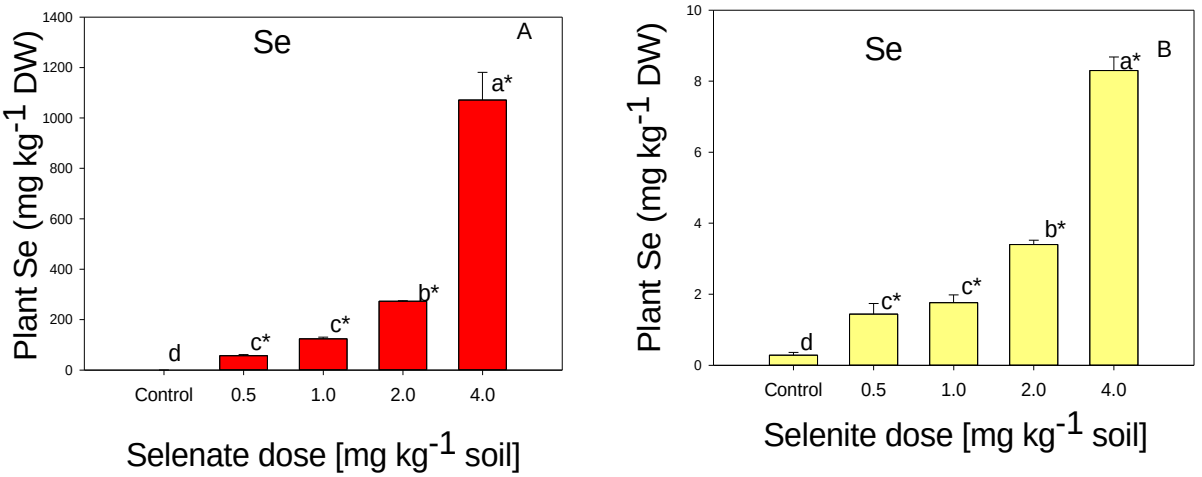


Figure 25. A. Effect of Se fertilizer type (A: selenate (Na_2SeO_4) and B: selenite (Na_2SeO_3)) and dose (0.5 to 4.0 mg Se kg^{-1} soil) on Se concentration in kenaf plants (mg Se kg^{-1} DW). Results are expressed as mean ± standard deviation of three independent samples. Statistical significance of differences between the different doses is denoted for each treatment using letters (Duncan's Multiple Range Test). Results significantly ($P \leq 0.05$) differing from the control according to LSD pairwise comparison were denoted with symbol (*).

7.4.3 Effect of Se fertilizer on metal pool in aboveground parts of kenaf plants

The accumulation of metals in the aboveground plant pool may differ from the way their concentration in the plant tissue is affected; as Se fertilization may also affect the biomass production (see Chapter 6). Therefore, the accumulation of metals in the aboveground part of the kenaf plants was calculated (Table 18). The metal accumulation in aboveground biomass was found to decrease significantly in plants grown on Se-fertilized soil. Significant effects were observed for Al, Cu, Fe, Mn, Zn, Se and Cd when plants were grown on soil fertilized with selenite, whereas Al, Cu, Fe, Zn, Se, Pb and Cd were significantly affected when plants were grown on selenate-fertilized soil ($p \leq 0.05$) at 4 mg Se kg^{-1} grown plants. The Se uptake in kenaf plants was higher when plants were grown on selenate-fertilized soils which is about 29-35% whereas, when they were grown on selenite-fertilized soils Se uptake is about 0.38 and 0.66 % from applied Se dose.. At the highest application dose (4 mg Se kg^{-1} soil), the trace metal uptake in the aboveground plant parts is lowest when using selenate as fertilizer.

Especially the accumulation of Al in the aboveground plant parts is significantly lower when selenite is supplied at 4 mg Se kg^{-1} soil.

Table 18. Elemental accumulation in the aboveground pool of kenaf plants as affected by Se fertilizer applied at four different doses and in two different Se forms, expressed in µg per pot. The percentage transferred from the soil in each pot to the aboveground plant parts is added between brackets

	Elemental uptake (µg/pot) (percentage uptake from soil)							
Dose	Al	Cu	Fe	Mn	Zn	Se	Cd	Pb
				Selenate				
0	73 (0.01)	9.0 (0.07)	252 (0.10)	123 (0.05)	105 (0.31)	0.7 (0.26)	1.5 (0.65)	1.2 (0.00)
0.5	76 (0.01)	7.7 (0.06)	169 (0.07)*	113 (0.04)	82 (0.24)*	148 (30)	1.3 (0.56)	1.1 (0.00)
1.0	60 (0.01)	7.6 (0.06)	150 (0.06)*	109 (0.04)	80 (0.24)*	348 (35)	1.4 (0.63)	0.8 (0.00)
2.0	58 (0.01)	9.1 (0.08)	133 (0.05)*	82 (0.03)*	69 (0.20)*	676 (34)*	0.9 (0.38)*	0.8 (0.00)
4.0	20 (0.00)*	5.2 (0.04)*	106 (0.04)*	31 (0.01)*	51 (0.15)*	1148 (29)*	0.4 (0.19)*	0.7 (0.00)
				Selenite				
0	73 (0.01)	9.0 (0.07)	252 (0.10)	123 (0.05)	105 (0.31)	0.7 (0.26)	1.5 (0.65)	1.2 (0.00)
0.5	60 (0.01)	8.2 (0.07)	189 (0.07)	125 (0.05)	84 (0.25)	3.3 (0.66)	1.5 (0.64)	1.5 (0.00)
1.0	51 (0.01)*	7.4 (0.06)	151 (0.06)	116 (0.05)	86 (0.26)	4.3 (0.43)	1.4 (0.61)	1.0 (0.00)
2.0	36 (0.01)*	5.8 (0.05)*	119 (0.05)*	116 (0.05)	70 (0.21)*	8.4 (0.42)	1.3 (0.58)	0.6 (0.00)
4.0	5 (0.00)*	4.8 (0.04)*	109 (0.04)*	101 (0.04)	68 (0.20)*	15 (0.38)*	1.0 (0.44)*	0.5 (0.00)*

A significant difference ($P \leq 0.05$) from the control according to pairwise comparison was denoted with symbol (*)

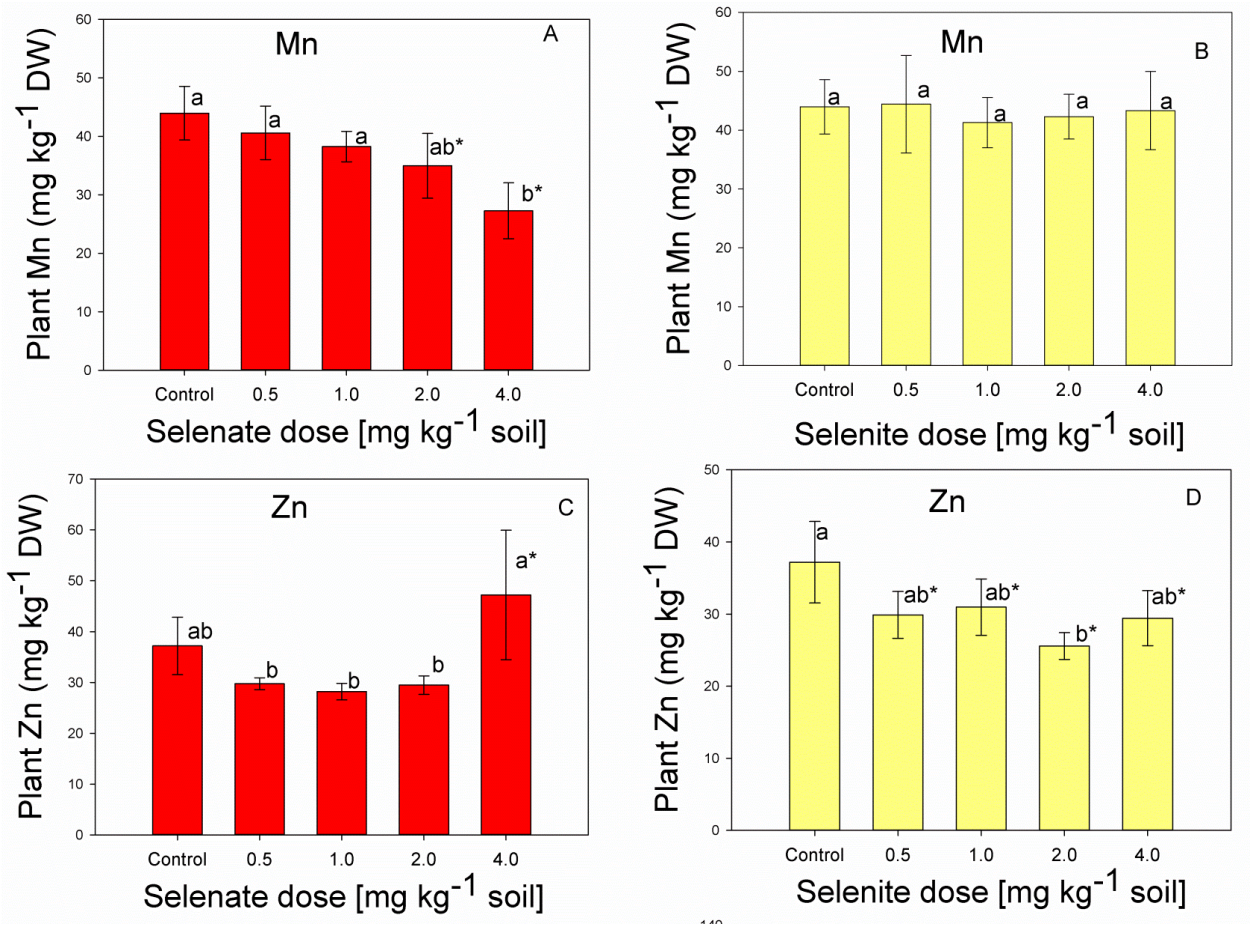


Figure 26. Effect of Se fertilizer type (selenate (Na_2SeO_4) and selenite (Na_2SeO_3)) and dose (0.5 to 4.0 mg Se kg^{-1} soil) on essential trace metal concentrations in kenaf plants (mg Se kg^{-1} DW). Results are expressed as mean ± standard deviation of three independent samples. Statistical significance of differences between the different doses is denoted for each treatment using letters (Duncan's Multiple Range Test). Results significantly ($P \leq 0.05$) differing from the control according to LSD pairwise comparison were denoted with symbol (*).

7.5 Discussion

7.5.1 Impact of Se fertilizer on metal uptake and accumulation in the plants

Effect of Se fertilizers on biomass was described in chapter 6. At higher dose of selenate fertilizer (4 mg Se kg^{-1} soil), a higher decrease in plant biomass was observed, while this was not the case for the selenite fertilizer. According to the present study, Se fertilization may decrease the concentration of metals in kenaf plants (Figure 26, 27). However, it may also increase trace metal concentrations (Zn and Cu), which was observed at the highest application dose of selenate (4 mg Se kg^{-1} soil). Antagonistic effects of low application doses of Selenite were also observed by He et al. (2004), who reported antagonist effects of Se on Zn concentrations in Chinese cabbage. Moreover, Landberg et al. (1994) reported that selenite decreased Cu and Cd uptake and selenate increased Cd and Cu uptake.

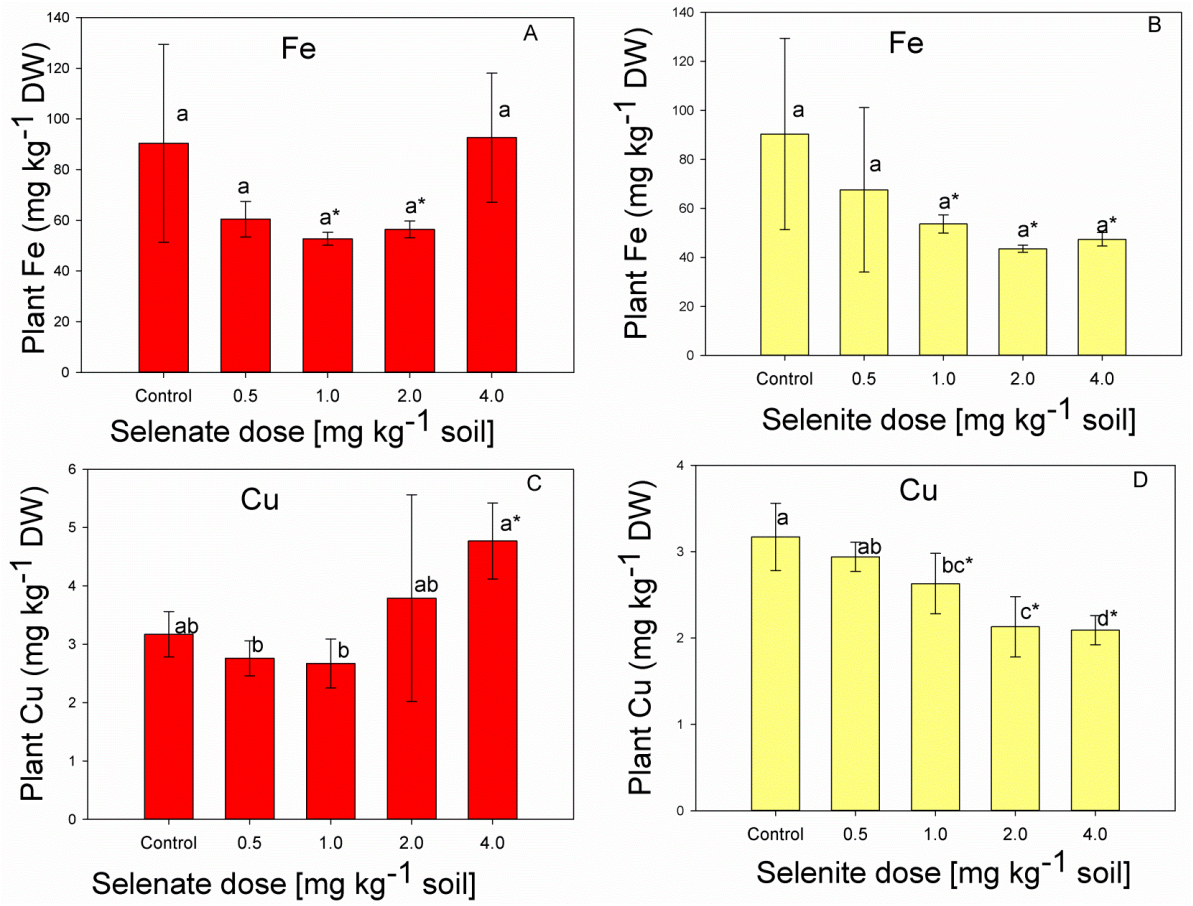


Figure 27. Effect of Se fertilizer type (selenate (Na_2SeO_4) and selenite (Na_2SeO_3)) and dose (0.5 to 4.0 mg Se kg^{-1} soil) on essential trace metal concentrations in kenaf plants (mg Se kg^{-1} DW). Results are expressed as mean ± standard deviation of three independent samples. Statistical significance of differences between the different doses is denoted for each treatment using letters (Duncan's Multiple Range Test). Results significantly ($P \leq 0.05$) differing from the control according to LSD pairwise comparison were denoted with symbol (*).

In kenaf, Cd uptake decreases with application dose in plants grown on soil fertilized with both forms of Se. However, a significant decrease was observed in plants grown on soils fertilized with selenite at 4 mg Se kg^{-1} and plants grown in soils fertilized with selenate at 2 and 4 mg Se kg^{-1} (Table 18). Although reduction in Cu uptake in kenaf grown on selenite-fertilized soils was in agreement with what was reported in a study on pea (*Pisum sativum*) and wheat (*Triticum aestivum*) (Landberg et al., 1994), there is no significant increase in Cu uptake by plants grown on selenate-fertilized soils (Table 18). The discrepancy in effects of Se between our study and the study from Landberg et al., (1994), implies that the influence of Se on metals may vary depending on plant species and Se forms present in the soil. A higher

concentration of Al in plants induces stress and reduces plant growth (Mossor-Pietraszewska, 2001). When Al stress is induced in plants, application of Se has shown antioxidant activity similar to *N*-acetylcysteine (NAC) at low concentration. However, at high concentrations, Se act as a ROS-promoting species, which was proven using seedlings of *Stylosanthes humilis* (Ribeiro et al., 2011). The antagonist effect was seen in Al uptake by kenaf plants grown on Se-enriched soils. The level of Al decrease was higher in plants grown on soils fertilized with selenite compared to plants grown on soils fertilized with selenate (Figure 28). In particular, when 4 mg kg^{-1} Se was added to the soil, the Al concentration is higher in plants grown on selenate-treated soils which could partly account for the decreased plant growth due to enhanced ROS generation by Al.

7.5.2 Factors affecting plant productivity at high selenate doses

Hajiboland and Amirazad (2010) suggested that Zn deficiency could lead to a decrease in plant growth when they were exposed at lower concentrations of 2.0 µM compared to 25 µM. This is in contrast to our study, where fertilization with Se-selenite decreased the Zn and Cu concentrations of the plants while it did not influence plant growth (Figure 26C, D and Figure 27C, D). A possible explanation for the discrepancy could be that the deficiency levels of Zn and Cu were not yet reached in our study. Requirements of Zn and Cu for kenaf were not previously described in literature. However, some other species started to show Zn and Cu deficiencies from 10-15 and 2-5 mg kg^{-1} DW (Schulte and Kelling, 2004).

Selenium may act as a healing antioxidant at low concentrations, but also as a harmful reactive oxygen species (ROS)-promoting compound at high concentrations (Ribeiro et al., 2011). In our study, it seemed that the highest Se concentrations (± 1000 mg kg^{-1} DW) became toxic and acted as growth-inhibiting agents (Figure 24, 25A). These highest concentrations were reached at the highest selenate doses. The reduced plant growth could be majorly attributed to higher Se concentrations in kenaf grown on selenate-fertilized soil. In addition, the slight difference in Fe concentration between kenaf grown on selenate-fertilized soil and kenaf grown on selenite-fertilized soil at 4 mg Se kg^{-1} could also be areason explanation for reduced plant growth. Similar to Se, the other ROS promoting metal in presence of higher concentrations in plants is Fe. The iron concentration in plants grown at

higher selenate doses is not significantly different from to the control group. However, a slight increase was observed at this selenate dose. When Fe accumulates too much in plants, it can act catalytically via the Fenton reaction to generate hydroxyl radicals, disturb the cellular mechanisms in plants and hamper plant growth (Connolly and Guerinot, 2002, Moran et al., 1994). In some species like rice, reduced plant growth is observed at 3000 mg Fe kg^{-1} DW (Mehraban et al., 2008). There is no available data on Fe essesntiality or toxicity of kenaf, the above conclusion is based on comparing control group.

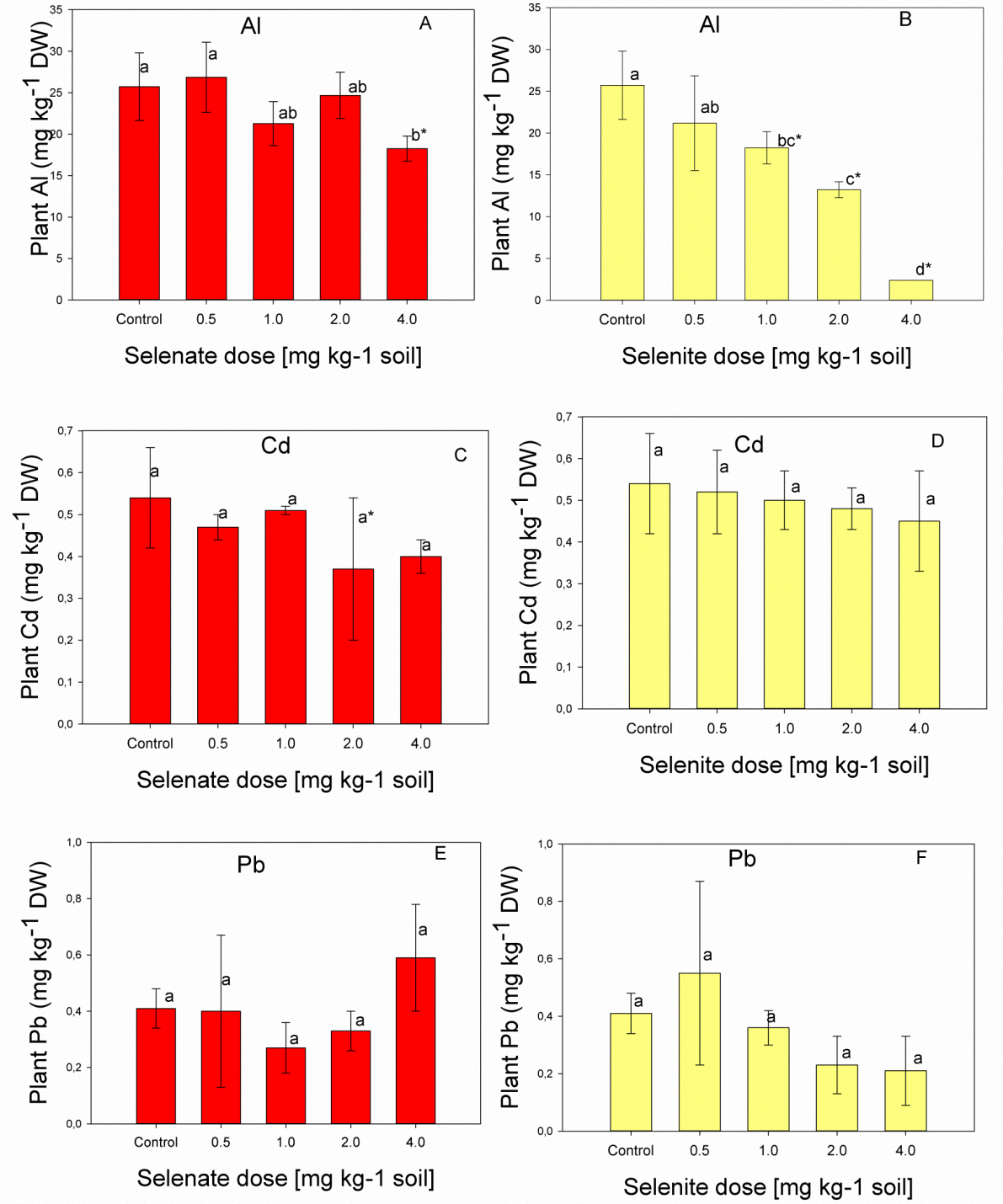


Figure 28. Effect of Se fertilizer type selenate (Na_2SeO_4) and selenite (Na_2SeO_3) and dose (0.5 to 4 mg Se kg^{-1} soil) on non-essential and toxic metal concentration in kenaf plants (mg Se kg^{-1} DW). Results are expressed as mean ± standard deviation of three independent samples. Statistical significance of differences between the different doses is denoted for each treatment using letters (Duncan's Multiple Range Test). Results significantly ($P \leq 0.05$) differing from the control according to LSD pairwise comparison were denoted with symbol (*).

Manganese deficiency is related to a reduction of plant physiological processes, such as photosynthesis and chlorophyll content (Arya and Roy, 2011, Singh et al., 2001) because Mn plays an important role in the catalytic centre for light-induced water oxidation in photosystem-II (Charles and Willigen, 2006). The deficiency of Mn is generally seen in baby leaves by interveinal chlorosis (yellowing between the veins of the leaves) while the veins themselves remain dark green. In the studied kenaf plants, the leaves developed at the highest selenate dose showed similar chlorosis symptoms as well as a significantly decreased chlorophyll content and photosynthesis rate (Figure 23). In plants, Mn contents of leaves range between 30-500 mg Mn kg^{-1} DW (Clarkson et al., 1971). In kenaf plants at higher selenate dose (4 mg Se kg^{-1}), the concentrations were below the normal range. This clearly indicates that the Mn deficiency at this dose could limit plant growth (Figure 26A).

From the previous chapter 6, it became evident that plant growth and physiological parameters such as net photosynthesis and chlorophyll content index are influenced by Se form and dose. Due to the pale yellowish color of leaves (Figure 24) at higher selenate doses, we hypothesized that the toxicity was not only attributed to higher Se concentrations in plants but also due to changes in other trace metals which are responsible for various plant physiological functions such as Mn, Zn and Cu. With our current experimental results, we conclude that higher Se concentrations clearly disrupt the pathway of other trace metals.

7.6 Conclusions

In conclusion, the present study showed that soil Se fertilizers influence the uptake of essential and toxic metals depending on applied fertilizer dose and Se form available in soils. For most tested doses and forms of Se, Se application to the soil showed antagonistic effects in kenaf plants for various metals. These results demonstrate that essential trace metal concentrations are not much influenced by Se fertilization, which is important for the consumer in terms of dietary needs. However, the uptake of the metals by the plants was strongly influenced even though biomass was not always different between kenaf grown at various soil Se doses. Reduction of plant growth at high doses of Se fertilizer could be due to variation in plant tissue concentrations of Se and Fe, inducing toxicity, and Mn, inducing deficiency.

Chapter 8. Influence of soil amendments on Selenium mobility and its uptake by wheat (*Triticum aestivum)*

8.1 Abstract

The influence of soil amendments and ageing on selenium (Se) mobility in soil and its availability to wheat (*Triticum aestivum*) was investigated to aid in the development of strategies for effective and environmentally safe Se fertilization. Wheat plants were grown on sandy loam soils spiked with 1 mg Se kg^{-1} in the form of sodium selenate or sodium selenite, each treated with 5% inorganic ($CaCO_3$) or organic (compost, cow manure and pig manure) amendments. The influence of soil ageing was assessed by growing the wheat plants at several time intervals after amending and spiking the soils. Additionally, mobility of Se in the soil was assessed using three soil extractions ($CaCl_2$, EDTA and aqua regia). During plant growth, pore water samples were collected using Rhizon samplers and analysed for Se concentrations. Plants grown on selenate-spiked soils had higher Se concentrations compared to plants grown on selenite-spiked soils. Organic amendments decreased Se concentrations in the plants. In the soils amended with cow and pig manure Se uptake by the plants decreased by 91 and 88% compared to the control when the soils were spiked with selenite, whereas it decreased by 95 and 89 % when the soils were spiked with selenate. Low plant uptake may result in accumulation in the soil and groundwater contamination, especially when Se is applied to the soil in the form of selenite or when soils are amended with cow or pig manure. It is obvious that careful soil management is needed when Se fertilizers are used to obtain Se-enriched food crops. In the current study, soils were treated with high doses of amendments under greenhouse conditions to assess potential effects and risks. However, future studies should also focus on effects of lower doses under field conditions.

8.2 Introduction

Selenium (Se) is considered as an essential micronutrient in human and animal health because of its biological role as component of the antioxidant enzyme glutathione peroxidase (GSH-Px). This enzyme scavenges hydrogen peroxide and lipid hydroperoxides to prevent oxidative damage in body tissues (Rotruck et al., 1973). Accordingly, the dietary Se requirement for humans ranges between 55 and 70 µg/day in Europe (El-Bayoumy, 2001, Rayman, 2004, Whanger, 2004). The deficiency of Se in humans over a period of time in

certain world regions has resulted in various diseases such as the alkali disease, white muscle disease, heart disorders (Keshan disease), and a bone and joint disease (Kashin-Beck disease) (Fordyce, 2005). Moreover, it was noted that dietary Se repletion may reduce cancer incidence in people at high risk who live in areas with low soil Se (Gissel-Nielsen et al., 1984; Gupta et al., 2000; Hartikainen, 2005a). It has been estimated that between 0.5 and 1 billion people globally may have an inadequate intake of Se, including populations in developed countries such as Western Europe (Combs, 2001). In Europe, Se intake by adults is in the range of 30-100 µg d^{-1} (Combs, 2001). In the Se deficient areas, a low Se uptake by humans is related to a low Se uptake by food crops from the soil, which in turn depends on the plant availability of soil Se levels and different Se forms that may occur in the soil. Over the last decade, the Se status of the UK population declined due to decreased Se contents in the wheat which is primarily consumed in the diet (Rayman, 2000). To increase Se contents in the food crops, Se may be supplemented to the soil as part of a Se fertilizer.

However, there are also regions in the world with very high soil Se concentrations, leading to Se toxicity (Banuelos and Dhillon, 2011). The fact that Se has a very narrow range between dietary deficiency and toxicity (40 µg d^{-1} to 400 µg d^{-1} per adult, respectively) (Fordyce, 2007) makes it necessary to control its intake by humans and animals, and hence it is important to understand the relationship between environmental exposure and health. It is important to develop strategies improving Se uptake in deficient regions without accumulating Se to levels that may be toxic to humans or the environment. In an attempt to increase Se intake by humans through crops, several strategies were tested, which includes foliar application, inorganic fertilization, seed treatment and soil incorporation of fly ash, municipal incinerator ash, or sewage sludge (Arthur et al., 1992; Logan et al., 1987).

Selenium absorption was significantly higher for wheat (81%) and garlic (78%) compared to fish (56%). Wheat and its products were reported to be among the most effective crops for Se supplementation when studying the efficiency of human Se absorption from three food sources (Hawkesford and Zhao, 2007; Lyons et al., 2003). Due to the high uptake of Se from wheat and the primary need to focus on staple food crops, wheat may be a good choice for enhancing the Se status of a Se-deficient population.

It is well know that the availability of trace elements to plants does not only depend on the contents of these elements in the soil, but also on soil factors such as pH, redox conditions, soil texture, mineralogy, organic matter content and the presence of competitive ions

(Fordyce, 2007). Some of these factors may be altered during field cultivation. However, it is not yet clear how some soil amendments and management practices may affect Se mobility, availability and its uptake by wheat after fertilizing the soil with different types of Se fertilizers, and how this is affected by ageing of the soil after its amendment. Therefore, we studied the impact of liming and application of compost, cow manure, and pig manure on the mobility, availability and uptake by wheat of Se supplied to the soil through two types of fertilizers (selenite and selenate). We also focused on the role of soil ageing by growing wheat plants on the amended soils at different time intervals after fertilizing and amending the soil.

8.3 Materials and methods

8.3.1 Pot experiment

Sandy loam soil originating from the upper 30 cm of an agricultural field was used for all treatments. It had a pH of 6.6, 3.5% organic matter, an electrical conductivity of 0.4 dS m^{-1} and a Se content of 0.3 mg Se kg^{-1} dry soil. Forty-five kg of homogenised air-dried soil was divided into two groups and each group was further divided into five sub-groups. Of these, one group was used as reference soil while the remaining four were amended with fresh compost, cow manure, pig manure and lime ($CaCO_3$), respectively. Before supplying organic matter or lime, the soils were fertilized with 1 mg Se kg^{-1} in the form of selenate or selenite. The fresh compost, cow manure and pig manure were oven-dried at 60^{o}C for 48 h to determine their dry weight contents, which were 78.3, 21.2 and 28.7%, respectively. In each soil receiving treatment with an organic amendment, an amount of fresh weight corresponding to 5% dry weight of the applied organic amendment was added to the soil.In the lime amended soil, lime was added to 5% of the soil dry weight. In each subgroup controls receiving the amendments without Se were also included. To ensure homogeneity, the soils were thoroughly mixed in open top plastic containers after receiving the amendment. No amendments were added to the reference soil. Each amended soil was distributed over one pot containing 1 kg soil used for soil and pore water sampling, as well as three pots containing 500 g soil used for growing the wheat after several time intervals (Figure 29).

The treatments were categorized as Group 1 (Treatment 1): the reference soil, Group 2 (Treatment 2): the compost amended soil, Group 3 (Treatment 3): the cow manure amended soil, Group 4 (Treatment 4): the pig manure amended soil, and Group 5 (Treatment 5): the lime amended soil. Each group contains soils who did not receive Se fertilizer (control) and two soils receiving different Se fertilizers (Na_2SeO_3 and Na_2SeO_4). In each group, subgroups were assigned and labeled as T-0, T-1 and T-2, representing the time periods at which the wheat was grown. T-0 represents the treatments in which wheat was grown after 10 days, and T-1 and T-2 represent the treatments in which wheat was grown after one and two months, respectively. At each of these time points, 10 seeds of wheat were sown in a pot used for growing the wheat, from which eight plantlets were kept in each recipient by removing the least developed plantlets after five days. At 30 days after sowing, the aboveground biomass was harvested. The different plants in each pot were pooled and fresh and dry weights of the aboveground plant parts were determined. Afterwards, this plant material was powdered for further analysis. The average minimum and maximum temperature during the entire growth period was 20 ^{o}C and 27 ^{o}C, respectively.

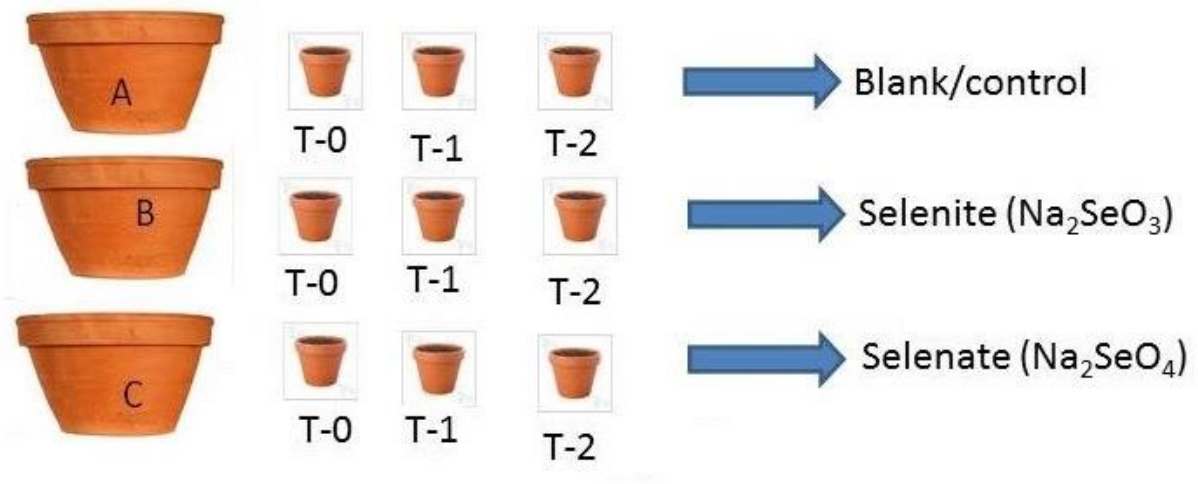


Figure 29. Experimental set up used for each type of soil amendment. Larger pots (A, B, C) were used to obtain pore water and soil samples during the entire experimental period of three months. The small pots were used to grow wheat. T-0, T-1 and T-2 refer to the pots used to grow wheat after 10 days, 1 month and 2 months of soil equilibration, respectively

8.3.2 Soil characterisation

Soil properties were determined on soil samples taken before fertilizing the soil with Se fertilizer. Soil pH was determined in a soil suspension (10 g soil: 25 mL deionized H_2O) using a calibrated pH meter (Thermo Scientific Orion 520A, Vantaa, Finland). The organic matter (OM) content and cation exchange capacity (CEC) were determined using the procedures described by Van Ranst et al. (1999). Total Se and sulphur (S) were determined in aqua regia digests using ICP-MS (PerkinElmer DRC-e, Waltham, MA, USA). Moreover, NHOAc-EDTA (pH 4.6) and $CaCl_2$-extractable Se contents were determined in soil samples taken before Se application and after harvest, according to Van Ranst et al. (1999). Selenium contents were measured in the extracts using ICP-MS as described by Lavu et al. (2012).

8.3.3 Porewater samples

Rhizon samplers type MOM having a pore size of about 0.15 µm (Rhizosphere Research Products, Wageningen, The Netherlands) were installed in the pots used for porewater and soil sampling. Porewater samples were taken by connecting vacuum tubes to the samplers. The porewater samples collected in the vacuum tubes were acidified with a drop of concentration HNO_3 and analysed by ICP-MS after dilution with internal standard (Indium).

8.3.4 Total Se and S determination in wheat plants

For the determination of total Se and S in the plant samples, 0.2 g sample was placed into a centrifuge tube followed by addition of 2.5 mL concentrated HNO_3 and 2.5 mL 30% H_2O_2. After 16 h, the tubes were capped and placed in a microwave oven. In a first step, the temperature was raised to 55 °C for 10 min at 600 W and 100% power. Afterward, the temperature was raised to 75 °C for 10 min. Finally, it was maintained at 100 °C for 30 min. After digestion, Se and S were measured.

8.3.5 Quality control

Certified reference material of spruce needles (BCR-CRM 101) and white clover (BCR-CRM 402) were analysed for S and Se, respectively, to control quality of the data. The measured S content of the spruce needles was 1568 ± 12, whereas 1690 ± 38 mg kg^{-1} is certified. The concentration of Se in the white clover was found to be 6.5 ± 0.28 mg kg^{-1}, whereas it was certified as 6.7 ± 0.27 mg kg^{-1}. As the experimental setup was not replicated, the analytical variability was assessed by analysing some randomly chosen plant samples three times for Se and S, which illustrated that the reproducibility of the analysis was high (Table 19).

Table 19. Results of replicate analysis of plant selenium and sulphur concentrations to assess the analytical variability (average ± standard deviation, n = 3)

Treatment	Sample	S/ mg kg^{-1}	Se/ µg kg^{-1}
Reference_blank_selenite	T-0	3852 ± 133	16483 ± 967
Cow manure_selenite	T-0	3139 ± 12	1605 ± 107
Cow manure_selenate	T-0	4090 ± 28	20924 ± 3230
Compost_blank	T-1	3240 ± 85	545 ± 192
Lime_selenate	T-1	9761 ± 488	303775 ± 31559
Reference_blank	T-2	3441 ± 43	445 ± 61
Pig maure_selenate	T-2	4920 ± 51	33868 ± 1118
Lime_selenite	T-2	4044 ± 282	14362 ± 125

8.3.6 Statistical data analysis

Simple correlation analysis was performed using SPSS 11.5 for Windows (SPSS Inc., USA). Pearson correlation coefficients were calculated to determine relationships between Se

contents in the wheat plants and Se contents which can be extracted from the soils using various extraction methods.

8.4 Results

8.4.1 Soil pH

In all soils, the initial soil pH was lower than the pH measured after one and two months (Table 20). Applying pig manure resulted in a decreased pH compared to the non-amended, cow manure amended and compost-amended soil. Applying cow manure resulted in the highest increase of soil pH. Initially, the pH was already higher in the soil amended with cow manure compared to the non-amended soil. Moreover, the pH still increased predominantly between the first and the second month. When lime was used, the pH was also higher compared to the non-amended soil and it also increased significantly with time, but it stabilised already after one month. The pig manure amended soils and the reference soils at the first sampling time had the lowest pH (6.6) while the soil amended with cow manure sampled after two months had the highest pH (8.7).

Table 20. Effect of amendments on soil pH (T-0: after two weeks, T-1 and T-2: after one and two months, respectively) and organic matter (%)

Amendment	Spike	pH				Organic matter (%)
		T-0	T-1	T-2	Avg ± stdev	Avg
Reference	Blank	6.6	7.5	7.4	7.1±0.5	3.4
	Selenite	6.6	7.5	7.2	7.1±0.4	3.5
	Selenate	6.6	7.5	7.3	7.1±0.5	3.4
Compost	Blank	6.7	7.6	7.5	7.2±0.4	4.6
	Selenite	6.7	7.5	7.5	7.2±0.5	5.0
	Selenate	6.8	7.5	7.6	7.2±0.5	5.2
Pig manure	Blank	6.6	6.9	7.1	7.3±0.4	6.9
	Selenite	6.6	6.9	7.0	6.9±0.2	6.1
	Selenate	6.8	6.7	7.1	6.8±0.2	5.9
Cow manure	Blank	7.3	8.1	8.5	6.9±0.2	6.9
	Selenite	7.2	7.8	8.7	7.9±0.6	7.6
	Selenate	7.2	7.9	8.1	7.9±0.7	7.3
Lime	Blank	7.0	7.6	7.9	7.8±0.5	3.4
	Selenite	7.0	7.8	7.9	7.5±0.5	3.4
	Selenate	7.0	7.8	7.8	7.5±0.5	3.5

8.4.2 Soil organic matter (OM) content

The organic matter content significantly increased when cow manure, pig manure and compost were added with cow manure resulting in the highest increase (Table 20). Lime treatment did not significantly increase the organic matter content compared to the non-amended soil. Significant negative correlations were found between the soil OM content on one hand and plant Se concentrations and plant Se uptake on the other hand (Table 23).

8.4.3 Soil-extractable Se

Aqua regia is used for extracting pseudo-total trace element contents, so logically it always extracted the highest Se amounts (Table 21). EDTA, a strong complexing agent, extracted more Se compared to $CaCl_2$. The highest $CaCl_2$- and EDTA-extractable concentrations were usually observed after one month of incubation. Overall, Se was recovered more in $CaCl_2$- and EDTA-extracts when selenate was used compared to when selenite was used, indicating a higher mobility of selenate compared to selenite. When soils spiked with selenate were amended with compost and lime, the $CaCl_2$ and EDTA extractability of Se increased compared to the non-amended soil, whereas the use of pig and cow manure decreased it.

8.4.4 Se concentrations in the porewater

The Se concentrations in the porewater of spiked soils generally decreased with time (Table 21). In the selenate-spiked soils, Se concentrations in the porewater were much higher compared to the selenite-spiked soils. The use of amendments resulted in lower Se concentrations in the porewater compared to non-amended soil. Cow manure was the most effective in reducing Se concentrations in the porewater, whereas liming and compost were the least effective. However, in the non-spiked soil, pig and cow manure resulted in an increase of Se concentrations in the porewater. Porewater concentrations usually decreased with time in the spiked soils, except in the compost-amended soil spiked with selenate (Table 21). Correlation analysis shows significant correlation with plant Se concentrations and uptake in wheat plants for both the selenite- and selenite-fertilized soils (Table 25).

Table 21. Selenium extracted from the soils using different extraction methods

Amendment	Spike	Aqua regia (µg kg^{-1})				$CaCl_2$ (µg kg^{-1})				EDTA (µg kg^{-1})				Pore water (µg L^{-1})			
		T-0	T-1	T-2	Avg ±stdev	T-0	T-1	T-2	Avg ±stdev	T-0	T-1	T-2	Avg ±stdev	T-0	T-1	T-2	Avg ±stdev
Reference	Blank	298	510	533	447±130	12	9	10	10±2	52	62	55	56±5	6.2	6.5	6.9	7±0
	Se1*	1398	1331	1533	1420±103	110	151	103	121±26	463	602	478	514±76	264	366	239	290±67
	Se2*	1478	1733	1637	1616±129	699	1276	902	959±293	1394	2322	1662	1793±478	4043	2204	2966	3017±924
Compost	Blank	542	444	498	495±49	12	8	7	9±3	64	60	48	57±8	6.2	2.3	3.7	4±2
	Se1*	1438	1280	1223	1314±111	119	126	87	111±21	511	520	413	481±59	207	187	160	185±24
	Se2*	1355	2359	1879	1864±502	769	1576	1213	1186±404	1658	3125	2152	2312±746	2684	2600	2793	2692±97
Pig manure	Blank	605	639	691	645±43	17	17	14	16±2	75	66	56	66±10	21.2	12.6	8.2	14±7
	Se1*	1457	1428	1787	1557±199	150	211	137	166±40	452	415	353	407±50	280	187	143	203±70
	Se2*	1577	1323	1886	1595±282	792	1906	1196	1298±564	1684	3215	2027	2309±803	1787	1410	1193	1463±301
Cow manure	Blank	570	486	502	519±45	14	14	11	15±2	76	72	63	70±7	17.3	15.1	16.2	16±1
	Se1*	1380	1192	1524	1365±166	65	70	56	64±7	415	322	324	354±53	122	90.6	54.3	89±34
	Se2*	1540	2854	1963	2119±671	236	497	327	353±132	587	978	621	729±217	1230	751	401	794±416
Lime	Blank	441	428	495	455±36	13	9	8	10±3	75	57	56	63±11	7.4	6.2	6.2	7±1
	Se1*	1486	1411	1542	1480±66	100	158	96	118±35	518	593	455	522±69	232	206	145	194±45
	Se2*	1371	1724	1481	1525±181	807	1484	721	1004±418	1778	2385	1373	1845±509	3769	2260	1237	2422±1274

Se1*: selenite treatment, Se2: selenate treatment

8.4.5 Se concentrations in wheat

The plants grown on selenate-spiked soils had the highest Se concentration followed by those grown on selenite-spiked soils (Table 22). The highest Se concentrations were seen in plants grown on lime-treated and reference soils spiked with selenate (Table 22). Wheat plants grown on lime-treated soils spiked with selenite fertilizer show higher Se concentrations compared to soils treated with organic amendments. They are however lower than those of the reference soil spiked with selenite. The plants grown on soils amended with cow and pig manure had lower Se concentrations compared to the plants grown on soils amended with compost (Table 22). The Se concentration in plants grown just after amending the Se-spiked soils (T-0) was generally higher than in those grown after 1 and 2 months (T-1 and T-2). However, when the plants were grown on non-amended soils, Se concentrations were higher after 1 and 2 months. Overall, the plants grown on non-amended soils spiked with selenite had higher Se concentrations compared to the plants grown on amended soils.

8.4.6 Sulphur concentrations in wheat plants and soils

The plants grown on soils spiked with selenate had a higher S concentration compared to plants grown on soils spiked with selenite (Table 22). The S concentrations in plants grown on soils spiked with selenite did not differ significantly from those of plants grown on soils that were not spiked. The S concentrations in the plants grown on soils amended with cow and pig manure were always lower than S concentrations in plants grown on limed soils and soils amended with compost. Plant S concentrations and uptake were significantly correlated with plant Se concentration and plant Se uptake. A higher correlation was observed for plants grown on selenate-treated soils compared to plants grown on selenite- treated soils (Table 23). The soil S concentrations are negatively correlated with plant Se concentrations and Se uptake by the plants (Table 23).

Table 22. Dry weight per pot (g), and plant Se and S concentrations of wheat plants grown at three different time periods

Amendment	Spike	Plant Se (mg kg^{-1})				Plant S (mg kg^{-1})				Dry weight (g) per pot			
		T-0	T-1	T-2	Avg ± stdev	T-0	T-1	T-2	Avg ± stdev	T-0	T-1	T-2	Avg ± stdev
Reference	Blank	0.2	1.2	0.5	0.6±0.5	3656	3347	3472	3492±155	0.8	1.3	0.8	1.0±0.3
	Selenite	15.8	19.5	18.0	17±2	3758	3970	3863	3864±106	0.8	1.1	0.6	0.8±0.3
	Selenate	375	318	436	376±59	6708	5840	8477	7008±1344	0.7	1.0	0.8	0.8±0.1
Compost	Blank	0.7	0.7	0.7	0.7±0.0	3565	3302	3576	3481±155	0.9	1.0	0.9	0.9±0.1
	Selenite	14.1	11.6	13.9	13±1	3645	3663	3950	3753±171	0.8	1.3	0.8	0.9±0.3
	Selenate	577	287	387	417±147	11761	6785	8691	9079±251	0.6	1.2	0.8	0.9±0.3
Pig manure	Blank	0.6	0.8	1.5	1.0±0.5	3525	3037	3453	3338±264	1.0	0.6	1.2	0.9±0.3
	Selenite	2.6	2.1	2.6	2.4±0.3	3720	3733	3452	3635±159	0.8	1.3	1.2	1.1±0.2
	Selenate	32.9	37.1	33.1	34±2	4683	6097	4884	5221±765	1.0	1.1	0.9	1.0±0.1
Cow manure	Blank	0.2	0.4	0.1	0.2±0.2	3102	3249	3127	3159±78	1.0	1.2	1.0	1.1±0.1
	Selenite	1.5	1.4	1.1	1.3±0.2	3131	3181	3033	3115±75	1.1	1.0	0.9	1.0±0.1
	Selenate	18.6	13.6	11.4	15±4	4070	4060	3205	3779±496	0.9	1.5	1.1	1.2±0.3
Lime	Blank	0.2	0.2	0.1	0.2±0.1	3623	3303	3422	3449±162	0.8	1.1	1.1	1.0±0.2
	Selenite	18.1	14.8	14.5	16±2	4178	3920	4244	4114±171	0.7	1.2	1.1	1.0±0.2
	Selenate	571	281	436	429±145	10194	10107	9599	9966±321	0.7	1.1	0.9	0.9±0.2

Table 23. Linear correlation coefficients (R) between soil characteristics and plant biomass on one hand, and plant Se concentrations and plant Se uptake on the other hand.

Soil & plant parameters	Plant Se concentration (mg kg^{-1})		Plant Se uptake (mg per pot)	
	Selenate	Selenite	Selenate	Selenite
OM	-0.389*	-0.451*	-0.407*	-0.431*
CEC	-0.067	-0.313	-0.082	-0.305
pH	-0.109	-0.080	-0.077	0.003
Soil S	-0.425*	-0.406*	-0.444*	-0.391*
Plant biomass	-0.415*	-0.167	-0.277	0.045
Plant S	0.938**	0.711**	0.909**	0.700**

* and ** indicate statistical significance at $p < 0.05$ and $p < 0.01$, respectively

8.4.7 Wheat biomass production

The average dry weight of the wheat plants per pot was calculated (Table 22). It was highest for the plants grown after one month of soil ageing and lowest for those grown just after amending the soils. A negative correlation between Se concentrations and biomass production was observed when the soils were fertilized with selenate (Table 23).

8.4.8 Impact of soil amendments on Se uptake by the wheat plants

Among organic amendments, cow manure amended soils of selenate and selenite shown lowest Se uptake (1.1 and 0.1% respectively). Compared to all treatments, higher Se uptake was shown in plants grown on lime treatment with selenate (34%). Application of lime increases Se uptake when soils were fertilized with selenate and such increase was not seen in plants grown on selenite fertilized soils upon lime application (Table 24).

Table 24. Effect of soil amendments on Se uptake in wheat plants

Treatments	Selenate fertilized		Selenite fertilized	
	Se µg/pot	% Se uptake	Se µg/pot	% Se uptake
Reference	313±33	26.9	15±5	1.6
Compost	330±16	26.9	12±2	1.4
Pig manure	33±7	3.8	1.8±0.5	0.2
Cow manure	16±4	1.1	1.1±0.3	0.1
Lime	355±45	34.3	15±2	1.5

8.4.9 Comparison of suitability of the soil extractions to predict concentrations and uptake of Se in the plants

Correlation analyses were performed to relate Se uptake by the wheat with extractable Se contents in soils (Table 25). The correlation analyses were performed considering all soils from the three different time points. Of the three soil extracts, EDTA-extractable Se was significantly correlated with Se concentrations and uptake in the wheat when grown on selenite treated soil whereas, aqua regia was correlated with Se uptake in plants when grown on selenate treated soils. However, $CaCl_2$ extraction was not significantly correlated with wheat Se concentration or uptake.

Table 25. Linear correlation coefficients (r) between Se extracted from the soils by different extraction methods and plant Se uptake and concentrations in wheat plants

Soil extraction method	Plant Se (mg kg^{-1})		Plant Se uptake (per pot)	
	Selenate	Selenite	Selenate	Selenite
Aqua regia	-0.269	0.285	0.739**	0.193
$CaCl_2$	0.080	0.097	0.167	0.241
EDTA	0.164	0.805**	0.116	0.883**
Pore water	0.770**	0.648**	-0.579*	0.627*

* and ** indicate statistical significance at the probability level of $p < 0.05$, and $p < 0.01$, respectively

8.5 Discussion

The present study indicates that Se concentrations in the pore water on Se-fertilized soil decreases when organic amendments are used. This effect depends on the type of organic amendment used, with cow manure and pig manure having a larger effect than compost.The porewater extractable Se was found to decrease with time. The high selenate concentration compared to the selenite concentration is due to the fact that selenate is weakly adsorbed on the soil than selenite on the other hand reacts strongly with the soil (Ylaranta, 1983). Selenate therefore will be leached more easily compared to selenite. The lowest values for the cow manure at the starting period (selenite 121 µg L^{-1} and selenate 1230 µg L^{-1}) are in conformity with the findings of Dhillon et al. (2010), who reported that incubating naturally occurring selenium forms with organic amendments led to a substantial decrease 20%-26% of easily available (water-soluble and extractable) forms of Se and a corresponding increase of 13%-62% in the less available Se forms (organic matter and metal oxide bound). The decreasing trend of Se availability to plants is in agreement with the study reported on wheat grown on seleniferous soils with farmyard manures (Dillon et al., 2010). A higher Se porewater concentration was obtained for selenate treatments irrespective of amendments compared to selenite treatments, which indicates that the selenate in these soils was weakly bound. Aside the effect of the organic treatments, variation in porewater metal concentrations may also be

related to the moisture regime of the soil (Du Laing et al., 2007). However, in the current study setup, equal moisture contents were maintained for the soils.

Selenium uptake in the wheat was influenced by various organic amendments. A clear increase in organic matter of cow and pig manure caused a greater decrease in Se concentrations in wheat. This effect of organic amendments was in agreement with what was reported in a study conducted by Dhillon et al. (2010). However, Dhillon et al. (2010) reported a lower effect with the decrease ranging between 21 and 28% for farm yard manure and 84-97% for other organic amendments when an application rate of 5% was used. Notably, the soil used in Dhillon et al. (2010) contained high Se concentrations (4.5 mg Se kg^{-1}). It has been reported that in presence of organic matter, organic anions may bind to Se meanwhile also promoting adsorption on the solid soil matrix and reducing the soluble fractions available for plant uptake (Wijnja and Schulthess, 2000). Moreover, use of organic amendments, especially cow manure and pig manure, may promote microbial activity, resulting in the production of microbial biomass and the establishment of reducing conditions.

In contrast to organic manures, compost treatment increases Se accumulation in plants when soils were treated with selenate. At time T-0 and T-1, Se accumulation increase to about 14 and 8% but at T-2 a decrease of 5% was observed. An increase in Se concentrations in compost-selenate (T-0 and T-1) due to higher Se availability and decrease over T-2 time point can probably be linked with the alternate wetting and drying of the soil which promoted rapid transformation of selenate into other forms such as selenite and organic Se that were adsorbed onto soil surfaces (Neal and Sposito, 1991). Moreover, the organic materials used in the current study were obtained from different sources and might have been in various decomposition states. During decomposition of organic manures, the differences in release of monocarboxylic and multicarboxylic organic ligands results in retention of various levels of Se in soils and made Se unavailable to plants (Ferri and Sangiorgio, 1999). In the current study, the organic amendments from various sources might had different S and P contents in various redox states, which could have contributed to differences in Se uptake. It was previously well documented that selenate in soil is taken up by plants via S transporters (Terry et al., 2000). Presence of high amounts of S ions inhibit Se uptake when selenate is the dominant soil Se form (Hopper and Parker, 1999). In contrast, there was no proven assimilation pathway like sulphur-selenate for P. However, it was reported in various studies that P levels in soils could determine the Se uptake particularly when selenite is the dominant

Se form in the soil. Some studies confirm an antagonistic effect of phosphate on selenite uptake in plants (Hopper and Parker, 1999; Broyer et al., 1972), whereas others found little effect (Mora et al., 2008; Nakamaru and Sekine, 2008; Yläranta, 1990b).

On the other hand, compared to reference spiked soils, lime treatment increases Se uptake in wheat plants at T-0 and T-2 time points of both selenate and selenite grown. Although there is a decrease in Se uptake at time point T-1 of 5 and 16% of selenate and selenite of lime amended soils. The increase of Se in lime amended soils decreases Se binding to soil chelates due to increase of OH^- ions concentration, which in turn leads to increase in Se uptake. Nevertheless, decrease of Se in T-1 aged soils was due to the binding of Se to clay, carbonate, extractable Al and Fe oxide content (Elsokkary, 1980, Levesque, 1974). However, the decrease in Se contents upon lime treatment is relatively lower compared to organic amendments.

Noticeable variations in Se uptake by the plants were observed between the various soil amendments. Significant correlations are observed between plant Se concentrations and uptake, and Se accumulation in these various treatments and pore water measurements. Thus, for Se fertilized soils pore water analysis could provide indirect assessment of the amount of Se available to the plant. The other soil extractions methods were not much successful to obtain significant correlations compared to porewater analysis. In fact, the mobility of Se was influence by the supply of organic matter, which was reflected in the results of soil extractions. However, these soil extraction methods such as $CaCl_2$ and EDTA-extractable Se concentrations may vary significantly due to the great impact of soil physical and chemical factors, which include $CaCO_3$, pH, silt content, clay content, available iron and organic matter (Zhao et al., 2005). Aqua regia, on the other hand, being a stronger extract reflects total Se contents. Of the three soil extracts, EDTA-extractable Se was most significantly correlated with Se concentrations in the wheat grown on selenite fertilized soils. This extraction was previously reported to result in a highly significant correlation with plant Se concentration for seleniferous organic soils (Williams and Thornton, 1973). For aqua regia, being strong extraction method, significant correlations were obtained for plant Se uptake when plants were grown on selenate-fertilized soils. The $CaCl_2$ extraction method resulted in no significant correlation with plant Se.

The soil amendments tested have an influence on pH. The shift of pH in each treatment was seen over the different time points. From table 20, it can be observed that there is an impact

of amendments and soil age on pH. Therefore, pH has an effect on Se bioavailability and subsequent uptake by plants (Fordyce, 2005). Almost all soils at the starting period had a neutral pH though with different values within the range. On average, they became slightly alkaline after the first month except for the pig manure amended soil which remained neutral. Liming resulted in a higher pH in the lime-amended soils compared to the non-amended soils. However, soil pH had no correlation with plant Se accumulations. Some soil properties, such as CEC and organic matter (OMshowed significant correlation with plant Se accumulation. The correlation of OM shows a negative relation with plant Se for both selenate and selenite fertilized soils. In these organic amended soils, there is an increase in CEC (data not shown). The organic content of the compost, cow and pig manure amended soils could be responsible for the high CEC (on average higher than 12.3 cmol + kg^{-1}) in these treatments compared to the reference Se and lime amended soils. In the surface horizon of mineral soils, soil organic matter is responsible for 25-90% of the total CEC (Van Dijk, 1971).

Dry weights of the aboveground plant parts were not significantly influenced by Se uptake. However, there is a significant negative correlation with Se concentrations in plants grown on selenate-fertilized soils. This confirms the fact that in wheat dry matter production is affected by high plant Se concentrations (Rani et al., 2005). In wheat, selenate follows the active sulphate pathway during uptake (Li et al., 2008), whereas selenite is taken up by plants via passive diffusion (Arvy, 1993). Accordingly, in our study Se and S are better correlated in plants grown on selenate-fertilized soil than in plants grown on selenite-fertilized soil (Table 23). The high S concentrations in the plants grown on soils spiked with selenate correspond with a high plant Se concentration. A synergistic relationship between Se and S was observed, which may be explained by the fact that elevated concentrations of Se in the root zone might increase plant S accumulation when sulphate concentrations are low in the root zone (Mikkelsen et al., 1988). The S concentrations in plants grown on lime-treated soil seem to be higher even though the S concentration in the soil was lower compared to organic amendments. Soil liming seems to enhance S uptake in the plants which leads to higher S and Se concentrations.

Soil ageing influenced Se uptake in wheat plants irrespective of soil amendments. These differences could be attributed to variations in soil conditions. An important parameter which might influence the variation in Se uptake by wheat plants is the quantity and quality of soil OM which changes upon ageing and has an impact on selenium retention in soils (Coppin et

al., 2006). Additionally, soil pH shifts were observed between the three different time points; however, there is no significant correlation with plant Se uptake. Moreover, wetting and drying of the soil has been reported to promote microbial changes which might lead to rapid transformation of Se into other insoluble Se and organic Se forms (Burger et al., 2005, Neal et al., 1991). These Se forms may differ in mobility and availability to plants.

8.6 Conclusion

Soil management practices seem to affect Se uptake by crops upon Se fertilization of soils due to their influence on soil pH and organic matter contents. In order to increase the Se status in wheat, amendments need to be chosen carefully. Cattle manure is not recommended to apply along with Se fertilizers when the aim is to enrich food crops with Se. Soil liming improves Se concentrations in crops especially when selenate fertilizers are used. Selenium accumulation in the soil when Se is applied to the soil in the form of selenite or when soils are amended with cow and pig manure poses an environmental risk. However, organic amendments may also supply the amount of Se available for plant uptake especially when compost is used as soil amendment. Long-term field studies monitoring Se mobility and bioavailability in soils amended with seleniferous crop residues and organic wastes are needed to be able to outweigh risks for Se accumulation in the soil against potential for Se supply to the crop. In the current study, higher doses of organic amendments were tested. Application of various organic amendments at this level shows a clear difference in Se uptake. However, Se uptake might be different at lower doses. Future studies should focus on lower doses, more relevant to field application, and evaluate the influence of microbial activity and inputs of S and P through these amendments.

Chapter 9. Selenium gastrointestinal bioaccessibility is matrix- and speciation dependent and is significantly increased by active colon microbiota

9.1 Abstract

Selenium (Se) is an essential nutrient for humans as it plays an important role in glutathione peroxidase (GPx) activity. Moreover, there is increasing evidence that a sufficient level of Se in the diet is effective in reducing cancer risks in both humans and animals. The objective of this work was to examine the bioaccessibility of selenium (Se) in three different Se-enriched food supplements and two different Se-enriched food crops, with reference to two pure Se standards (sodium selenate and SeMet), and their speciation changes. This was done using an in vitro gastrointestinal digestion procedure that mimics stomach, small intestine and colon. Additionally, the impact on microbial activity was investigated by measuring presence of short chain fatty acids and also the role of colon microbes in Se bioaccessibility was assessed by using heat-inactivated colon microbiota in comparison with normal SHIME suspension as presence of active colon microbes. Clear differences in bioaccessibility patterns were observed between the different Se containing matrices with Se-enriched food crops showing the highest Se bioaccessibility upon colon digestion. The impact of microorganisms on Se bioaccessibility in the colon was demonstrated by the significantly lower Se bioaccessibility values upon digestion with heat-inactivated colon microbiota. While selenite was found to be highly stable throughout the entire digestion, incubation of SeMet resulted in the production of two minor metabolites, identified as MeSeCys and Se(O)-methionine. In conclusion, a clear contribution of colon microbiota towards Se bioaccessibility was observed and possible biotransformation of Se species was highlighted. The higher proportion of Se reaching the colon phase from Se-enriched foods compared to Se supplements could have a possible impact on their role in inducing colon health beneficial effects.

9.2 Introduction

Selenium (Se) enters the food chain through plants, which take up Se from the soil. Geographic variations in soil Se concentrations around the world were previously reported, ranging from high concentrations in soils of the USA and Venezuela to low concentrations in Korea, some regions of China and some parts of Europe (Brtkova and Brtko, 1996, Ferguson et al., 2004, Rayman, 2000, 2005). The recommended daily intake for healthy adults is 55

µg/day in the USA. It ranges between 55 and 70 µg/day in Europe (El-Bayoumy, 2001, Rayman, 2004, Whanger, 2004). In Se-deficient populations, Se-enriched food crops and Se-enriched food supplements are often recommended to overcome Se deficiency. In particular, Se-enriched wheat, cabbage, mushroom, pumpkin, broccoli, onions, chives and garlic have been recommended as Se-fortified food crops (Dumont et al., 2006a, Govasmark et al., 2010, Kapolna et al., 2007). Among these, *Brassica* and *Allium* species were often reported as preferential crops due to their higher potential for accumulating specific organic Se species such as MeSeCys and γ-glut-cyst, that were previously reported to induce beneficial health effects (Thomson, 2004). However, it is technically more feasible to produce Se-enriched yeast as a Se-enriched food supplement. Such yeast also has the potential to accumulate organic species, particularly some yeast species such as baker's yeast (*S.cerevisiae*) (Reyes et al., 2006). Selenium supplements containing Se-enriched yeast are commercially available as tablets with SeMet as major compound.

An association between Se deficiency in the diet and cancer risk was already reported more than 40 years ago (Shamberger and Frost, 1969). In some human epidemiological studies conducted during the last years the relationship between dietary Se intake and cancer risk was studied (Connelly-Frost et al., 2009, Duffield-Lillico et al., 2002, Rudolf et al., 2008). The Nutritional Prevention of Cancer (NPC) trial by Clark and co-workers showed that Se supplementation reduced the risk for colon, rectum, prostate and lung cancers (Clark et al., 1996, Letavayová et al., 2006). Moreover, epidemiological studies conducted earlier have shown a geographical correlation between Se deficiency and high incidence of particular types of cancer, especially colorectal adenomas (Dworkin et al., 1988, Psathakis et al., 1998, Rumi et al., 1992). Dietary intake in 27 countries showed a significant inverse correlation with age-adjusted mortality for colon, prostate, breast, ovary and lung cancers, but there was a weak correlation for pancreas, skin and bladder cancers (Letavayová et al., 2006). In addition to epidemiological studies, *in vivo* experiments with rats suggested that ingestion of Se-enriched broccoli significantly reduced colon cancer compared to other Se forms, such as selenite (Finley et al., 2000). From this study it was concluded that Se from the Se-enriched broccoli does not accumulate in the body more rapidly compared to other supplementation forms of Se, but it could be more efficient in decreasing the formation of polyps/tumors in the colon and improving GPx activity in epithelial cells (Gong et al., 2012).

It is well-known that absorption and bioavailability of Se may depend on the chemical forms in which it occurs, i.e. its speciation. In recent years, the (potential) bioavailability of Se in

different food sources has been demonstrated by using various in vitro intestinal digestion procedures to assess bioaccessibility (Reyes et al., 2006). The term "bioaccessibility" has been defined as the fraction of a compound that is released from its matrix in the gastrointestinal tract and thus becomes available for intestinal absorption (and may enter the blood stream) (Fernandez-Garcia et al., 2009). Some studies already reported differences in bioaccessibility between different sources of Se (Brandt-Kjelsen et al., 2012, Govasmark et al., 2010, Moreda-Pineiro et al., 2011, Reyes et al., 2006). These *in vitro* studies were performed with Se-enriched food crops, Se supplements (tablets) and pure Se standard compounds, and usually mimic the stomach and small intestine (Moreda-Pineiro et al., 2011). However, no study reported on the bioaccessibility of Se in the large intestine (colon) yet. Moreover, it is not well understood how much Se from Se-enriched food sources becomes bioaccessible during colon digestion. We hypothesized that Se bioaccessibility in the colon after ingestion of some Se-enriched foods would be higher in upper intestine where a substantial amount of Se reaches the colon and has beneficial effects on colon epithelial cells and colon microbial flora. Therefore, we aimed to assess Se bioaccessibility in the colon in the presence of colon microbiota. Meanwhile, we also evaluated the bioaccessibility of Se from food supplements, Se-enriched food crops and pure standard Se compounds in the stomach and small intestine to be able to assess how much of the ingested Se may reach the colon environment. This was done using an *in vitro* gastrointestinal digestion procedure. For the two standard compounds, speciation changes during digestion were also studied.

9.3 Materials and methods

9.3.1 Reagents and standards

For sample preparation, protease XIV and the Se reference compounds sodium selenate (Na_2SeO_4) and SeMet were purchased from Sigma Aldrich, while concentrated HNO_3 and H_2O_2 were purchased from Chemlab (Zedelgem, Belgium). MilliQ® (MQ) water from Water Systems Ltd. (Brussels, Belgium) was used throughout the experiment. Chromatographic standards and other solutions were prepared freshly every day. To prepare gastric and small intestinal fluids, pepsin and pancreatin (porcine pancreas) were purchased from Sigma

Aldrich, and dehydrated galpowder (Difco TM Oxgall) and sodium bicarbonate from VWR (Leuven, Belgium).

9.3.2 Instrumentation

An inductively coupled plasma mass spectrometer (ICP-MS, PerkinElmer DRC-e, Sunnyvale, CA, USA) was used for total Se and as element-specific detector in speciation analysis. The ICP-MS was fitted with a Babington nebulizer and a cyclonic spray chamber. For speciation analysis, the ICP-MS was coupled to a liquid chromatography system (Series 200 HPLC, PerkinElmer, Sunnyvale, CA, USA). It consisted of a P680 HPLC pump and an ASI-100 automated sample injector. A Hamilton PRP-X100 anion exchange column and Altima C_8 column (250 mm × 4.6 mm I.D., 5 μm, 120 Å) were used as stationary phase. Both columns were equipped with a guard column containing the same stationary phase material. The extracts were analysed using the anion exchange column. However, the reversed phase column was also used to confirm the absence of the oxidised form of SeMet (SeMet oxide). HPLC-ICP-MS conditions as described in (see chapter 3). Extraction of Se for speciation analysis and batch incubations for bioaccessibility assessment was carried out using a shaker fitted in an incubator chamber from Sartorius (Goettingen, Germany). The samples were centrifuged on a Sigma 2-16PK centrifuge (Germany). For total Se determination, a microwave digestion apparatus from Mars (North Carolina, USA) was used. The short chain fatty acid analysis was performed using a gas chromatographic method (Van de Wiele et al., 2007).

9.3.3 In vitro gastrointestinal digestion with active microbiota in the colon phase of Se-enriched food crops and supplements

Two lyophilized and powdered samples of Se-enriched food crops (leek and kenaf) grown on soil fertilized with Na_2SeO_4 (see chapter 3 and 6) and three food supplements (tablets), i.e. SelenoPrecise tablets (SP, Se-enriched yeast), a Se+ACE-vitamins mixture (ACE), and a Se-enriched yoghurt–based tablet (YB), obtained from commercial available sources were used

for assessment of Se bioaccessibility. Three grams of lyophilized and powdered samples of Se-enriched kenaf and leek were boiled with 10 mL of deionized water for 3 min to mimic the food preparation process applied prior to human consumption. After cooling, 3 g of the obtained suspension was transferred into 100 mL amber colored bottles. Thirty mL of simulated gastric juice (10 g L^{-1} pepsin adjusted to pH 2.0 with 2M HCl) was added to the bottles and they were capped with a rubber stopper and aluminum seal. They were placed on a mechanical shaker (100 rpm) in an incubator (37 °C) for 1 h. After 1 h of incubation 5 mL was sampled with a syringe. The sample was considered to represent the gastric phase. Afterwards, 12.5 mL of small intestine fluid was added and the mixture was shaken in the incubator for 2 h again. The small intestine fluid was prepared by weighing 0.75 g dehydrated bile powder, 0.5 g of pancreatin and 1.5 g sodium bicarbonate into 100 mL of deionized water. After 2 h, 5 mL of sample, representing the small intestine was sampled. Subsequently, 25 mL of colon suspension, sampled from the colon compartments of the SHIME® (Simulator of the Human Intestinal Microbial Ecosystem) (Figure 30), was added to mimic the colon conditions. Thse SHIME is a dynamic model of the human gastrointestinal tract that mimics the physicochemical, enzymatic and microbiological conditions of the stomach, duodenum, colon ascendens, colon transversum and colon descendens in five consecutive compartments (Van de Wiele et al., 2004, Molly et al., 1994, Possemiers et al., 2006).

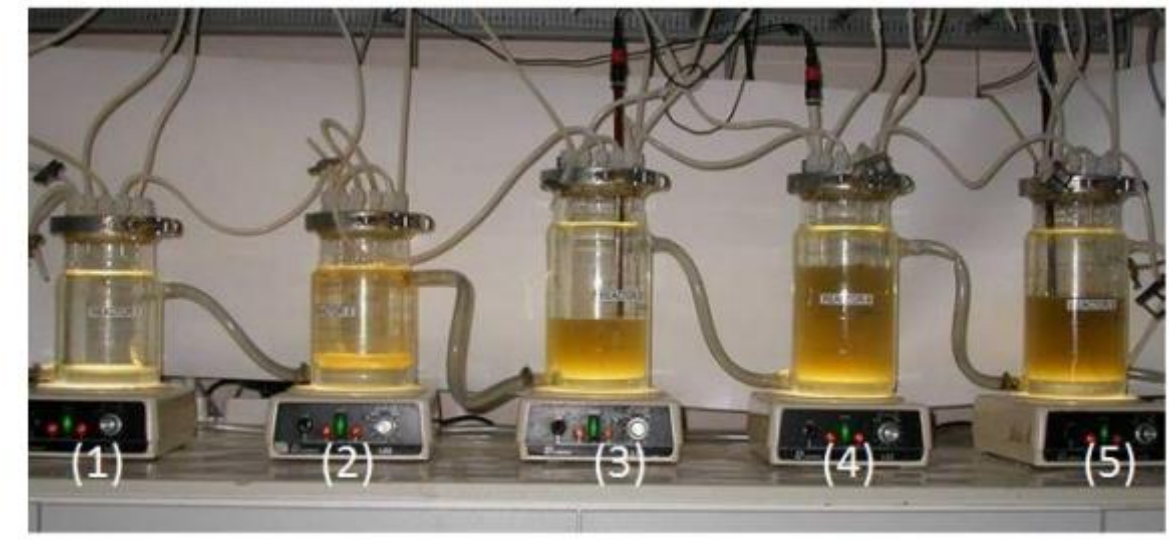


Figure 30. SHIME reactor. 1) stomach, 2) small intestine, 3) ascending colon, 4)transverse colon, 5) descending colon

The bottles were capped and flushed with nitrogen gas to create anaerobic conditions and immediately sampled after shaking which is considered as 0 h (T0) sample. The time between addition of SHIME suspension and nitrogen flushing is approximately 5-10 mins. They were again shaken in the incubator and sampled after 2 h (T2), 24 h (T24) and 48 h (T48). The samples collected in each step were placed in 10 mL polypropylene tubes and centrifuged at 10,000 g for 10 minutes. The supernatant collected after centrifugation was filtered (0.45 µm) and stored at -80 ºC. The collected filtrates and pellets (residues) were analysed for total Se using ICP-MS. For digestion of the pellet prior to analysis, microwave digestion with concentrated HNO_3 and H_2O_2 was used. A similar procedure was conducted for Se supplements (tablets) by weighing 0.3 g powdered samples which were prepared by crushing 20 tablets using mortar and pestle in order to obtain homogeneity of Se. They were used in the gastric phase, followed by the next digestion phases. The entire procedure was repeated using pure Na_2SeO_4 and SeMet spiked into the gastric solution to a concentration of 2.4 mg L^{-1}. All experiments were conducted in triplicate.

The relative bioaccessibility of Se was calculated for each digestion phase as

$$\% \text{ Bioaccessibility} = \frac{\text{Se in collected supernatant}}{\text{Total Se in the suspension}} \times 100$$

9.3.4 In vitro gastrointestinal digestion with inactivated microbiota in the colon phase

The procedure described in 9.3.3 was repeated for SelenoPrecise and pure SeMet. However, the SHIME suspension collected for use in the colon phase was now autoclaved twice for 30 min (121°C, 1 bar overpressure) to inactivate colon microbiota.

9.3.5 Short chain fatty acids analysis

To assess the microbial metabolic activity in the working conditions of our study short chain fatty acids (SCFA) analysis, reflecting the activity and ability of colonic microbiota to ferment carbohydrates and proteins in a sample (Jouany *et al.*, 1982), was conducted. The analysis was performed by gas chromatography using thawed samples of colonic phase at time point T2.

9.3.6 Stability of Se species during digestion

The reference Se species SeMet and selenate (2400 µg L^{-1}) were subjected to an in vitro digestion to monitor speciation changes in the bioaccessible fraction. The collected supernatants from each digestion phase were analysed by HPLC-ICP-MS

9.3.7 Selenium speciation of crops used for bioaccessibility study

Se-enriched crops (leek and kenaf), previously obtained by growing them on selenite fertilized soils, were used to assess Se bioaccessibility in the crops, which was compared with bioaccessibility in commercial food supplements and pure Se reference compounds. The speciation data of leek and kenaf were reported in chapter 3 and 6 (grown on lowest Se applied dose).

9.3.8 Statistical analysis

Results are expressed as mean±SD and one way analysis of variance (ANOVA) was carried out using a statistical analysis system (SAS version 9.2). Differences in the concentrations of Se in samples of the various digestion phases were tested with ANOVA. Significance of

differences was evaluated at the 0.05 level. Tukey's multiple-comparison test was used to compare differences in the mean values

9.4 Results

9.4.1 Bioaccessibility of Se from Se-enriched food crops, Se-enriched food supplements, and pure Se reference compounds

For both crops, the bioaccessibility of Se is higher in the small intestine compared to the stomach and it generally decreases with time in the colon (Figure 31A). The bioaccessibility did not differ between the crops in the stomach and small intestine, but it clearly differs in the colon, where a higher bioaccessibility was observed for leek as compared to kenaf.

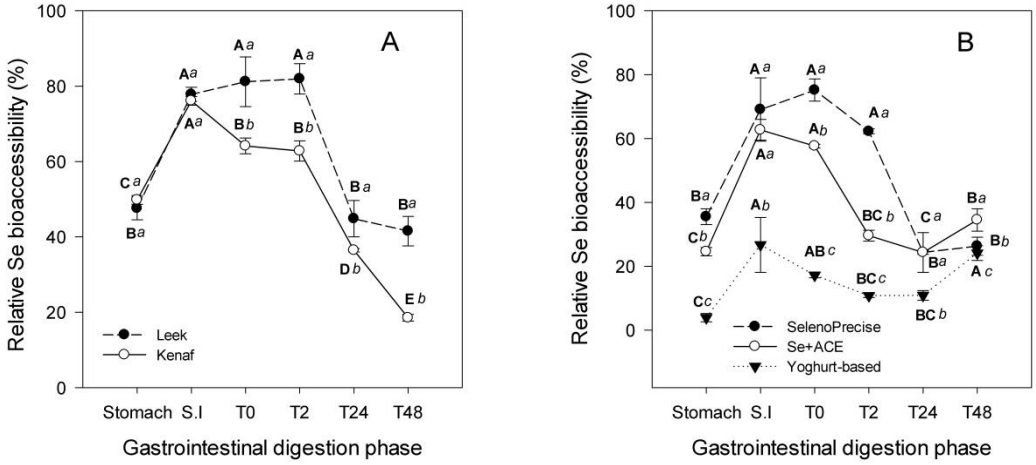


Figure 31. (A) Relative bioaccessibility of Se for (A) two Se-enriched crops and (B) three Se-enriched food supplements in different steps of an in vitro simulation of gastrointestinal digestion (T0, T2, T24 and T48 refer to 0, 2, 24 and 48 hours after starting colon incubation, respectively; S.I. refers to small intestine). The capital letters indicate Tukey's multiple-comparison test for comparison of means between the various digestion phases for each crop/tablet, whereas small letters indicate comparison of means between the different crops/tablets in each digestion phase. A same letter indicates no significant difference ($p > 0.05$).

The bioaccessibility of Se in the yoghurt-base Se supplement (YB) is significantly lower compared to the bioaccessibility of Se in the other Se supplements (SP and ACE) (Figure

31B). This is the case for all digestion phases. Moreover, the bioaccessibility is higher for SP compared to ACE in the first 2 hours of colon incubation. However, at 24 and 48 hours after starting the colon incubation differences between the different food supplements were much smaller. Whereas the bioaccessibility decreased in the first 24 hours of colon incubation, it slightly increased again afterwards. However, YB showed a significantly lower Se bioaccessibility compared to SP and ACE throughout all digestion phases whereas small intestine and T24 in the colon phase showed no significant difference between SP and ACE.

The relative bioaccessibility of the pure Se reference compounds SeMet and selenate does not significantly differ in upper intestinal phases. However, significant differences were observed between these two Se forms in the colon, with the bioaccessibility being much lower when SeMet is used (Figure 32). Compared to food supplements and food crops (Figure 31), the bioaccessibility of reference compounds in the upper intestinal tract is higher (Figure 32). This should be attributed to the absence of a matrix, allowing instantaneous solubilization of these reference compounds when subjected to gastrointestinal conditions.

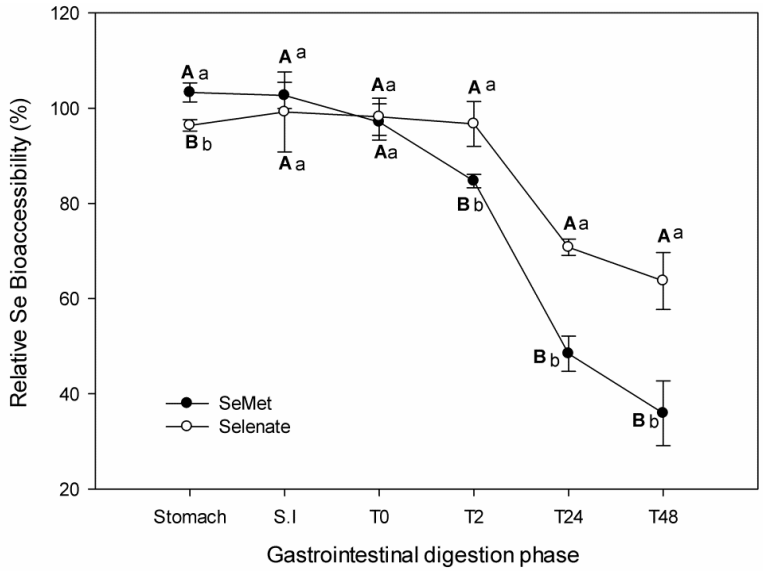


Figure 32. Relative bioaccessibility of Se for two Se reference compounds (selenate and SeMet) in different steps of an in vitro simulation of gastrointestinal digestion (T0, T2, T24 and T48 refer to 0, 2, 24 and 48 hours after starting colon incubation, respectively; S.I. refers to small intestine). The capital letters indicate Tukey's multiple-comparison test for comparison of means between the various digestion phases for each Se compound, whereas small letters indicate comparison of means between the different Se compounds in each digestion phase. A same letter indicates no significant difference ($p > 0.05$)

9.4.2 Role of colon microbiota

In a second experiment, SP and SeMet were incubated with inactivated colon microbiota, using autoclaved colon suspension. Colon microbiota were found to play a prominent role in reducing Se bioaccessibility in the colon environment (Figure 33A,B). The presence of inactivated microbiota resulted in a higher bioaccessibility compared to the presence of an active microbial community. For the inactivated microbiota, no significant difference is observed between SeMet and SP.

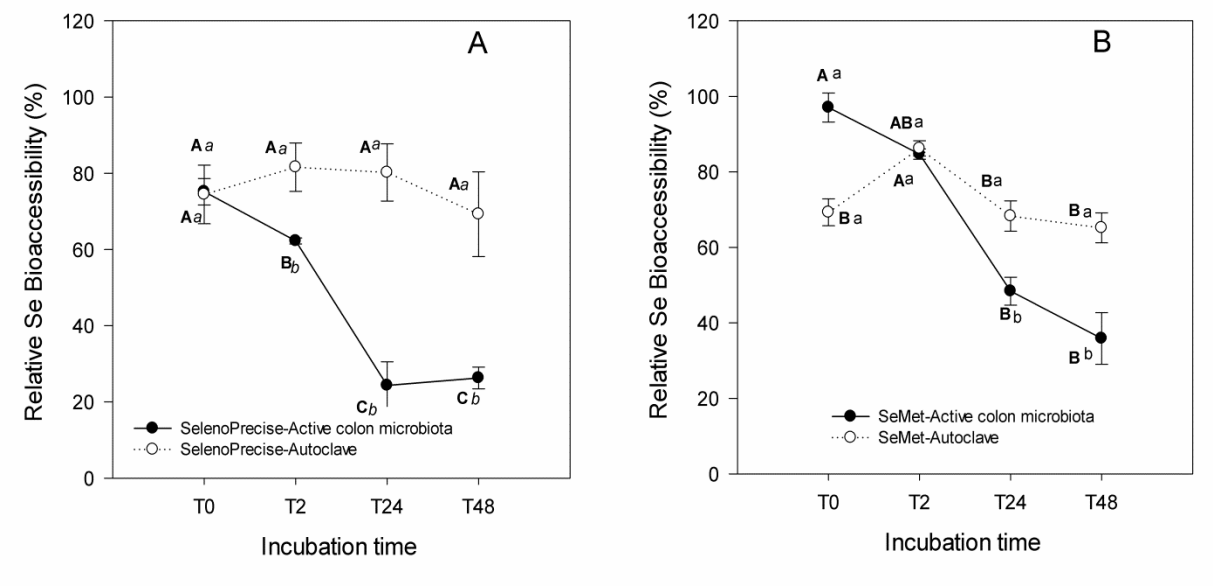


Figure 33. Comparison of relative Se bioaccessibility in the colon phase at various incubation time points (T0, T2, T24 and T48) for (A) SelenoPrecise supplement (SP) and (B) SeMet, in presence of active and inactivated (autoclaved) colon microbiota. The capital letters indicate Tukey's multiple-comparison test for comparison of means between the various digestion phases for each Se tablet/compound, whereas small letters indicate comparison of means between the different Se tablet/compound in each digestion phase. A same letter indicates no significant difference ($p > 0.05$).

9.4.3 Stability of Se species during gastrointestinal digestion

Biotransformation of SeMet was observed in the colon. SeMet concentrations decreased, whereas MeSeCys was formed to some extent (Figure 34). Moreover, Se-methionine oxide (SeMetO) was detected in the small intestine when incubating SeMet (Figure 34). In contrast, selenate was highly stable and only selenate was identified in the bioaccessible fraction in the colon at various time points when incubating this compound. No unknown peaks were observed.

9.5 Discussion

In our study, bioaccessibility of all Se reference compounds, Se-enriched food supplements and food crops was highest in the small intestine. Nevertheless, the Se reference compounds were found to be more bioaccessible in the gastric phase (Figure 31A,B and 32), which should be attributed to the fact that they should not be released anymore from a food matrix and can be instantaneously solubilized. The effects of food microstructure on the bioaccessibility of several nutrients has previously been reviewed (Parada and Aguilera, 2007), and also the bioaccessibility of Se was reported to differ according to the food matrix (Palafox-Carlos et al., 2011). The incorporation of Se during enrichment of food products was found to play a very important role (de Leon et al., 2002).

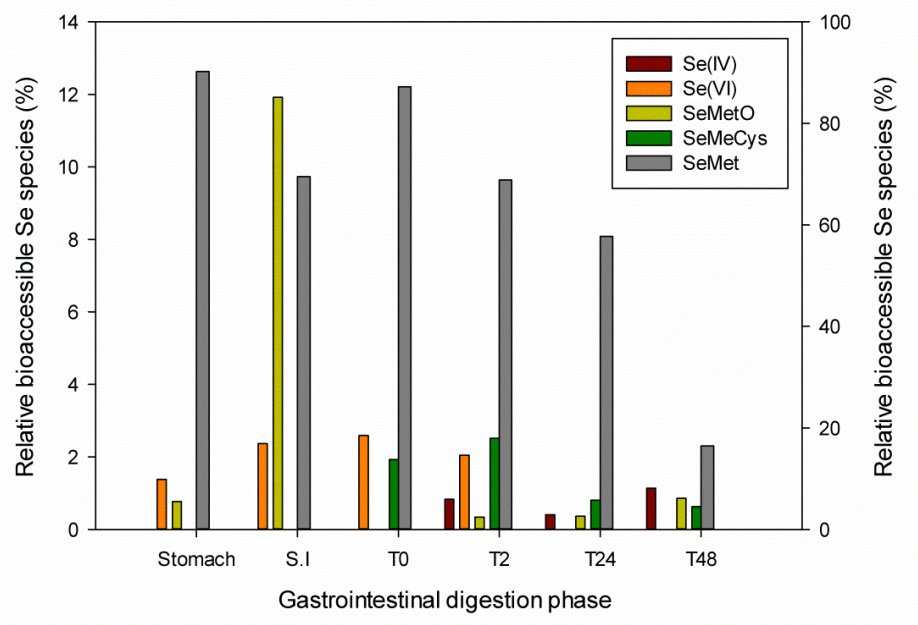


Figure 34. Relative percentage of Se species of total Se present in the bioaccessible fraction when incubating SeMet in different steps of an in vitro simulation of gastrointestinal digestion (T0, T2, T24 and T48 refer to 0, 2, 24 and 48 hours after starting colon incubation, respectively) represented using two different y-axes (right axis for SeMet and left axis for other Se species)

In the current study, Se was more easily released from the SelenoPrecise tablets in the upper intestine compared to the Se-ACE tablets. Compared to the SelenoPrecise and Se-ACE tablets, the yoghurt-based supplement exhibits a much lower Se bioaccessibility, possibly due to the presence of nano- or microparticles of elemental Se. These are formed by microbiota in the yoghurt (Mounicou et al., 2009). Among the two Se-enriched food crops, leek and kenaf were found to have a similar bioaccessibility in the stomach and small intestine. For both crops, the Se bioaccessibility in the upper intestine was found to be higher compared to the bioaccessibility of Se in the food supplements but lower compared to the Se bioaccessibility of the pure reference compounds. This implies that the efficiency of Se uptake by the human body may be slightly higher when consuming Se-enriched food crops compared to Se-enriched food supplements. Food preparation (cooking) prior to consumption may probably promote the release of Se from the food crop matrix in the intestine. Besides, the incorporation of Se in the food matrix could also lead to differences in bioacessibility between yeast supplement and food crops. In yeast, the majority of Se was reported to be

incorporated as SeMet whereas in food crops, it can differ widely based on the plant species (Mounicou et al., 2009).

Previous studies also reported that Se bioaccessibility is different in various digestion phases and between various food products (Brandt-Kjelsen et al., 2012, Dumont et al., 2004). For different Se supplementation sources it was demonstrated that the majority of Se becomes bioaccessible in upper gastrointestinal tract (Dumont et al., 2004). Recent studies indicate that Se-containing or Se-enriched food products like meat products, chives and wheat have a higher Se bioaccessibility in the small intestine compared to the gastric phase, due to the presence of enzymes. There is a probability that pancreatin may have a stronger influence on Se extraction efficiency into intestinal fluids than compounds present in the gastric phase. The ratio of different enzymes in an extract plays an important role, as was illustrated for extraction of Se species from Se-enriched yeast (Yang et al., 2004).

Although the majority of nutrients is taken up by the body in the stomach and small intestine, some nutrients and water are still available in the colon (Sandle, 1998). Selenium may be partly transferred to the large intestine when not taken up by the body in the small intestine, where it may also induce beneficial effects. In the colon, Se may be taken up by the bacterial fraction, resulting in a decreased bioaccessibility (Heider and Bock, 1993, Turner et al., 1998). When Se-enriched kenaf is used, part of the Se is very rapidly removed from solution when moving from small intestine to colon (T0), whereas this is not the case for Se-enriched leek. This should probably be attributed to the fact that kenaf and leek contain and release different Se species, which are adsorbed to a different extent to the biomass when moving to the colon. The bioaccessibility of pure SeMet was indeed found to decrease with about 10% when moving from the small intestine to the colon, whereas this was not the case for pure selenate (Figure 32). However, it should be noted that this change was not statistically significant due to the relatively high standard deviations in the small intestine and during early colon incubation. The Se-ACE and yoghurt-based food supplements also exhibited some (less significant) removal when moving from small to large intestine (Figure 31B).

Following the early physical processes in the stomach, when moving from small intestine to colon, Se is actively taken up by the bacterial cell fractions of gut contents and feces which was earlier reported in rats (Kim and Combs, 1997). In our study, the differences in behavior when incubating SelenoPrecise tablets and pure SeMet in non-autoclaved colon suspensions have shown lower bioaccessibility than autoclaved suspensions (Figure 33). The uptake of Se

by colon microbiota should be attributed to the fact that microbes also require Se as micronutrient for their metabolic processes (Baesman et al., 2007, Kasaikina et al., 2011, Stolz et al., 2006). We observed that the uptake of SeMet by colon microbiota is much more efficient compared to the uptake of selenate. The bioaccessibility of selenate decreases with about 30% during 48 hours of colon incubation, whereas it decreases with about 60% when incubating SeMet (Figure 32). In case of leek and kenaf, a decrease of bioaccessibility by about 42 and 19% after 48 hours of colon phase incubation is observed. The highest final Se bioaccessiblity remaining after 48 hours of incubation is observed for Se-enriched leek, which makes leek most suitable for biofortification purposes when beneficial health effects in the colon are targeted.

Colon incubation of the Se-ACE supplement and selenized yeast (SelenoPrecise) resulted in a decrease of less than 35% and about 50%, respectively, whereas incubation of the yoghurt-based supplement did not result in significant Se uptake by the microbial biomass. The Se-ACE supplement resulted in a significant decrease of bioaccessibility already after 2 hours of incubation, whereas a significant drop was observed for SelenoPrecise only after 24 hours. Presence of vitamins in the Se-ACE supplement probably promoted Se uptake by the microbiota (Dumont et al., 2004). However, all food supplements resulted in a similar residual bioaccessibility after 48 hours of colon incubation.

During colon incubation conducted in our experiments, some formation of MeSeCys from SeMet was observed (Figure 34). Numerous studies elaborated hypotheses on prevention of colon cancer with Se supplementation, and some could also confirm the role of Se (Brigelius-Flohe, 2008). One of these studies conducted by Clark et al. (1996) suggested that an intake of 250-300 µg Se day^{-1} through Se-enriched yeast results in a decreased incidence of colon cancer. These reports show the importance of Se species in colon carcinogenesis. Moreover, Se-enriched food crops like *Allium* and *Brassica* species were found to reduce the risk of colon cancer, compared to other sources of Se (Ip et al., 2000). This pointed towards a role of MeSeCys and γ–Glu-SeMeCys, species which particularly occur in high concentrations in Se-enriched *Allium* and *Brassica* species, but also occur in Selenized yeast at very minute amounts (Pyrzynska, 2009, Goenaga et al. 2004,). Our study gives evidence that Se is subjected to speciation changes during colon digestion.

Limited studies have focused on a possible relationship between Se supplementation and the microbial community composition in the colon (Kasaikina et al., 2011, Molan et al., 2009).

These studies did not only emphasize the role Se may play in enhancing beneficial microbiota in the colon, but also illustrate the positive influence Se may have on epithelial cells of the gut (Gong et al., 2012). In our study, we investigated the effect of Se supplementation on the microbial activity by conducting SCFA analysis. The survey data from different population studies show that fecal SCFA are in the order of acetate > propionate ≥ butyrate (Topping and Clifton. 2001). No negative effects towards colon microbial metabolism were observed (data not shown). SCFA was not significantly different between Se supplements and control samples. Hence, the role of microbial activity is evident through SCFA analysis. The active microbial colon phase incubation of SeMet results in the formation of other Se species, such as MeSeCys (Figure 34). This is probably a biotransformation process, as MeSeCys is not expected to be produced purely chemically. It illustrates how Se entering the colon becomes involved in biochemical processes of colon microbiota.

Next to biotransformations in the colon, some chemical species transformations were also observed in the small intestine. Pure SeMet is chemically oxidized to *Se*-methionine oxide (SeMetO) under the oxic conditions in the small intestine, which has previously also been reported (Dumont et al., 2004). To what extent this change in speciation will also occur when incubating food supplements releasing SeMet and to what extent it will affect the bioavailability, i.e. the uptake by the bloodstream, should be subject of future research.

9.6 Conclusion

In vitro bioaccessibility studies using SHIME suspension could be used to assess bioaccessibility of Se in the colon phase, as an indirect approach to assess the potential of Se supplements and Se-enriched food crops in colon health. In our study, clear differences in bioaccessibility patterns were observed between the different Se containing matrices with Se-enriched food crops showing the highest Se bioaccessibility upon colon digestion. The impact of microorganisms on Se bioaccessibility in the colon was demonstrated by the significantly lower Se bioaccessibility values upon digestion with heat-inactivated colon microbiota. While selenite was found to be highly stable throughout the entire digestion, incubation of SeMet resulted in the production of two minor metabolites, identified as MeSeCys and Se(O)-methionine.

Chapter 10. General Discussion

10.1 Soil selenium in Flanders (Belgium)

The metalloid selenium (Se) is an essential micronutrient for many organisms, including humans and animals, and the soil Se concentration is important in providing optimum Se levels to humans and animals through the diet. In general, soils with Se concentrations below 0.6 mg Se kg^{-1} are considered as Se deficient soils. As such, soils in the Flemish region of Belgium can be considered as overall deficient in Se, with concentrations ranging from 0.05 to 0.4 mg Se kg^{-1} across Flanders. Moreover, extractability of Se from the soil was previously considered as relatively poor in Belgian soils (Sillanpää and Jansson, 1992). Although soil properties such as pH, OM, EC, etc., were reported to have an influence on Se bioavailability, the field data generated by growing leek on these Se deficient soils showed relatively weak correlations between Se uptake and soil properties among the full range of soils studied (chapter 4).

10.2 Use of Se fertilizers to obtain Se-enriched food crops

The human population in Belgium was previously reported to have a deficient to suboptimal Se intake, which could be due to low Se concentrations in agricultural products obtained from the Se deficient soils (Robberecht, 1994). The dietary average intake of Belgium was reported to be 45 µg Se d^{-1}. Moreover, the average plasma Se concentration in the Belgian population (84.3±9.4 µg Se L^{-1}) is lower than 120 µg Se L^{-1}, which was reported to be a plasma Se level that minimizes cancer risks (Combs, 2001). These observations suggest that Se supplementation in the Belgian population may be useful. One alternative to overcome the Se deficiency can be the addition of Se in fertilizers for pastures and food crops, similar to what has previously been done in Finland, the UK and Australia (Aro et al., 1995, Broadley et al., 2010, Eurola et al., 1991, Lyons et al., 2004). In our study, focus on biofortified Se-enriched leek was chosen to improve the Se status. Leek, being an *Allium* family species, could provide particular Se species which are considered to be beneficial for human health, such as MeSeCys and γ-glut-cyst. Moreover, it is highly cultivated as an important commercial crop in Belgium and other European countries. Based on the results reported in chapter 3, it can be concluded that leek responds to Se fertilization when the soil is fertilized

with two forms of Se, selenite and selenate, at various doses. Use of selenate clearly results in the highest accumulation in the crop. The Se speciation data reveal that leek provides a higher relative amount of organic species when fertilized with selenite compared to selenate. In *Allium* species, MeSeCys and its derivatives were previously reported to be present in higher concentrations compared to SeMet. These species were also found to occur in leek, but to a lesser extent compared to the other *Allium* family species.

On the other hand, Kenaf was also chosen as a study crop because kenaf as a feed crop was successfully tested on beef cattle and small ruminants before (Lopez et al., 2006, Xiccato et al., 1998), and Kenaf leaves are also sometimes used in human diets (Adebayo, 2010). A recent survey on grasslands in Belgium reported that most grasses contain 83 µg Se kg^{-1} (Hambuckers et al., 2010). It was previously reported that Se deficiency in livestock generally occurs when soil Se concentrations are below 0.6 mg Se kg^{-1} which would lead to less than 100 µg Se kg^{-1} in plants (Hambuckers et al., 2010). The recommended Se concentration for forages fed to dairy cattle ranges between 100 and 300 µg Se kg^{-1}, whereas the toxicity threshold level is close to 2 mg Se kg^{-1} (Buchanan-Smith et al., 2001). From chapter 6, we can conclude that kenaf contains already 300 µg Se kg^{-1}, i.e. an adequate concentration for feed, when it is grown on a soil containing 0.3 mg Se kg^{-1}. As full replacement of grass by kenaf is not realistic, we focus on use of this crop as feed additive or as intercropping in grasslands. As was the case for leek, kenaf also responds significantly to Se fertilization. Moreover, Se toxicity to the plants was observed when they were grown at 4 mg Se kg^{-1} soil supplied in the form of selenate, which was not observed for leek, but no toxic effect was seen when a similar dose was supplied in the form of selenite (see chapter 6). Similar to humans, Se supplementation with organic Se species can be also considered as beneficial for the health of livestock (Steen et al, 2008). The speciation data presented in chapter 6 reveal that a higher relative amount of organic species is present when selenite fertilizer is used, compared to when selenate fertilizer is used. However, although both leek and kenaf contain more relative amounts of organic species when fertilized with selenite, longer-term usage of selenite fertilizers may need to be avoided due to their much lower uptake by the plant, and thus also higher potential for accumulation in the soil.

10.3 Calculation of daily Se intake through leek in fertilized and non-fertilized

Based on the data from the field study (Chapter 4), the amount of Se taken up through consumption of non-fertilized leek and Se-fertilized leek. The percentage of the recommended daily allowance (RDA) taken up through Se-enriched leek does not exceed 0.66 % when assuming a consumption rate of 0.345 kg/person/day. The consumption of Se-enriched leek at this rate could not provide a Se dose exceeding the upper limit of the RDA (400 µg d^{-1}) (Table 26).

The daily intake of Se by humans was calculated using the following equation:

$$\text{Daily intake of Se } (\mu g) = \frac{M \times K \times I}{W} * 1000$$

where M: the concentration of Se in plants (mg kg^{-1} DW); K: conversion factor to convert fresh to dry weight (0.11: 11% dry weight content); I: daily intake of vegetables (fresh weight); W: average body weight. The average body weight was considered to be 55.9 kg, while the average daily vegetable intake for adults was considered to be 0.345 kg/person/day, respectively (Amin et al., 2013).

Table 26. Amount of Se taken up through consumption of non-fertilized leek and Se-fertilized leek obtained from the study fields

Plot No	Non-fertilized leek		Se-fertilized leek	
	Daily intake (µg kg^{-1} BW)	RDA (%)	Daily intake (µg kg^{-1} BW)	RDA (%)
1	0.05	0.08	0.46	0.66
2	0.12	0.16	0.27	0.39
3	0.05	0.08	0.21	0.30
4	0.07	0.11	0.46	0.65
5	0.14	0.19	0.23	0.33
6	0.04	0.06	0.22	0.31
7	0.07	0.11	0.25	0.35
8	0.06	0.09	0.29	0.41
9	0.05	0.07	0.18	0.26
10	0.12	0.16	0.17	0.24
11	0.03	0.04	0.05	0.08
12	0.07	0.10	0.45	0.64
13	0.00	0.00	0.09	0.13
14	0.03	0.04	0.14	0.20
15	0.03	0.04	0.07	0.09
16	0.00	0.00	0.16	0.23
17	0.06	0.09	0.30	0.43
18	0.03	0.04	0.08	0.11
19	0.03	0.05	0.16	0.23
20	0.05	0.08	0.16	0.23
21	0.03	0.04	0.30	0.43
22	0.11	0.16	0.30	0.43
23	0.05	0.07	0.23	0.33
24	0.03	0.05	0.16	0.23
25	0.06	0.09	0.22	0.32
26	0.10	0.14	0.13	0.18

% of RDA based on a RDA of 70 µg Se d^{-1} (National Research Council 1989)

10.4 Estimation of Se fertilizer application doses to obtain safe Se-enriched food crops

In recent years, use of wheat was studied in the UK to improve the Se status of the human population. In our study, we investigated whether Se-enriched vegetables could be an alternative for wheat. For humans, a Se intake of 55 Se µg d^{-1} is the recommended dose according to the Scientific Committee on Food of the European Commission (SCF, 2000) and the US Food and Nutrition Board (cited in SCF, 2000). A mean dietary Se intake estimation of 60 µg Se d^{-1} was reported for the Belgian population in a recent study (Waegeneers et al., 2013), Table 27). However, this study highlighted serious concerns about the Se intake of vegetarians and vegans in Belgium. When meat and fish products were excluded, the dietary intake of Se was estimated to be only 29 µg Se d^{-1}. Vegetarians replace these meat and fish products in their diet by other ingredients, which however often contain much less Se. Roekens et al. (1986) even estimated the daily Se intake of Belgian vegetarians in the 1980s to be as low as 13 µg Se d^{-1} (Table 27). We estimated the feasibility of using Se-fortified leek to improve the Se intake of the Belgian population.

In general, the vegetable consumption per day was estimated to be 350 g d^{-1} (based on fresh weight). It can be assumed that Se-enriched leek could be consumed up to three times per week. To compensate for a daily intake deficiency of 26 µg Se, coinciding with a weekly deficiency of 182 µg, one would need to consume leek with a concentration of 2 µg Se g^{-1}.

Soil fertilization doses needed to obtain this concentration were assessed based on the field and greenhouse experiments that were conducted. Therefore, the average percentage of Se uptake from the soil was obtained from the pot experiment in which leek was grown on various fertilizer types and doses (Table 5), whereas the average biomass production was obtained from the field experiment (chapter 4). Combining both data, a Se concentration was calculated that may be expected in leek grown on the field for all Se fertilization doses used in the pot experiments, assuming that total Se uptake by a plant depends on the fertilization

Table 27. Mean usual Se intake (µg d^{-1}) by the Belgian adult population (>15 years), by food group (adopted from Waegeneers et al., 2013)

Food group	Mean usual intake (µg Se day^{-1})
Bread, toast(rusk) and breakfast cereals	6.9
Potatoes and potato products	0.6
Pasta and rice	6.9
Vegetables (excluding soups and juices)	1.8
Fruits (excluding juices and olives)	-
Meat and meat products	18.7
Fish and shell fish	11.6
Eggs	2.3
Cheese	5.5
Yoghurt and custard	0.3
Milk and dairy drinks	2.3
Drinks	2.8
Total	59.6
Total excluding meat and fish products	29.3

dose rather than on the biomass production. Afterwards, regression analysis was performed to assess the dose of fertilizer that would be needed to obtain about a concentration of 2 µg Se kg^{-1} DW of leek in the field (Figure 35).

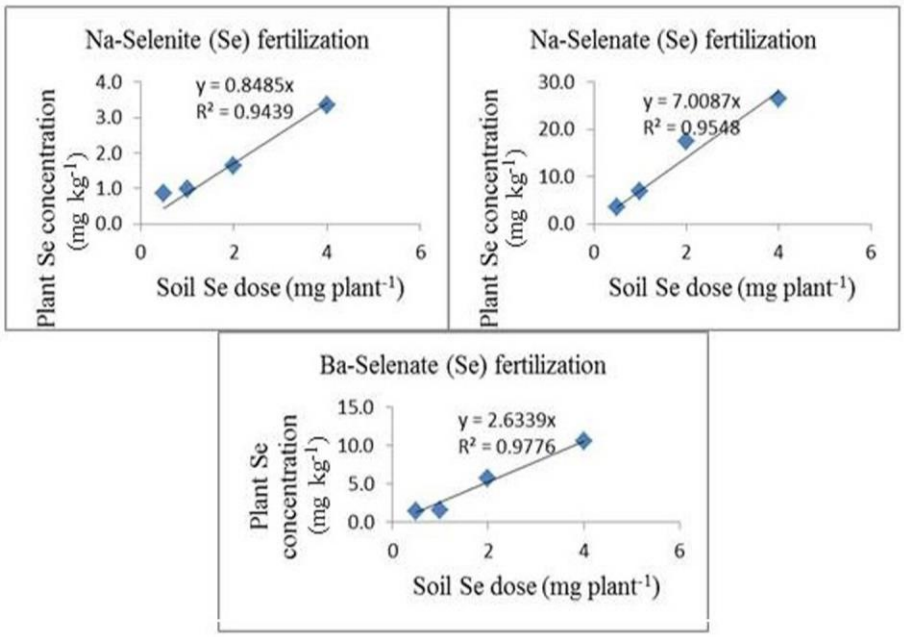


Figure 35. Regressions predicting Se concentration in leek grown on the field from soil Se fertilizer doses tested in greenhouse experiments

According to this calculation, a selenate dose of 0.29 mg Se per plant and a selenite dose of 2.36 mg Se per plant would be needed to obtain a concentration of about 2 µg Se kg^{-1} DW in the leek. Although this calculation is useful to assess approximate amounts of Se fertilizer needed, it should be emphasized that it is based on quite some assumptions. The relative uptake of Se by the leek could change with varying biomass production, soil properties and climate conditions. In addition, the assessment was based on the assumption that concentrations in white part and green part of the leek are similar, as the entire plant was analysed in the greenhouse experiment.

Based on the calculated dose of Se fertilizer needed per plant, the Se dose needed per hectare was also calculated. The average number of leek plants per hectare is at maximum 170,000, which coincides with 170,000 (leek plants) × 2.36 (mg Se per plant) = 400,707 mg (401 g) Se needed in the form of Na-selenite fertilizer, 170,000 (leek plants) × 0.29 (mg Se per plant) = 48,511 mg (49 g) Se needed in the form of Na-selenate fertilizer and 170,000 (leek plants) × 0.76 (mg Se per plant) = 129,200 mg (129 g) Se needed in the form of Ba-selenate fertilizer. If an individual substitute part of his diet with with three servings per week of Se-enriched leek (2 µg g^{-1}) obtained from above recommended fertilization dose, Se intake would increase. The estimated increase was calculated and presented in Table 28.

Table 28. Estimated increase of Se intake with three servings of Se-enriched leek (2 µg g^{-1}) per week

Reference	Reported Se intake	Estimation of Se intake after leek consumption
Robberecht, 1994	Lowest intake 28 µg Se d^{-1}	[a]54 µg Se d^{-1}
	Mean intake 45 µg Se d^{-1}	[b]71 µg Se d^{-1}
	Highest intake 61 µg Se d^{-1}	[b]87 µg Se d^{-1}
Waegeneers et al., 2012	Average intake 60 µg Se d^{-1}	[b]86 µg Se d^{-1}
	[1]Without meat and fish intake 29 µg Se d^{-1}	[c]55 µg Se d^{-1}

[1]Substracting Se intake through meat and fish from average Se intake estimated by Waegeneers et al., 2012. [a]Not yet adequate Se intake based on 55 µg Se d^{-1} recommendation from SCF of the European Commission and the US Food and Nutrition Board; [b]Selenium intakes were within the range of 80-100 µg Se d^{-1} recommendation from Combs 2001 to obtain plasma Se concentrations (120 µg L^{-1}) in countries with low-moderate Se intakes; [c]based on above µg Se d^{-1} intake recommendation.

In order to determine required Se dietary levels from leek and kenaf, Se uptake data from pot experiments were considered where biomass from field experiments (leek) and based on literature (kenaf) were used. Compared to field experiments, pot experiment differs in Se uptake due to differences in soil conditions and availability of nutrients. For instance, leek in pot experiment obtained higher Se uptake compared to field experiments. However, the higher level of Se uptake in field experiment was within the range of Se uptake obtained in pot experiment studies could provide best approximation of required Se fertilizer doses.

For kenaf, a similar calculation can be done using the uptake data for Se fertilizer doses (mg Se kg^{-1} soil translated to amount of Se per hectare) provided in chapter 6 and 7. The hay production (DW) of kenaf that was used to recalculate Se uptake by the plant to concentrations that can be expected on the field was 0.5 ton per acre (1.2 ton ha^{-1}). This value was taken from literature (Knowles et al., 1999). For kenaf, we focused on obtaining Se

concentrations similar to commercial Se feed additives, i.e., 600 mg Se kg^{-1} DW. Regression analysis was performed to assess the dose of fertilizer that would be needed to obtain such concentration in the kenaf in the field (Figure 36). This calculation resulted in a recommended fertilizer dose of 110 g Se ha^{-1} when using selenate fertilizer and 136364 g Se ha^{-1} when using selenite fertilizer.

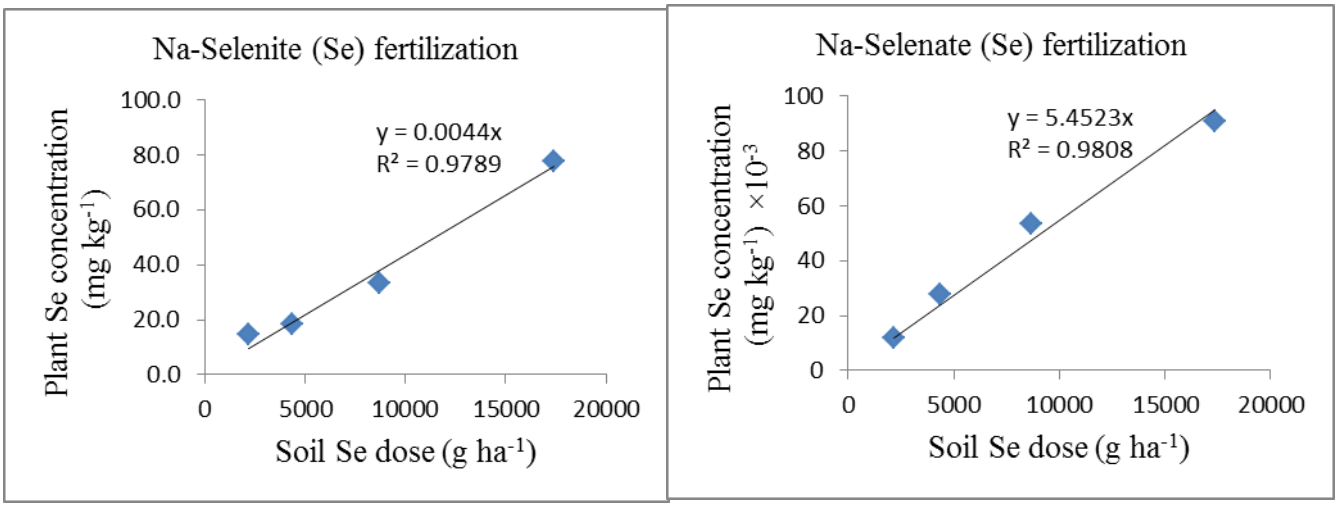


Figure 36. Regressions predicting Se concentration in kenaf grown on the field from soil Se fertilizer doses tested in greenhouse experiments

It should be noted that a much higher dose of selenite needs to be applied to reach the targeted concentrations compared to when selenate is used. This is due to its lower uptake by crops, which may form a risk for the environment by long-term accumulation in the soil, and render the use of selenite to be technically not feasible and economically not viable. These disadvantages may not offset the higher amount of organic species in the crops when selenite fertilizer is used.

10.5 Se biofortification: economic evaluation and potential for commercial application of Se-enriched leek and kenaf

The costs of Se fertilizers applied at the doses calculated above were estimated and presented in Table 29. A cost of 571 € kg^{-1} was used for sodium selenate, 566 € kg^{-1} for sodium selenite, and 3483 € kg^{-1} for barium selenate. Starting from the cost of the salts, a cost per unit of Se was calculated.

Table 29. Estimated required Se fertilizer doses and cost of soil-applied Se to achieve targeted Se concentrations in kenaf and leek

	Leek		Kenaf	
Se fertilizer type	Se fertilizer dose (g Se ha^{-1})	Cost (€ ha^{-1})	Se fertilizer dose (g Se ha^{-1})	Cost (€ ha^{-1})
Na-Selenate	24	33	110	355
Na-Selenite	201	249	136364	168966
Ba-Selenate	129	1320	-	-

Pricing of selenium salts were according to Lyons et al. (2004) and Sigma Aldrich.

Based on the recommended Se intake, the economic viability of Se-enriched leek was assessed by comparing it with commercially available food supplements containing selenized yeast (SelenoPrecise tablets). The cost of each tablet containing 200 µg Se (per tablet) was 0.1 € (cost converted to € from £). Using the above recommended Se fertilization dose for different Se forms, the Se amount obtained in leek plant was estimated. An amount of 18700 mg Se was obtained in consumable white part per hectare. An amount similar to the amount of Se in a SelenoPrecise tablet (200 µg) can be obtained in leek at a cost of 0.0021 €, 0.0004 € and 2.7 € for Na-selenite, Na-selenate and Ba-selenate, respectively, when recommended fertilization doses were applied. In case of kenaf, a feed supplement containing 600 mg Se

kg^{-1} in the form of selenized yeast was compared with Se-enriched kenaf. The cost of this supplement for 600 mg Se was 5 € (converted to € from USD $). In kenaf, similar Se concentrations can be obtained with a cost of 0.30 € for selenate fertilizer and a cost of 141 € for selenite fertilizer.

It is clear that the cost for supplementing food and feed crops is generally lower compared to commercially available food and feed supplements containing selenized yeast, except when kenaf is fertilized with selenite. However, commercially available supplements based on selenized yeast contain organic Se species, mainly SeMet, as a major entity, whereas in the crops Se speciation depends on the form of Se supplied to the soil. Selenized yeast approved as feed supplement by the European Commission is supposed to contain at least 63 % of the Se in an organic form (Commission of the european communities., 2006). If crops were fertilized with selenite, approximately 30 % of species were organic. These species include more MeSeCys and its derivatives, which were previously reported to potentially have anticarcinogenic properties. On the other hand, when selenate was used as fertilizer, higher percentages (upto 50%) of inorganic Se species were observed, which should be offset against the advantage of its lower cost. In any case, when the aim is to bring a large population from suboptimal to optimal Se intake, only agronomic biofortification seems to be a feasible option because the risk for over supplementation and toxicity to occur in some cases is too high when supplying selenized yeast as a food or feed supplement to a whole population.

10.6 Soil management practices affecting Se uptake

Soil management practices may influence Se mobility in the soil and its uptake by the plants when soils are fertilized with Se. It is previously reported that Se uptake in plants decreases with application of soil organic amendments such as poultry and farmyard manure on seleniferous soils (Dhillon et al., 2010). In fact, organic matter was expected to decrease Se concentrations in food crops when the dominant form of Se in soil is selenite. Upon soil ageing selenate is expected to be slowly converted to selenite which can be adsorbed onto soil surfaces. This could decrease Se uptake. In a similar way, soil pH may affect Se uptake by plants (Mukherjee, 2007; Geering et al., 1968). In low pH soils, Se uptake was lower

compared to high pH soils (Johnsson, 1991). In order to assess the influence of organic matter and lime on the fate of two inorganic Se forms during soil ageing, we conducted an experiment in which porewater samples were taken and extractability of Se from the soil was studied as function of time after amending the soil and applying Se fertilizer. Moreover, wheat was used as study crop to assess Se bioavailability. The results showed that organic amendments may decrease Se uptake by wheat irrespective of the Se form used in the fertilizer. However, for all soil amendments, Se uptake by the wheat is higher when selenate is supplied to the soils compared to when selenite is used. Although a higher Se uptake was obtained for the wheat grown on compost and lime amended soils spiked with selenate (40 – 48 %), less Se was taken up by wheat grown on pig and cow manure amended soils spiked with selenate (2-4 %). Irrespective of the soil amendment, a lower Se uptake was observed in the plants grown on soils spiked with selenite (less than 1-2%). Such very low Se transfer from soil to crop may lead to Se accumulation in the soil on longer term. Impact of soil ageing on Se availability and mobility was also assessed by porewater analysis. However, no clear trends could be observed. Longer-term studies are needed to completely assess the fate of residual Se remaining in the soil after fertilization, and factors affecting its mobility and availability.

10.7 Bioaccessibility of Se in Se-enriched food crops versus food supplements

Bioaccessibility is a key concept to ascertain nutritional efficiency of food and food formula developed with the aim of improving human health. It is essential to prove that supplied nutrients are also bioaccessible in the intestine. For example, it was previously discovered that wheat flour fortified with iron may not be effective because the iron it contains is not bioaccessible (Hurrell, 2004). In vitro bioaccessibility assessment is considered as a useful tool to compare Se-enriched food crops and food supplements for their bioaccessibility and nutritional efficiency. Numerous studies previously reported the bioaccessibility of Se from food supplements and food crops to be relatively high in the upper intestinal tract (stomach and small intestinal phase) although there are also substantial evidences that Se supplementation in the colon (large intestine) could help in reducing colon carcinogenesis. However, no literature is yet available focused on the fate of Se in the colon when it is supplied through Se-enriched food crops and food supplements. The results obtained in our

study provide insights in the bioaccessibility of Se in stomach and small intestine, as well as in its fate in the colon. We observed that the majority of Se in all tested Se-enriched products is bioaccessible in the small intestine phase. Similar to what was reported previously by others, the bioaccessibility of Se from Se-enriched food crops was found to be higher in the small intestine compared to the stomach (Figure 31 and 32) due to enzymatic digestion occurring in the small intestine.

Among the commercially available Se supplements, a new yoghurt based (YB) Se supplement showed a much lower Se bioaccessibility (not exceeding 25%), which was attributed to the presence of elemental Se. Moreover, it was observed that microbes in the colon alter the bioaccessibility of Se. These observations highlight the need for assessing Se speciation and bioaccessibility when evaluating the efficacy of new food supplements and fortified food products being brought to the market. Thus, speciation and bioaccessibility measurements may help to optimize Se supplementation strategies in Se deficient populations.

10.8 Conclusions

We can conclude that the soils in Flanders (Belgium) can be considered as Se deficient. These low levels of soil Se result in crops with low Se levels, leading to low Se intakes in humans and livestock and tending to give low blood Se concentrations. When Se fertilizer is added to the soil, the concentration and speciation of Se in leek depend on the form and dose of Se fertilizer used, with use of selenate resulting in the highest accumulation in the crop. Its uptake by the leek ranges between 5-10% and 36-48% of the amount added to the soil for supply of selenite and selenate, respectively. Accordingly, the use of selenite as fertilizer results in a higher risk for Se accumulation in the soil on longer term. Among 20 different leek cultivars tested in a field experiment, some cultivars seem to be superior in accumulating Se. In a field experiment conducted on different field plots across Flanders with selenite as fertilizer, a negative correlation between soil organic carbon and Se uptake by the leek is observed. Moreover, organic amendments seem to decrease Se availability and its concentration in wheat. In soils amended with cow and pig manure, Se uptake by the plants decreases by 91-95% and 88-89%, respectively, when the soils are spiked with selenite or

selenate. Soil liming improves Se concentrations in crops especially when selenate fertilizers are used.

As was observed for leek, the uptake of Se by kenaf is also highest when the soil is fertilized with selenate, whereas also the speciation of Se in kenaf differs when different fertilizers are used, with a higher percentage of organic species being formed in the crop when soils are fertilized with selenite. At higher doses of selenate fertilizer, plant growth is negatively affected, whereas this is not the case when selenite was used at a same Se application dose.

The majority of Se can be considered as bioaccessible in the small intestine, and a significant fraction of Se contained in the crops also has good chances to reach the colon, where it seems to be taken up by the microbial community and may also induce positive health effects. However, further research is needed to assess whether this is actually the case.

It is concluded that Se-enriched leek or kenaf can be used to increase intake of Se by humans and animals from suboptimal levels to levels which have been reported to promote beneficial health effects. However, long-term field studies monitoring Se mobility and bioavailability in soils amended with Se fertilizers are needed to be able to outweigh the risk for Se accumulation in the soil against the benefit of supplying Se to the crop.

10.9 Recommendations for future research

Study the environmental impact and long-term fate of selenite fertilizers and options to increase selenite uptake by crops

Given our finding that selenite is the best fertilizer to increase fraction of organic species in crops, but the majority of the selenite remains in the soil after fertilization, there is a clear need to understand the environmental implications if selenite fertilization and the longer-term environmental fate of selenite fertilizers. Moreover, in this context it may also be interesting to study options to increase the efficiency of the uptake of selenite fertilizers by the crops.

Effect of Se intake on Se status and human health upon consumption of biofortified food crops

Limited studies previously focused on assessing the impact of consumption of Se biofortified food crops on human health, in comparison to food supplements containing Se, so there is an urgent need to conduct research on the effect of consuming biofortified food crops on Se status and human health.

Impact of Se supplementation on the colon environment

Clinical studies have shown that supplementing Se may decrease the incidence of colon cancer. However, the mechanism behind this is not yet clear. Given our finding that Se bioaccessibility decreases in presence of colon microbes, it would be interesting to evaluate the impact of Se on the colon environment, including its effect microbial activity and community composition.

References

Adebayo A. O., 2010. Effect of processing methods on chemical and consumer acceptability of kenaf and corchorus vegetables. Journal of American Science. 6: 165-170.

Adams M. L., Lombi E., Zhao F.-J. and Mcgrath S. P., 2002. Evidence of low selenium concentrations in UK bread-making wheat grain. Journal of the Science of Food and Agriculture. 82: 1160-1165.

Alfthan G., Aro A., Arvilommi H. and Huttunen J. K., 1991. Selenium metabolism and platelet glutathione peroxidase activity in healthy Finnish men: Effects of selenium yeast, selenite, and selenate. American Journal of Clinical Nutrition. 53: 120-125.

Alemi M. H., Goldhamer D. A., Nielsen D. R., 1991. Modeling selenium transport in steady-state, unsaturated soil columns. Journal of Environmental Quality. 20: 89-95.

Allaway W. H., 1968. The chemistry of selenium: Proceedings semi-annual meeting. Animal Feed Manufacturers Association. 126: 355-361.

Amin N.U., Hussain A., Alamzeb S. and Begum S., 2013. Accumulation of heavy metals in edible parts of vegetables irrigated with waste water and their daily intake to adults and children, district Mardan, Pakistan. Food Chemistry. 136: 1515-1523.

Anderson J. W., 1993. Selenium interactions in sulphur metabolism. In: De Kok LJ (editor) Sulphur nutrition and assimilation in higher plants—regulatory, agricultural and environmental aspects. SPB Academic publishing, The Hague, Netherlands. 49–60.

Arnon D. I. and Stout P. R., 1939. The essentiality of certain elements in minute quantity for plants with special reference to copper. Plant Physiology. 14: 371-375.

Aro A., Alfthan G. and Varo P., 1995. Effects of supplementation of fertilizers on human selenium status in Finland. Analyst. 120: 841-843.

Arteel G. E. and Sies H., 2001. The biochemistry of selenium and the glutathione system. Environmental Toxicology and Pharmacology. 10: 153-158.

Arthur J. R., 2003. Selenium supplementation: Does soil supplementation help and why? Proceedings of Nutrition Society. 62: 393-397.

Arthur M. A., Schneider R. E., Weinstein L. H. and Rubin G., 1992. Uptake and accumulation of selenium by terrestrial plants growing on a coal fly ash landfill. Part 3. Forbs and grasses. Environmental Toxicology and Chemistry. 11: 1301-1306.

Arvy M. P., 1993. Selenate and selenite uptake and translocation in bean-plants (*Phaseolus-vulgaris*). Journal of Experimental Botany. 44: 1083-1087.

Arya S. K. and Roy B. K., 2011. Manganese induced changes in growth, chlorophyll content and antioxidants activity in seedlings of broad bean (*Vicia faba* L.). Journal of Environmental Biology. 32: 707-711.

Aubert, H. and Pinta, M. (1977). Trace Elements in Soils. Amsterdam - Oxford, New York, Elsevier Scientific Publishing Company.

Bada B. S. and Raji, K.A., 2010. Phytoremediation potential of kenaf (*Hibiscus cannabinus* L.) grown in different soil textures and cadmium concentrations. African Journal of Environmental Science and Technology. 4: 250-255.

Baesman S. M., Bullen T. D., Dewald J., Zhang D., et al., 2007. Formation of tellurium nanocrystals during anaerobic growth of bacteria that use Te oxyanions as respiratory electron acceptors. Applied and Environmental Microbiology. 73: 2135-2143.

Balistrieri L. S., Chao T. T., 1990. Adsorption of selenium by amorphous iron oxyhydroxide and manganese dioxide. Geochimica et Cosmochimica Acta. 54: 739-751.

Banuelos G. S., Ajwa H. A., Mackey B., Wu L., et al., 1997a. Evaluation of different plant species used for phytoremediation of high soil selenium. Journal of Environmental Quality. 26: 639-646.

Banuelos G. S., Ajwa H. A., Terry N. and Zayed A., 1997b. Phytoremediation of selenium laden soils: A new technology. Journal of Soil and Water Conservation. 52: 426-430.

Banuelos G. S. and Dhillon K. S., 2011. Developing a sustainable phytomanagement strategy for excessive selenium in Western United States and India. International Journal of Phytoremediation. 13 Suppl 1: 208-228.

Banuelos G. S., Lin Z. Q., Arroyo I. and Terry N., 2005. Selenium volatilization in vegetated agricultural drainage sediment from the San Luis Drain, Central California. Chemosphere. 60: 1203-1213.

Banuelos G. S., Zayed A., Terry N., Wu L., et al., 1996. Accumulation of selenium by different plant species grown under increasing sodium and calcium chloride salinity. Plant and Soil. 183: 49-59.

Bedwal R.S., Nair N., Sharma M.P., Mathur R.S., 1993. Selenium—Its biological perspectives. Medical Hypotheses. 41: 150-159.

Benoit F. and Ceustermans N., 1994. Ecological growing of leeks with plastics. Plasticulture. 101: 45-49.

Bernaert N., De Paepe D., Bouten C., De Clercq H., et al., 2012. Antioxidant capacity, total phenolic and ascorbate content as a function of the genetic diversity of leek (*Allium ampeloprasum var. Porrum*). Food Chemistry. 134: 669-677.

Bernaert N., Goetghebeur L., De Clercq H., De Loose M., Daeseleire E. et al., 2012a. Influence of cultivar and harvest time on the amounts of isoalliin and methiin in Leek (*Allium ampeloprasum* var. *porrum*). Journal of Agricultural and Food Chemistry. 60: 10910−10919**.**

Bhasin P., Singla N. and Dhawan D. K., 2012. Protective role of zinc during aluminum-induced hepatotoxicity. Environmental Toxicology. doi: 10.1002/tox.21760

Birringer M., Pilawa S. and Flohe L., 2002. Trends in selenium biochemistry. Natural Product Reports. 19: 693-718.

Bisbjerg B., 1972. Studies on selenium in plants and soils. Riv Report No. 200, Danish Atomic Energy Commission.

Eric B., Naganathan S., Putman D. and Zhao S. H., 1992. *Allium* chemistry: HPLC analysis of thiosulfinates from onion, garlic, wild garlic (ramsoms), leek, scallion, shallot, elephant garlic, chive and chinese chive. Uniquely high allyl methyl ratios in some garlic samples. Journal of Agricultural and Food Chemistry. 40: 2418-2430.

Borowska K., 1998. Selenium, zinc and iron accumulation in soils and alfalfa (*Medicago sativa*) from selected plantations. Microelements and trace elements. Anke M., Arnhold W., Bergmann H. et al. 04205 Leipzig, Verlag Harold Schubert**:** 602-607.

Borowska K., Koper J., 2011. Dynamics of changes of selenium content in soil and red clover (*Trifolium pretense* L.) affected by long-term organic fertilization on the background of selected soil oxidoreductases. Polish Journal of Environmental Studies. 20: 1403-1410**.**

Brandt-Kjelsen A., Govasmark E., Vegarud G., Haug A., et al., 2012. In vitro digestion of selenium from selenium-enriched chicken. Pure and Applied Chemistry. 84: 249-258.

Brenda B. C. and Robert B. J., 1997. Plant competition underground. Annual Review of Ecology and Systematics. 28: 545-570.

Brigelius-Flohe R., 2008. Selenium compounds and selenoproteins in cancer. Chemistry & Biodiversity. 5: 389-395.

Broadley M. R., Alcock J., Alford J., Cartwright P., et al., 2010. Selenium biofortification of high-yielding winter wheat (*Triticum aestivum* L.) by liquid or granular Se fertilisation. Plant and Soil. 332: 5-18.

Broadley M. R., White P. J., Bryson R. J., Meacham M. C., et al., 2006. Biofortification of UK food crops with selenium. Proceedings of the Nutrition Society. 65: 169-181.

Broadley M. R., White P. J., Hammond J. P., Zelko I. and Lux A., 2007. Zinc in plants. New Phytologist. 173: 677-702.

Brown K. M. and Arthur J. R., 2001. Selenium, selenoproteins and human health: A review. Public Health Nutrition. 4: 593-599.

Broyer T. C., Johnson C. M., Huston, R P., 1972. Selenium and nutrition of *Astragalus*. Plant and Soil. 36: 651-669.

Brtkova A. and Brtko J., 1996. Selenium: Metabolism and endocrines (minireview). Endocrine Regulation. 30: 117-128.

Bryman A. and Cramer D., 1997. Quantitative data analysis with SPSS for windows. A Guide for Social Scientists. London: Routledge.

Buchanan-Smith J. G., Berger C., Ferrel. C. L., Fox D. G., Gulgean M. L., Hutcheson D. P., Klopfenstein T. J. and Spears J.W., 2001. Nutrient requirements of dairy cattle. Washington, D.C.:National Academy Press.

Burger M., Jackson L., Lundquist E., Louie D., et al., 2005. Microbial responses and nitrous oxide emissions during wetting and drying of organically and conventionally managed soil under tomatoes. Biology and Fertility of Soils. 42: 109-118.

Cankur O., Yathavakilla S. K. and Caruso J. A., 2006. Selenium speciation in dill (*Anethum graveolens* L.) by ion pairing reversed phase and cation exchange HPLC with ICP-MS detection. Talanta. 70: 784-790.

Cartes P., Gianfreda L. and Mora M. L., 2005. Uptake of selenium and its antioxidant activity in ryegrass when applied as selenate and selenite forms. Plant and Soil. 276: 359-367.

Cartes P., Gianfreda L., Paredes C., Mora, M. L., 2011. Selenium uptake and its antioxidant role in ryegrass cultivars as affected by selenite seed pelletization. Journal of Soil Science and Plant Nutrition. 11: 1-14.

Cary E. E., Wieczorek G. A. and Allaway W. H., 1967. Reactions of selenite-selenium added to soils that produce low-selenium forages. Soil Science Society American Journal. 31: 21-26.

Cassens R. G., 1997. Composition and safety of cured meats in the USA. Food Chemistry. 59: 561-566.

Cauwenbergh R. V., Robberecht H., Van Vlaslaer V., De Smet A., et al., 2007. Plasma selenium levels in healthy blood bank donors in the Central-Eastern part of Belgium. Journal of Trace Element in Medicine and Biology. 21: 225-233.

Cauwenbergh R. V., Robberecht H., Van Vlaslaer V. and Deelstra H., 2004. Comparison of the serum selenium content of healthy adults living in the Antwerp region (Belgium) with recent literature data. Journal of Trace Element in Medicine and Biology. 18: 99-112.

Charles D. G. and Willigen R. T. V., 2006. Manganese: The oxygen-evolving complex & models. Encyclopedia of Inorganic Chemistry, John Wiley & Sons, Ltd.

Chen, A., Yang, F., Chen, J., Chen, X., Wen, Z., Ge, K. 1980. Studies on the relation of selenium and Keshan disease. Biological Trace Element Research. 2: 91-107.

Chen C., Yu H., Zhao J., Li B., et al., 2006. The roles of serum selenium and selenoproteins on mercury toxicity in environmental and occupational exposure. Environmental Health Perspectives. 114: 297-301.

Cheng O. T., 2002. Selenium deficiency and cardiomyopathy. Journal of the Royal Society of Medicine. 95: 219–220.

Cheng Z., Lu B.-R., Sameshima K., Fu D.-X. and Chen J.-K., 2004. Identification and genetic relationships of kenaf (*Hibiscus cannabinus* L.) germplasm revealed by AFLP analysis. Genetic Resources and Crop Evolution. 51: 393-401.

Chilimba A. D. C., Young S. D., Black C. R., Meacham M. C., et al., 2012. Agronomic biofortification of maize with selenium (se) in Malawi. Field Crops Research. 125: 118-128.

Cirelli A., Ciardi M., de Simone C., Sorice F., et al., 1991. Serum selenium concentration and disease progress in patients with HIV infection. Clinical Biochemistry. 24: 211-214.

Cerella C., Mareike K., Elodie V., Mario D., Claus J. and Marc D., 2011. Naturally occurring organic sulfur compounds: an example of a multitasking class of phytochemicals in anti-cancer research, phytochemicals - bioactivities and impact on health, Prof. Iraj Rasooli (Ed.).

Clark L. C., Combs G. F., Jr., Turnbull B. W., Slate E. H., 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. Journal of American Medical Association. 276: 1957-1963.

Clarkson D. T. and Sanderson J., 1971. Inhibition of the uptake and long-distance transport of calcium by aluminium and other polyvalent cations. Journal of Experimental Botany. 22: 837-851.

Codex alimentarius commission (FAO/WHO)., 2001 Food additives and contaminants. Joint FAO/WHO food standards program; Alinorm 01/12a, 1-289.

Coleysmith, J. R. A., 1986. Comparison of flavor and odor compounds of onion, leek, garlic and *Allium-fistulosum* in relation to germination of sclerotia of *Sclerotium-cepivorum*. Plant Pathology. 35: 370−376.

Combs G. F., Jr., 2001. Selenium in global food systems. The British Journal of Nutrition. 85: 517-547.

Commission of the European Communities., 2006. Commission regulation (ec) no 1750/2006 of 27 november 2006 Concerning the authorisation of selenomethionine as a feed additive No additive[online].Availableat:http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:330:0009:0011:EN:PDF.

Connelly-Frost A., Poole C., Satia J. A., Kupper L. L., et al., 2009. Selenium, folate, and colon cancer. Nutrition and Cancer. 61: 165-178.

Connolly E. L. and Guerinot M. L., 2002. Iron stress in plants. Genome Biology. 3.

Coppin F., Chabroullet C., Martin-Garin A., Balesdent J. and Gaudet J. P., 2006. Methodological approach to assess the effect of soil ageing on selenium behaviour: First results concerning mobility and solid fractionation of selenium. Biology and Fertility of Soils. 42: 379-386.

Curtin D., Hanson R., Lindley T. N. and Butler R. C., 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.

Daniels L. A., 1996. Selenium metabolism and bioavailability. Biological Trace Element Research. 54: 185-199.

Davies B. E. and Watkinson J. H., 1966. Uptake of native and applied selenium by pasture species II. Effects of sulphate and soil type on uptake by clover. New Zealand Journal of Crop and Horticultural Science. 9: 641-652.

de Leon C. A. P., Bayon M. M., Paquin C. and Caruso J. A., 2002. Selenium incorporation into *Saccharomyces cerevisiae* cells: A study of different incorporation methods. Journal of Applied Microbiology. 92: 602-610.

de Souza M. P., Lytle C. M., Mulholland M. M., Otte M. L. and Terry N., 2000. Selenium assimilation and volatilization from dimethylselenoniopropionate by Indian mustard. Plant Physiology. 122: 1281-1288.

Department of health., 1991. Dietary reference values for food, energy and nutrients for the uk. London, Uk, her majesty‟s stationery office (HMSO).

Dhillon K. S. and Dhillon S. K., 1991. Accumulation of selenium in sugarcane (S*achharum-officinarum* L.) in seleniferous areas of Punjab, India. Environmental Geochemistry and Health. 13: 165-170.

Dhillon K. S., Dhillon S. K. and Dogra R., 2010. Selenium accumulation by forage and grain crops and volatilization from seleniferous soils amended with different organic materials. Chemosphere. 78: 548-556.

Dhillon K. S. and Dhillon S. K., 2009. Accumulation and distribution of selenium in some vegetable crops grown in selenate-Se treated clay loam soil. . Frontiers of Agriculture in China. 3: 366-373.

Du Laing G., Vanthuyne D. R., Vandecasteele B., Tack F. M. and Verloo M. G., 2007. Influence of hydrological regime on pore water metal concentrations in a contaminated sediment-derived soil. Environmental Pollution. 147: 615-625.

Duffield-Lillico A. J., Reid M. E., Turnbull B. W., Combs G. F., Jr., et al., 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 and Biomarkers Preview. 11: 630-639.

Dumont E., Ogra Y., Vanhaecke F., Suzuki K. and Cornelis R., 2006a. Liquid chromatography–mass spectrometry (lCP–MS): A powerful combination for selenium speciation in garlic (*Allium sativum*). Analytical and Bioanalytical Chemistry. 384: 1196-1206.

Dumont E., Vanhaecke F. and Cornelis R., 2004. Hyphenated techniques for speciation of Se in in vitro gastrointestinal digests of *Saccharomyces cerevisiae*. Analytical and Bioanalytical Chemistry. 379: 504-511.

Dumont E., Vanhaecke F. and Cornelis R., 2006b. Selenium speciation from food source to metabolites: A critical review. Analytical and Bioanalytical Chemistry. 385: 1304-1323.

Dworkin B. M., Rosenthal W. S., Mittelman A., Weiss L., et al., 1988. Selenium status and the polyp-cancer sequence - a colonoscopically controlled-study. American Journal of Gastroenterology. 83: 748-751.

El-Bayoumy K., 2001. The protective role of selenium on genetic damage and on cancer. Mutation Research. 475: 123-139.

Ellis D. R. and Salt D. E., 2003. Plants, selenium and human health. Current Opinion in Plant Biology. 6: 273-279.

Elrashidi M. A., Adriano D. C., Workman S. M. and Lindsay W. L., 1987. Chemical equilibria of selenium in soils: A theoretical development. Soil Science. 144: 141-152.

Elsokkary I. H., 1980. Selenium distribution, chemical fractionation and adsorption in some Egyptian alluvial and lacustrine soils. Zeitschrift für Pflanzenernährung und Bodenkunde. 143: 74-83.

Eric B., 2010. Garlic and other *Alliums*: The Lore and the Science. Published by the Royal Society of Chemistry. ISBN: 978-0-85404-190-9.

Eurola M., Alfthan G., Aro A., Ekholm P. I., et al., 2003. Results of the Finnish selenium monitoring program of 2000-2001. In: Agrifood Research Reports 36. Agriculture Food Research Finland. 1-36.

Eurola M., Ekholm P. I., Ylinen M., Koivistoinen P. and Varo P., 1989. Effects of selenium fertilization on the selenium content of selected Finnish fruits and vegetables. Acta Agriculturae Scandinavica. 39: 345 - 350.

Eurola M. H., Ekholm P. I., Ylinen M. E., Varo P. T. and Koivistoinen P. E., 1991. Selenium in Finnish foods after beginning the use of selenate-supplemented fertilisers. Journal of the Science of Food and Agriculture. 56: 57-70.

Eurola M., Hietaniemi V., Kontturi M., Tuuri H., Kangas A., Niskanen M. and Saastamoinen M., 2004. Selenium content of Finnish oats in 1997–1999: effect of cultivars and cultivation techniques. Agricultural and Food Science. 13: 46–53.

Fairweather-Tait S. J., Bao Y., Broadley M. R., Collings R., et al., 2011. Selenium in human health and disease. Antioxid Redox Signal. 14: 1337-1383.

Fattorusso E., Lanzotti V., Taglialatela-Scafati O. and Cicala C., 2001. The flavonoids of leek, *Allium porrum*. Phytochemistry. 57: 565-569.

Ferguson L. R., Philpott M. and Karunasinghe N., 2004. Dietary cancer and prevention using antimutagens. Toxicology. 198: 147-159.

Fernandez-Garcia E., Carvajal-Lerida I. and Perez-Galvez A., 2009. In vitro bioaccessibility assessment as a prediction tool of nutritional efficiency. Nutrition Research. 29: 751-760.

Feroci G., Badiello R. and Fini A., 2005. Interactions between different selenium compounds and zinc, cadmium and mercury. Journal of Trace Element in Medicine and Biology. 18: 227-234.

Ferri T., Sangiorgio P., 1999. Voltammetric study of the interaction between Se(IV) and dissolved organic matter in environmental aqueous matrices. Analytica Chimica Acta. 385: 337-343.

Filley S. J., Peters A., Bouska C., Pirelli G. and Oldfield J., 2007. Selenium fertilization of pastures for improved forage selenium content. Journal of Animal Science. 23: 144-147.

Finley J. W. and Davis C. D., 2001. Selenium (se) from high-selenium broccoli is utilized differently than selenite, selenate and selenomethionine, but is more effective in inhibiting colon carcinogenesis. Biofactors. 14: 191-196.

Finley J. W., Davis C. D. and Feng Y., 2000. Selenium from high selenium broccoli protects rats from colon cancer. Journal of Nutrition. 130: 2384-2389.

Fleming G. A., 1962. Selenium in Irish soils and plants. Soil Science. 94: 28-35.

Fleming G. A., 1980. Essential micronutrients ii: Iodine and selenium. In: Davis, B.E. (editor). Applied Soil Trace Elements. John Wiley and Sons, New York, USA. 199-234.

Fordyce F., 2005. Selenium deficiency and toxicity in the environment. In: Selinus, O., Alloway, B.J., (editor). Essentials of medical geology: Impacts of the natural environment on public health. Academic Press, London, U.K. 373-415.

Fordyce F., 2007. Selenium geochemistry and health. AMBIO: A Journal of the Human Environment. 36: 94-97.

Gailer J., George G. N., Pickering I. J., Prince R. C., et al., 2000. A metabolic link between arsenite and selenite: The seleno-bis(*S*-glutathionyl) arsinium ion. Journal of the American Chemical Society. 122: 4637-4639.

Geering H. R., Cary E. E., Jones L. H. P. and Allaway W. H., 1968. Solubility and redox criteria for the possible forms of selenium in soils. Soil Science Society of America Journal. 32: 35-40.

Geoffroy L., Gilbin R., Simon O., Floriani M., et al., 2007. Effect of selenate on growth and photosynthesis of *Chlamydomonas reinhardtii*. Aquatic Toxicology. 83: 149-158.

Gibson B. D., Blowes D. W., Lindsay M. B. and Ptacek C. J., 2012. Mechanistic investigations of Se(VI) treatment in anoxic groundwater using granular iron and organic carbon: An EXAFS study. Journal of Hazardous Material. 241-242: 92-100.

Gissel-Nielsen G., 1971. Influence of pH and texture of the soil on plant uptake of added selenium. Journal of Agricultural and Food Chemistry. 19: 1165-1167.

Gissel-Nielsen G., Gupta U. C., Lamand M. and Westermarck T., 1984. Selenium in soils and plants and its importance in livestock and human nutrition. Advances in agronomy. N. C. Brady, Academic Press. 37**:** 397-460.

Gissel-Nielsen G., 1998. Effects of selenium supplementation of field crops. In: Frankenberger WT, Engberg RA (Eds.) Environmental Chemistry of Selenium. New York, USA, Dekker, pp 99-112.

Goenaga Infante H., O'Connor G., Rayman M., Wahlen R., et al., 2004. Selenium speciation analysis of selenium-enriched supplements by HPLC with ultrasonic nebulisation ICP-MS and electrospray MSMS detection. Journal of Analytical Atomic Spectrometry. 19: 1529-1538.

Gong G., Meplan C., Gautrey H., Hall J. and Hesketh J. E., 2012. Differential effects of selenium and knock-down of glutathione peroxidases on TNF alpha and flagellin inflammatory responses in gut epithelial cells. Genes and Nutrition. 7: 167-178.

Govasmark E., Brandt-Kjelsen A., Szpunar J., Bierla K., et al., 2010. Bioaccessibility of Se from Se-enriched wheat and chicken meat. Pure and Applied Chemistry. 82: 461-471.

Greenberg A., Parkinson D. K., Fetterolf D. E., Puschett J. B., et al., 1986. Effects of elevated lead and cadmium burdens on renal function and calcium metabolism. Archives of Environmental Health. 41: 69-76.

Greger J. L., 1999. Nutrition versus toxicology of manganese in humans: Evaluation of potential biomarkers. Neurotoxicology. 20: 205-212.

Gupta U. C. and Gupta S. C., 2000. Selenium in soils and crops, its deficiencies in livestock and humans: Implications for management. Communications in Soil Science and Plant Analysis. 31: 1791-1807.

Gupta U. C. and Gupta S. C., 2002. Quality of animal and human life as affected by selenium management of soils and crops. Communications in Soil Science and Plant Analysis. 33: 15-18.

Gupta U. C. and Gupta S., 2010. Selenium deficiency in soils and crops and its impact on animal and human health. Current Nutrition and Food Science. 6: 268-280.

Hambuckers A., Dotreppe O. and Istasse L., 2010. Problem of applying sodium selenate to increase selenium concentration in grassland plants in Southern Belgium. Communications in Soil Science and Plant Analysis. 41: 1283-1292.

Hajiboland R. and Amirazad F., 2010. Growth, photosynthesis and antioxidant defense system in Zn-deficient red cabbage plants. Plant Soil and Environment. 56: 209-217.

Hart D. J. and Scott K. J., 1995. Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. Food Chemistry. 54: 101-111.

Hartikainen H., 2005a. Biogeochemistry of selenium and its impact on food chain quality and human health. Journal of Trace Elements in Medicine and Biology. 18: 309-318.

Havey M. J., Galmarini C. R., Gokce A. F. and Henson C., 2004. QTL affecting soluble carbohydrate concentrations in stored onion bulbs and their association with flavor and health-enhancing attributes. Genome. 47: 463-468.

Hawkesford M. J. and Zhao F. J., 2007. Strategies for increasing the selenium content of wheat. Journal of Cereal Science. 46: 282-292.

Hays S. M., 1989. Kenaf tops equal high-quality hay. Agricultural Research. 37: 18-18.

He P. P., Lv X. Z. and Wang G. Y., 2004. Effects of Se and Zn supplementation on the antagonism against Pb and Cd in vegetables. Environment International. 30: 167-172.

Heider J. and Bock A., 1993. Selenium metabolism in micro-organisms. Advance Microbial Physiology. 35: 71-109.

Hira C. K., Partal K. and Dhillon K. S., 2004. Dietary selenium intake by men and women in high and low selenium areas of Punjab. Public Health Nutrition. 7: 39-43.

Hoffmann J. E. and King M. G., 1997. Selenium and selenium compounds. Kirk-Othmer Encyclopedia of Chemical Technology. 21: 686-719.

Hoffmann P. R. and Berry M. J., 2008. The influence of selenium on immune responses. Molecular Nutrition and Food Research. 52: 1273-1280.

Hopper J. L. and Parker D. R., 1999. Plant availability of selenite and selenate as influenced by the competing ions phosphate and sulphate. Plant and Soil. 210: 199-207.

Hotz C. and McClafferty B., 2007. From harvest to health: Challenges for developing biofortified staple foods and determining their impact on micronutrient status. Food and Nutrition Bulletin. 28: S271-279.

Huber J. R. E. and Criddle R. S., 1967. Comparison of the chemical properties of selenocysteine and selenocystine with their sulphur analogs. Archives of Radiochemistry and Riophysics. 122: 164-173.

Ip C., 1988. Differential effect of dietary methionine on the biopotency of selenomethionine and selenite in cancer chemoprevention. Journal of the National Cancer Institute. 80: 258-262.

Ip C., Birringer M., Block E., Kotrebai M., et al., 2000. Chemical speciation influences comparative activity of selenium-enriched garlic and yeast in mammary cancer prevention. Journal of Agricultural and Food Chemistry. 48: 2062-2070.

Ip C. and Lisk D. J., 1995. Efficacy of cancer prevention by high-selenium garlic is primarily dependent on the action of selenium. Carcinogenesis. 16: 2649-2652.

Issa A. A. and Adam M. S., 1999. Influence of selenium on toxicity of some heavy metals in the green alga *Scenedesmus obliquus*. Folia Microbiologica. 44: 406-410.

Jackson M. J., Dillon S. A., Broome C. S., Mcardle A., Hart C. A. and Mcardle F., 2004. Are there functional consequences of a reduction in selenium intake in UK subjects? Proceedings of the Nutrition Society. 63: 513-517.

Jackson M. I. and Combs G. F., 2008. Selenium and anticarcinogenesis: underlying mechanisms. Current Opinion in Clinical Nutrition & Metabolic Care. 11: 718–726.

Jaiswal S. K., Prakash R., Acharya R., Nathaniel T.N., Reddy A.V.R. and Prakash T. N., 2012. Bioaccessibility of selenium from Se-rich food grains of the seleniferous region of Punjab, India as analyzed by instrumental neutron activation analysis. CyTA - Journal of Food. 10: 160-164.

Johnsson L., 1991. Selenium uptake by plants as a function of soil type, organic-matter content and pH. Plant and Soil. 133: 57-64.

Jouany J. P., 1982. Volatile fatty acids and alcohol determination in digestive contents, silage juice, bacterial cultures and anaerobic fermentor contents. Science Aliments. 2: 131–144.

Kahakachchi C., Boakye H. T., Uden P. C. and Tyson J. F., 2004. Chromatographic speciation of anionic and neutral selenium compounds in se-accumulating *Brassica juncea* (Indian mustard) and in selenized yeast. Journal of Chromatography A. 1054: 303-312.

Kapolna E. and Fodor P., 2007. Bioavailability of selenium from selenium-enriched green onions (*Allium fistulosum*) and chives (*Allium schoenoprasum*) after '*in vitro*' gastrointestinal digestion. International Journal of Food Sciences and Nutrition. 58: 282-296.

Kápolna E., Shah M., Caruso J. A. and Fodor P., 2007b. Selenium speciation studies in se-enriched chives (*Allium schoenoprasum*) by HPLC-ICP-MS. Food Chemistry. 101: 1398-1406.

Kasaikina M. V., Kravtsova M. A., Lee B. C., Seravalli J., et al., 2011. Dietary selenium affects host selenoproteome expression by influencing the gut microbiota. FASEB Journal. 25: 2492-2499.

Kim B. W. and Sung K. I., 2007. Effects of planting density, cultivar and growing day on the dry matter yield and forage quality of kenaf (*Hibiscus Cannabinus* L) in the northern area of South Korea. Journal of Dairy Science. 90: 39-39.

Kim J. and Combs G. F., Jr., 1997. Effects of selenium on colonic fermentation in the rat. Biological Trace Element Research. 56: 215-224.

Kim Y. Y. and Mahan D. C., 2001. Comparative effects of high dietary levels of organic and inorganic selenium on selenium toxicity of growing-finishing pigs. Journal of Animal Science. 79: 942-948.

Kinnear P. R. and Gray C. D., 1999. Spss for windows made simple. Third edition. United Kingdom: Psychology Press Ltd.

Klein E. A., Thompson I. M. Jr., Tangen C. M., Crowley J. J., Lucia M. S et al., 2011.Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). Journal of the American Medical Association. 306: 1549-56.

Kljai K. and Runje R., 2001. Selenium and glycogen levels in diabetic patients. Biological Trace Element Research. 83: 223-229.

Knowles T. C., Wright N. and Sherrill C., 1999. Growth characteristics, hay yield, and feed quality of kenaf grown in Mohave Valley. Forage and Grain Report; [cited 2013 Mar 19]. Available from: http://ag.arizona.edu/pubs/crops/az1147/.

Kopsell D. A. and Randle W. M., 1997. Selenate concentration affects selenium and sulphur uptake and accumulation by `Granex 33' onions. Journal of the American Society for Horticultural Science. 122: 721-726.

Kotrebai M., Tyson J. F., Block E. and Uden P. C., 2000. High-performance liquid chromatography of selenium compounds utilizing perfluorinated carboxylic acid ion-pairing agents and inductively coupled plasma and electrospray ionization mass spectrometric detection. Journal of Chromatography A. 866: 51-63.

Kubmarawa D., Andenyang, I. F. H., Magomya, A. M., 2009. Proximate composition and amino acid profile of two non-conventional leafy vegetables (*Hibiscus cannabinus and Haematostaphis barteri*). African Journal of Food Science. 3: 233-236.

Landberg T. and Greger M., 1994. Influence of selenium on uptake and toxicity of copper and cadmium in pea (*Pisum sativum*) and wheat (*Triticum aestivum*). Physiologia Plantarum. 90: 637-644.

Larsen E., Lobinski R., Burger-Meÿer K., Hansen M., et al., 2006. Uptake and speciation of selenium in garlic cultivated in soil amended with symbiotic fungi (mycorrhiza) and selenate. Analytical and Bioanalytical Chemistry. 385: 1098-1108.

Lavu R. V., Du Laing G., Van De Wiele T., Pratti V. L., et al., 2012. Fertilizing soil with selenium fertilizers: Impact on concentration, speciation, and bioaccessibility of selenium in leek (*Allium ampeloprasum*). Journal of Agricultural and Food Chemistry. 60: 10930-10935.

Lemly A. D., 1997. Environmental implications of excessive selenium: A review. Biomedical Environmental Science. 10: 415-435.

Letavayová L., Vlčková V. and Brozmanová J., 2006. Selenium: from cancer prevention to DNA damage. Toxicology. 227: 1-14.

Levander O. A., Alfthan G., Arvilommi H., Gref C. G., et al., 1983. Bioavailability of selenium to Finnish men as assessed by platelet glutathione peroxidase activity and other blood parameters. American Journal of Clinical Nutrition. 37: 887-897.

Levesque M., 1974. Some aspects of selenium relationships in Eastern Canadian soils and plants. Canadian Journal of Soil Science. 54: 205-214.

Lewis B. G., Johnson C. M. and Broyer T. C., 1974. Volatile selenium in higher plants the production of dimethyl selenide in cabbage leaves by enzymatic cleavage of Se-methyl selenomethionine selenonium salt. Plant and Soil. 40: 107-118.

Li H. F., McGrath S. P. and Zhao F. J., 2008. Selenium uptake, translocation and speciation in wheat supplied with selenate or selenite. New Phytologist. 178: 92-102.

Liang Zhu Y., Pilon-Smits E. A., Jouanin L. and Terry N., 1999. Overexpression of glutathione synthetase in indian mustard enhances cadmium accumulation and tolerance. Plant Physiology. 119: 73-80.

Lingle J. C., Tiffin L. O. and Brown J. C., 1963. Iron uptake-transport of soybeans as influenced by other cations. Plant Physiology. 38: 71-76.

Liu Q., Wang D. J., Jiang X. J., Cao Z. H., 2004. Effects of the interactions between selenium and phosphorus on the growth and selenium accumulation in rice (*Oryza sativa*). Environmental Geochemistry and Health. 26: 325-330.

Liu J. T., Li Z., Wei S., Shan W. and Wei-min Z., 2006. Research progress of bioactive constituent and molecular biology in *Allium tuberosum*. Food Science and Technology International. 8: 67–70.

Logan T. J., Chang A. C., Page A. L. and Ganje T. J., 1987. Accumulation of selenium in crops grown on sludge-treated soil. Journal of Environmental Quality. 16: 349-352.

Look M. P., Rockstroh J. K., Rao G. S., Kreuzer K. A., et al., 1997. Serum selenium versus lymphocyte subsets and markers of disease progression and inflammatory response in human immunodeficiency virus-I infection. Biological Trace Element Research. 56: 31-41.

Lopez R. G., Vinent E. and Herrera J., 2006. Green protein in 60 days, kenaf (*Hibiscus cannabiscus*), an alternative for cattle rearing. Cuban Journal of Agricultural Science. 40: 143-146.

Lu J. and Jiang C., 2005. Selenium and cancer chemoprevention: hypotheses integrating the actions of selenoproteins and selenium metabolites in epithelial and non-epithelial target cells. Antioxidants & Redox Signaling. 7: 1715–1727.

Lyi S. M., Heller L. I., Rutzke M., Welch R. M., et al., 2005. Molecular and biochemical characterization of the selenocysteine se-methyltransferase gene and Se-methylselenocysteine synthesis in broccoli. Plant Physiology. 138: 409-420.

Lyons G. H., Judson G. J., Ortiz-Monasterio I., Genc Y., Stangoulis J. C. R. and Graham R. D., 2005. Selenium in Australia: Selenium status and biofortification of wheat for better health. Journal of Trace Elements in Medicine and Biology. 19: 75-82.

Lyons G., Stangoulis J. and Graham R., 2003. High-selenium wheat: Biofortification for better health. Nutrition Research Review. 16: 45-60.

Lyons G. H., Lewis J., Lorimer M. F., Holloway R. E., et al., 2004. High selenium wheat: Agronomic biofortification strategies to improve human nutrition. Journal of Food Agriculture and Environment. 2: 171-178.

Madentzidou E., Gerasopoulos D., Siomos A. and Bloukas I., 2012. Salt-stressed fresh cut leek accelerates CO_2 and C_2H_4 production and enhances the development of quality characteristics of traditional Greek sausages during storage. Meat Science. 92: 789-794.

Malik J. A., Kumar S., Thakur P., Sharma S., et al., 2011. Promotion of growth in mungbean (*Phaseolus aureus R*oxb.) by selenium is associated with stimulation of carbohydrate metabolism. Biological Trace Element Research. 143: 530-539.

Masscheleyn P. H., Delaune R. D. and Patrick W. H., 1990. Transformations of selenium as affected by sediment oxidation-reduction potential and pH. Environmental Science and Technology. 24: 91-96.

Mayland H.F., Gough L.P. and Stewart K.C., 1991. Selenium mobility in soils and its absorption, translocation, and metabolism in plants, pp. 55-64. In: Severson, R.C and Fisher Jr, .E and Gough, L.P. (eds.) Proceedings of 1990 Reclamation Symposium.

Mazej D., Osvald J. and Stibilj V., 2008. Selenium species in leaves of chicory, dandelion, lamb's lettuce and parsley. Food Chemistry. 107: 75-83.

Mazzafera P., 1998. Growth and biochemical alterations in coffee due to selenite toxicity. Plant and Soil. 201: 189-196.

Mehraban P., Zadeh A. A. and Sadeghipour H. R., 2008. Iron toxicity in rice (*Oryza sativa* L.), under different potassium nutrition. Asian Journal of Plant Sciences. 7: 251-259.

Micetic-Turk D., Rossipal E., Krachler M. and Li F., 2000. Maternal selenium status in Slovenia and its impact on the selenium concentration of umbilical cord serum and colostrum. European Journal of Clinical Nutrition. 54: 522-524.

Mikkelsen R. L., Page A. L. and Haghnia G. H., 1988. Effect of salinity and its composition on the accumulation of selenium by alfalfa. Plant and Soil. 107: 63-67.

Molan A. L., Flanagan J., Wei W. and Moughan P. J., 2009. Selenium-containing green tea has higher antioxidant and prebiotic activities than regular green tea. Food Chemistry. 114: 829-835.

Molly K., Vandewoestyne M., Desmet I. and Verstraete W., 1994. Validation of the simulator of the human intestinal microbial ecosystem (SHIME) reactor using microorganism-associated activities. Microbial Ecology in Health and Disease. 7: 191-200.

Mondy N., Duplat D., Christides J. P., Arnault I. and Auger J., 2002. Aroma analysis of fresh and preserved onions and leek by dual solid-phase microextraction-liquid extraction and gas chromatography-mass spectrometry. Journal of Chromatography A. 963: 89-93.

Mora M., Pinilla L., Rosas A., Cartes P., 2008. Selenium uptake and its influence on the antioxidative system of white clover as affected by lime and phosphorus fertilization. Plant and Soil. 303: 139-149.

Moran J., Becana M., Iturbe-Ormaetxe I., Frechilla S., et al., 1994. Drought induces oxidative stress in pea plants. Planta. 194: 346-352.

Moreda-Pineiro J., Moreda-Pineiro A., Romaris-Hortas V., Moscoso-Perez C., et al., 2011. In-vivo and in-vitro testing to assess the bioaccessibility and the bioavailability of arsenic, selenium and mercury species in food samples. Trace-Trends in Analytical Chemistry. 30: 324-345.

Mossor-Pietraszewska T., 2001. Effect of aluminium on plant growth and metabolism. Acta Biochimica Polonica. 3: 673-686.

Mounicou S., Dernovics M., Bierla K. and Szpunar J., 2009. A sequential extraction procedure for an insight into selenium speciation in garlic. Talanta. 77: 1877-1882.

Nakamaru Y., Tagami K., Uchida S., 2006. Effect of phosphate addition on the sorption-desorption reaction of selenium in Japanese agricultural soils. Chemosphere. 63: 109-115.

Nakamaru Y. M., Sekine K., 2008. Sorption behavior of selenium and antimony in soils as a function of phosphate ion concentration. Soil Science & Plant Nutrition. 54: 332-341.

Navarro-Alarcon M. and Cabrera-Vique C., 2008. Selenium in food and the human body: A review. Science of The Total Environment. 400: 115-141.

National Research Council. 1989, Recommended dietary allowances 10th ed. Washingtom, DC. National Academy Press.

Neal R. H. and Sposito G., 1991. Selenium mobility in irrigated soil columns as affected by organic carbon amendment. Journal of Environmental Quality. 20: 808-814.

Ochoka T., Razniewska M. and G J., 2000. Blood serum selenium levels in children and adults in Poland. Dustri, Deisenhofen, Allemagne. 17: 3.

Ogra Y., Ishiwata K., Iwashita Y. and Suzuki K. T., 2005. Simultaneous speciation of selenium and sulphur species in selenized odorless garlic (*Allium sativum* L. Shiro) and shallot (*Allium ascalonicum*) by HPLC-inductively coupled plasma-(octopole reaction system)-mass spectrometry and electrospray ionization-tandem mass spectrometry. Journal of Chromatographic A. 1093: 118-125.

Paganga G., Miller N. and Rice-Evans C. A., 1999. The polyphenolic content of fruit and vegetables and their antioxidant activities. What does a serving constitute? Free Radical Research. 30: 153-162.

Palafox-Carlos H., Ayala-Zavala J. F. and Gonzalez-Aguilar G. A., 2011. The role of dietary fiber in the bioaccessibility and bioavailability of fruit and vegetable antioxidants. Journal of Food Science. 76: R6-R15.

Pallud S., Lennon A.-M., Ramauge M., Gavaret J.-M., et al., 1997. Expression of the type ii iodothyronine deiodinase in cultured rat astrocytes is selenium-dependent. Journal of Biological Chemistry. 272: 18104-18110.

Parada J. and Aguilera J. M., 2007. Food microstructure affects the bioavailability of several nutrients. Journal of Food Science. 72: R21-R32.

Parr R. M., Aras N. K. and Iyengar G. V., 2006. Dietary intakes of essential trace elements: Results from total diet studies supported by the IAEA. Journal of Radioanalytical and Nuclear Chemistry. 270: 155-161.

Pedrero Z., Encinar J. R., Madrid Y. and Camara C., 2007. Identification of selenium species in selenium-enriched Lens esculenta plants by using two-dimensional liquid chromatography-inductively coupled plasma mass spectrometry and [Se-77] selenomethionine selenium oxide spikes. Journal of Chromatography A. 1139: 247-253.

Phillips W. A., Rao S. C., Fitch J. Q. and Mayeux H. S., 2002. Digestibility and dry matter intake of diets containing alfalfa and kenaf. Journal of Animal Science. 80: 2989-2995.

Pilon-Smits E. A., Quinn C. F., Tapken W., Malagoli M. and Schiavon M., 2009. Physiological functions of beneficial elements. Current Opinion in Plant Biology. 12: 267-274.

Poley, W.E., Wilson W.O., Moxon A.L. and Taylor J.B., 1941. The effect of selenized grains on the rate of growth in chicks. Poultry Science. 20: 171-179.

Possemiers S., Bolca S., Grootaert C., Heyerick A., et al., 2006. The prenylflavonoid isoxanthohumol from hops (*Humulus lupulus* L.) is activated into the potent phytoestrogen 8-prenylnaringenin *in vitro* and in the human intestine. Journal of Nutrition. 136: 1862-1867.

Prasad A. S., 2009. Impact of the discovery of human zinc deficiency on health. Journal of the American College of Nutrition. 28: 257-265.

Proteggente A. R., Pannala A. S., Paganga G., Van Buren L., et al., 2002. The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. Free Radical Research. 36: 217-233.

Psathakis D., Wedemeyer N., Oevermann E., Krug F., et al., 1998. Blood selenium and glutathione peroxidase status in patients with colorectal cancer. Diseases of the Colon & Rectum. 41: 328-335.

Pyrzynska K., 2009. Selenium speciation in enriched vegetables. Food Chemistry. 114: 1183-1191.

Rani N., Dhillon K. S. and Dhillon S. K., 2005. Critical levels of selenium in different crops grown in an alkaline silty loam soil treated with selenite-Se. Plant and Soil. 277: 367-374.

Rayman M. P., 2000. The importance of selenium to human health. Lancet. 356: 233-241.

Rayman M. P., 2004. The use of high-selenium yeast to raise selenium status: How does it measure up? Brazilian Journal of Nutrition. 92: 557-573.

Rayman M. P., 2005. Selenium in cancer prevention: A review of the evidence and mechanism of action. Proceedings of Nutritional Society. 64: 527-542.

Rayman M. P. and Rayman M. P., 2002. The argument for increasing selenium intake. Proceedings of Nutritional Society. 61: 203-215.

Reilly C., 1998. Selenium: A new entrant into the functional food arena. Trends in Food Science & Technology. 9: 114-118.

Rengel Z. and Graham R., 1995. Importance of seed Zn content for wheat growth on Zn-deficient soil. Plant and Soil. 173: 259-266.

Reyes L. H., Encinar J. R., Marchante-Gayon J. M., Alonso J. I. and Sanz-Medel A., 2006. Selenium bioaccessibility assessment in selenized yeast after "*in vitro*" gastrointestinal digestion using two-dimensional chromatography and mass spectrometry. Journal of Chromatography A. 1110: 108-116.

Ribeiro D. M., Mapeli A. M., Antunes W. C. and Barros R. S., 2011. A dual role of selenium in the growth control of seedlings of *Stylosanthes humilis*. Scientific Research, Open access: DOI: 10.4236/as.2011.22012. 2: 78-85.

Robberecht H., Hendrix P., Cauwenbergh R. and Deelstra H., 1994. Actual daily dietary intake of selenium in Belgium, using duplicate portion sampling. Zeitschrift für Lebensmittel-Untersuchung und Forschung. 199: 251-254.

Robberecht H., Vanden Berghe D., Deelstra H. and Van Grieken R., 1982. Selenium in the Belgian soils and its uptake by rye-grass. Science of The Total Environment. 25: 61-69.

Roekens E. J., Robberecht H. J. and Deelstra H. A., 1986. Dietary selenium intake in Belgium for different population groups at risk for deficiency. Z Lebensm Unters Forsch. 182: 8-13.

Rossi A., Poverini R., Di Lullo G., Modesti A., et al., 1996. Heavy metal toxicity following apical and basolateral exposure in the human intestinal cell line caco-2. Toxicology in Vitro. 10: 27-36.

Rotruck J. T., Pope A. L., Ganther H. E., Swanson A. B., et al., 1973. Selenium - biochemical role as a component of glutathione peroxidase. Science. 179: 588-590.

Ruby M. V., Davis A., Schoof R., Eberle S. and Sellstone C. M., 1996. Estimation of lead and arsenic bioavailability using a physiologically based extraction test. Environmenatal Science and Technology. 30: 422-430.

Rudolf E., Kralova V. and Cervinka M., 2008. Selenium and colon cancer--from chemoprevention to new treatment modality. Anticancer Agents in Medicinal Chemistry. 8: 598-602.

Rumi G., Imre I., Sulle C., Sarudi I., et al., 1992. Selenium in the blood of patients with colorectal-cancer and neoplastic polyp. Acta Physiologica Hungarica. 80: 275-279.

Sandle G. I., 1998. Salt and water absorption in the human colon: A modern appraisal. Gut. 43: 294-299.

Schulte E. E., Kelling K. A., 2004. Understanding plant nutrients: Soil and Applied copper. Online webpage acccesed: 11/08/2013: http://dev.soils.wisc.edu/extension//pubs/A2528.pdf.

Schulte E. E., Kelling K. A., 2004. Understanding plant nutrients: Soil and Applied Zinc. Online webpage acccesed: 11/08/2013: http://dev.soils.wisc.edu/extension//pubs/A2528.pdf.

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

Selenius M., Rundlof A.K., Olm E., Fernandes A.P. and Bjornstedt M., 2010. Selenium and the selenoprotein thioredoxin reductase in the prevention, treatment and diagnostics of cancer. Antioxidants & Redox Signaling. 12: 867–880.

Shah M., Kannamkumarath S. S., Wuilloud J. C. A., Wuilloud R. G. and Caruso J. A., 2004. Identification and characterization of selenium species in enriched green onion (*Allium fistulosum*) by HPLC-ICP-MS and ESI-MS. Journal of Analytical Atomic Spectrometry. 19: 381-386.

Shamberger R. J. and Frost D. V., 1969. Possible protective effect of selenium against human cancer. Cancer Medical Association Journal. 100: 682.

Sharma S., Bansal A., Dhillon S. K. and Dhillon K. S., 2010. Comparative effects of selenate and selenite on growth and biochemical composition of rapeseed (*Brassica napus* L.). Plant and Soil. 329: 339-348.

Shrift A., 1973. Metabolism of selenium by plants and microorganisms organic selenium compounds: Their chemistry and biology. (Klayman, D. L & Gu"nther, W.H.H., eds.). 763–814.

Sillanpää M. and Jansson H., 1992. Status of cadmium, lead, cobalt, and selenium in soils and plants of 30 countries. Geneva, Switzerland: FAO.

Simpson H. L., Roberts C. L., Rhodes J. M. and Campbell B. J., 2012. PMO-030 soluble plant fibres, particularly leek and plantain, inhibit adherence of diarrhoea-associated pathogens c difficile and enterotoxigenic escherichia coli to intestinal epithelial cells. Gut. 61: A85.

Singh B. R., 1991. Selenium content of wheat as affected by selenate and selenite contained in a Cl^- or So_4-based npk fertilizer. Fertilizer research. 30: 1-7.

Singh M. and Singh N., 1978. Selenium toxicity in plants and its detoxification by phosphorus. Soil Science. 126: 255-262.

Singh P., Misra A. and Srivastava N. K., 2001. Influence of Mn deficiency on growth, chlorophyll content, physiology, and essential monoterpene oil(s) in genotypes of spearmint (*Mentha spicata* L.). Photosynthetica. 39: 473-476.

Slavik P., Illek J., Brix M., Hlavicova J., Rajmon R. and Jilek F., 2008. Influence of organic versus inorganic dietary selenium supplementation on the concentration of selenium in colostrum, milk and blood of beef cows. Acta Veterinaria Scandinavica,50:43. doi:10.1186/1751-0147-50-43.

Smith F. W., Ealing P. M., Hawkesford M. J. and Clarkson D. T., 1995. Plant members of a family of sulphate transporters reveal functional subtypes. Proceedings of the National Academy of Sciences of the United States of America. 92: 9373–9377.

Sors T. G., Ellis D. R. and Salt D. E., 2005. Selenium uptake, translocation, assimilation and metabolic fate in plants. Photosynthesis Research. 86: 373-389.

Souza. M. P. D., Pilon-Smits. E. A. H., Lytle. C. M., Hwang. S., et al., 1998. Rate-limiting steps in selenium assimilation and volatilization by Indian mustard. Plant Physiology. 117: 1487-1494.

Srivastava M., Ma L. Q. and Cotruvo J. A., 2005. Uptake and distribution of selenium in different fern species. International Journal of Phytoremediation. 7: 33-42.

Steen A., Strom T. and Bernhoft A., 2008. Organic selenium supplementation increased selenium concentrations in ewe and newborn lamb blood and in slaughter lamb meat compared to inorganic selenium supplementation. Acta Veterinaria Scandinavica. 50: 7.

Stolz J. F., Basu P., Santini J. M. and Oremland R. S., 2006. Arsenic and selenium in microbial metabolism. Annual Review in Microbiology. 60: 107-130.

Suzuki K. T., Doi C. and Suzuki N., 2006a. Metabolism of 76Se-methylselenocysteine compared with that of 77Se-selenomethionine and 82Se-selenite. Toxicology and Applied Pharmacology. 217: 185-195.

Suzuki K. T., Kurasaki K., Ogawa S. and Suzuki N., 2006b. Metabolic transformation of methylseleninic acid through key selenium intermediate selenide. Toxicology and Applied Pharmacology. 215: 189-197.

Suzuki K. T., Tsuji Y., Ohta Y. and Suzuki N., 2008. Preferential organ distribution of methylselenol source Se-methylselenocysteine relative to methylseleninic acid. Toxicology and Applied Pharmacology. 227: 76-83.

Tamas M., Mandoki Z. S. and Csapo J., 2010. The role of selenium content of wheat in the human nutrition. Acta Universitatis Sapientiae, Alimentaria. 3: 5−34.

Terry N., Zayed A. M., De Souza M. P. and Tarun A. S., 2000. Selenium in higher plants. Annual Review of Plant Physiology and Plant Molecular Biology. 51: 401-432.

Toole O. D., Raisbeck M. F., Diagn J. V., 1995. Pathology of experimentally induced chronic selenosis (alkali disease) in yearling cattle. Journal of Veterinary Diagnostic Investigation. 7: 364-373.

Topping D. L. and Clifton P. M., 2001. Short-Chain Fatty Acids and Human Colonic Function: Roles of Resistant Starch and Nonstarch Polysaccharides. Physiological Reviews. 81: 1031-106.

Thomson C. D., 2004. Assessment of requirements for selenium and adequacy of selenium status: A review. European Journal of Clinical Nutrition. 58: 391-402.

Thomson C. D. and Robinson M. F., 1980. Selenium in human health and disease with emphasis on those aspects peculiar to New Zealand. American Journal of Clinical Nutrition. 33: 303-323.

Thomson C. D. and Robinson M. F., 1996. The changing selenium status of New Zealand residents. European Journal of Clinical Nutrition 50: 107-114.

Thomson C. D., Robinson M. F., Butler J. A. and Whanger P. D., 1993. Long-term supplementation with selenate and selenomethionine: Selenium and glutathione peroxidase in blood components of New Zealand women. British Journal of Nutrition. 69: 577-588.

Thomson C. D., Robinson M. F., Campbell D. R. and Rea H. M., 1982. Effect of prolonged supplementation with daily supplements of selenomethionine and sodium selenite on glutathione peroxidase activity in blood of New Zealand residents. American Journal of Clinical Nutrition. 36: 24-31.

Tiwary A. K., Stegelmeier B. L., Panter K. E., James L. F. and Hall J. O., 2006. Comparative toxicosis of sodium selenite and selenomethionine in lambs. Journal of Veterinary Diagnostic Investigation. 18: 61-70.

Turner R. J., Weiner J. H. and Taylor D. E., 1998. Selenium metabolism in escherichia coli. Biometals. 11: 223-227.

Van de Wiele T., Boon N., Possemiers S., Jacobs H. and Verstraete W., 2007. Inulin-type fructans of longer degree of polymerization exert more pronounced *in vitro* prebiotic effects. Journal of Applied Microbiology. 102: 452-460.

Van de Wiele T., Boon N., Possemiers S., Jacobs H. and Verstraete W., 2004. Prebiotic effects of chicory inulin in the simulator of the human intestinal microbial ecosystem. FEMS Microbiology Ecology. 51: 143-153.

Van Dijk H., 1971. Cation binding of humic acids. Geoderma. 5: 53-67.

Van Ranst E., Verloo M., Demeyer A. and Pauwels J. M., 1999. Manual for the soil chemistry and fertility laboratory. University of Gent. ISBN: 90-766603-01-4.

Varo P., Alfthan G., Ekholm P., Aro A. and Koivistoinen P., 1988. Selenium intake and serum selenium in Finland: effects of soil fertilization with selenium. The American Journal of Clinical Nutrition. 48: 324-329.

Vrca V. B., Skreb F., Cepelak I., Romic Z. and Mayer L., 2004. Supplementation with antioxidants in the treatment of graves' disease; the effect on glutathione peroxidase activity and concentration of selenium. Clinical Chimica Acta. 341: 55-63.

Waegeneers N., Thiry C., De Temmerman L. and Ruttens A., 2013. Predicted dietary intake of selenium by the general adult population in Belgium. Food Additive and Contamination Part-A Chemical and Analytical Control Exposure Risk Assessment. 30: 278-285.

Watkinson J. H., 1981. Changes of blood selenium in New Zealand adults with time and importation of Australian wheat. American Journal of Clinical Nutrition. 34: 936-942.

Webber I. C. L., 1993. Crude protein and yield components of six kenaf cultivars as affected by crop maturity. Industrial Crops and Products. 2: 27-31.

Weiss H. V., Koide M. and Goldberg E. D., 1971. Selenium and sulphur in a greenland ice sheet: Relation to fossil fuel combustion. Science. 172: 261-263.

Welch R. M. and Graham R. D., 1999. A new paradigm for world agriculture: Meeting human needs: Productive, sustainable, nutritious. Field Crops Research. 60: 1-10.

Whanger P. D., 2004. Selenium and its relationship to cancer: An update. Brazilian Journal of Nutrition. 91: 11-28.

Whanger P. D., Ip C., Polan C. E., Uden P. C. and Welbaum G., 2000. Tumorigenesis, metabolism, speciation, bioavailability, and tissue deposition of selenium in selenium-enriched ramps (*Allium tricoccum*). Journal of Agriculture and Food Chemistry. 48: 5723-5730.

Whelan B. R. and Barrow N. J., 1994. Slow-release selenium fertilizers to correct selenium deficiency in grazing sheep in Western-Australia. Fertilizer Research. 38: 183-188.

White P. J., Bowen H. C., Parmaguru P., Fritz M., et al., 2004. Interactions between selenium and sulphur nutrition in *arabidopsis thaliana*. Journal of Experimental Botany. 55: 1927-1937.

WHO/FAO/IAEA. 1996. Trace elements in Human nutrition and health. Geneva. World Health Organization.

Wijnja H. and Schulthess C. P., 2000. Vibrational spectroscopy study of selenate and sulphate adsorption mechanisms on fe and al (hydr)oxide surfaces. Journal of Colloid and Interface Science. 229: 286-297.

Wilber C. G., 1980. Toxicology of selenium: A review. Clinical Toxicology. 17: 171-230.

Williams C. and Thornton I., 1973. The use of soil extractants to estimate plant-available molybdenum and selenium in potentially toxic soils. Plant and Soil. 39: 149-159.

Williams P. N., Villada A., Deacon C., Raab A., et al., 2007. Greatly enhanced arsenic shoot assimilation in rice leads to elevated grain levels compared to wheat and barley. Environmental Science and Technology. 41: 6854-6859.

Wilson L. G. and Bandurski R. S., 1958. Enzymatic reactions involving sulphate, sulphite, selenate, and molybdate. Journal of Biological Chemistry. 233: 975-981.

Wróbel K., Wróbel K., Kannamkumarath S. S., Caruso J. A., et al., 2004. HPLC-ICP-MS speciation of selenium in enriched onion leaves – a potential dietary source of se-methylselenocysteine. Food Chemistry. 86: 617-623.

Xiccato G., Trocino A. and Carazzolo A., 1998. Ensiling and nutritive value of kenaf (*Hibiscus cannabinus*). Animal Feed Science and Technology. 71: 229-240.

Ximenez-Embun P., Alonso I., Madrid-Albarran Y., Camara C., 2004. Establishment of selenium uptake and species distribution in lupine, Indian mustard, and sunflower plants. Journal of Agricultural and Food Chemistry. 52: 832-838.

Yang L., Sturgeon R. E., McSheehy S. and Mester Z., 2004. Comparison of extraction methods for quantitation of methionine and selenomethionine in yeast by species specific isotope dilution gas chromatography-mass spectrometry. Journal of Chromatography A. 1055: 177-184.

Yao X., Chu J. and Wang G., 2009. Effects of selenium on wheat seedlings under drought stress. Biological Trace Element Research. 130: 283-290.

Yazan L. S., Foo J. B., Ghafar S. A. A., Chan K. W., et al., 2011. Effect of kenaf seed oil from different ways of extraction towards ovarian cancer cells. Food and Bioproducts Processing. 89: 328-332.

Ylaranta T., 1983. Sorption of selenite and selenate in the soil. Annales Agriculturae Fenniae. 22: 29-39.

Ylaranta T., 1984. Raising the selenium content of spring wheat and barley using selenite and selenate. Annales Agriculturae. 23: 75-84.

Ylaranta T., 1990. The Selenium content of some agricultural crops and soils before and after the addition of Selenium to fertilizers in Finland. Annales Agriculturae Fenniae. 29: 131−139.

Zeng H., 2009. Selenium as an essential micronutrient: Roles in cell cycle and apoptosis. Molecules. 14: 1263-1278.

Zeng H. W., Botnen J. H. and Johnson L. K., 2008. A selenium-deficient caco-2 cell model for assessing differential incorporation of chemical or food selenium into glutathione peroxidase. Biological Trace Element Research. 123: 98-108.

Zhang P. C. and Sparks D. L., 1990. Kinetics of selenate and selenite adsorption desorption at the goethite water interface. Environmental Science and Technology. 24: 1848-1856.

Zhao C., Ren J., Xue C. and Lin E., 2005. Study on the relationship between soil selenium and plant selenium uptake. Plant and Soil. 277: 197-206.

Zwolak I. and Zaporowska H., 2012. Selenium interactions and toxicity: A review. Selenium interactions and toxicity. Cell Biology and Toxicology. 28: 31-46.

Appendices

Appendix 1. Elemental concentrations (mg kg^{-1}) in soils of the study fields

Field.No	Cr	Cu	Mn	Ni	Pb	Zn	Al	Fe	Ca	Mg	Se
1	18.1	24.5	377	8.27	21.6	60.4	7038	10925	1476	1430	0.34
2	14.8	19.8	165	5.35	10.6	52.9	6746	8231	1984	1092	0.32
3	13.7	16.5	209	4.93	9.3	33.3	5328	6525	1223	798	0.21
4	15.1	17.0	192	4.65	14.4	40.3	6543	6489	1313	727	0.3
5	17.1	21.9	320	8.14	12.0	41.7	6646	7893	1830	1049	0.28
6	21.4	18.6	242	7.22	11.6	46.0	6682	13008	4422	1554	0.29
7	19.7	13.7	191	6.88	13.8	37.2	6275	9889	2101	1132	0.16
8	12.2	18.4	100	5.05	17.9	48.7	4570	5548	1529	757	0.28
9	22.9	14.0	172	6.52	11.4	35.7	6555	11803	1475	1286	0.24
10	26.1	54.6	428	10.8	149	169	5949	11970	5483	1031	0.25
11	9.12	25.1	117	4.41	62.0	55.8	3363	4679	1068	428	0.28
12	16.4	21.7	201	6.95	14.9	48.9	6778	8837	2665	1088	0.26
13	15.9	24.5	214	6.86	14.5	45.4	6878	8519	3160	1111	0.43
14	31.3	18.2	199	9.93	24.3	66.0	9153	18492	2625	2265	0.2
15	23.0	20.9	324	9.44	22.2	76.4	7669	13429	2209	1545	0.33
16	22.7	20.6	188	6.98	14.8	50.3	7999	12666	3155	1411	0.35
17	21.1	20.7	135	6.08	20.4	43.0	5857	12410	1825	1231	0.27
18	20.7	12.6	102	4.91	11.6	31.3	6696	13581	1222	1248	0.46
19	14.2	15.9	148	4.84	20.5	53.6	5999	7147	1375	915	0.18
20	15.5	21.9	274	5.54	16.0	51.3	7014	7910	1224	1071	0.21
21	17.6	11.1	249	8.38	16.9	42.4	8684	10205	1933	1532	0.23
22	29.3	20.1	278	10.1	40.5	82.5	8081	19905	3335	2025	0.33
23	16.5	13.0	118	5.43	13.1	36.4	7753	8026	1978	939	0.33
24	12.8	22.8	274	4.08	22.3	44.4	5164	6061	1647	864	0.2
25	26.8	20.3	240	12.4	37.5	67.5	13075	16342	2701	2032	0.37
26	18.3	11.4	128	6.15	14.4	39.8	10256	11532	2733	1226	0.39

Appendix 2: Extratable S and P (mg kg^{-1}) in soils of the study fields

Field.No	P	S
1	1.9	30
2	3.7	10
3	3.3	10
4	2.5	10
5	2.6	10
6	2.2	20
7	1.6	10
8	4.2	10
9	2.6	30
10	5.9	10
11	6.3	10
12	2.6	40
13	2.9	60
14	1.2	370
15	1.0	280
16	2.0	50
17	0.7	30
18	0.4	40
19	1.1	50
20	1.1	30
21	1.1	10
22	1.0	50
23	0.9	220
24	4.0	10
25	0.9	90
26	0.6	120

Apeendix 3: Average S (µg plant^{-1}) in various leek cultivars after application of different Se doses forms

	Se dose and form			
Name of cultivar	0.5 mg Se selenite	2.0 mg Se selenite	4.0 mg Se selenate	4.0 mg Se selenite
Albana	127	67	161	304
Miracle F1	129	104	118	148
Zeus F1	104	46	111	153
Striker F1	97	51	134	122
Breugel F1	121	130	115	121
Tadorna	114	42	177	167
Alcazar	105	68	106	112
Belton F1	79	59	98	143
Pretan F1	84	82	137	169
VLimberg R	74	130	126	124
Coolidge F1	108	169	200	76
Artico	62	89	89	77
Farinto	66	102	183	97
Arkansas	129	70	94	155
Gavia	100	79	175	116
Toledo	70	63	153	164
Uytterhoe E	96	155	123	266
Engels P	106	128	103	65
Harston F1	161	74	86	142
Fahrenheit F1	83	154	106	90

CURRICULUM VITAE

Rama Venkata Srikanth Lavu
Erpelsteeg 46, 9000 Gent,
Belgium.
Tel: +32 49 24 00228
e-mail: srikanthlavu1@gmail.com
Date of Birth: 27-06-1986

EDUCATION

2008- 2013 **Doctoral studies**
Faculty of Biosciences Engineering, Ghent University, Belgium.
Scientific supervisors: Prof. Filip Tack, Prof. Gijs du Laing
Research topic: "*Biofortification, speciation and bioaccessibility of selenium in food and feed crop"*

2007-2008 **Post graduate studies in Pharmaceutical Research**
at the Department of Pharmaceutical Analysis of the Catholic University of Leuven, Belgium.
Project Title: "*Comparative & stability study of the quality of 51 pharmaceutical products containing Clopidogrel by HPLC-UV and capillary electrophoresis (CE-UV)*".

2003-2007 **Bachelors in Pharmacy**
Bharat Institute of Technology and Pharmacy affiliated to Jawaharlal Nehru Technological University, Hyderabad, India.
Project Title: "*Synthesis, characterization and antimicrobial screening of 1-substituted-3-methy-4-(arylhydrazono)-2-pyrazolin-5-ones*"

PROFESSIONAL ACTIVITIES

2008-2013 PhD researcher in Applied Biological Sciences (Laboratory of Analytical Chemistry and Applied Ecochemistry)

2010-2011 Involved in project "Screening trace metal contaminants in food supplements, food crops and medicinal plants grown in Indian (peri) urban areas"

2009-2012 Services: responsible for selenium speciation analysis on HPLC-ICP-MS (Se-enriched yeast, food crops and fertilizers) at the Laboratory of Analytical Chemistry and Applied Ecochemistry

2009-2013 Tutor for three students (during their Master's thesis)

ANALYTICAL EXPERTISE

Trace metal Analysis:	ICP-MS, ICP-OES
Separation techniques:	HPLC, Capillary Electrophoresis
Detection techniques	Ultraviolet-visible spectroscopy (UV), Mass Spectrometry (MS), Infrared Spectroscopy (IR)

ACHEVIEMENTS

- Received oral presentation award in IInd International Conference on Selenium-2011, China on 'Bioaccessibility and conversion of selenium species in human intestinal tract (In-vitro)''
- Received best student poster award in European COST action meeting-2010, Turkey on ''In-vitro conversion of selenium species in human intestinal tract''

ORAL PRESENTATION

- R.V. Srikanth Lavu, et al., "Bio-accessibility and conversion of selenium species in human intestinal tract (In-vitro)'', IInd International Conference on Selenium, China 2011

POSTER PRESENTATIONS

- R.V. Srikanth Lavu, et al., "Effect of selenium speciation in soil on selenium accumulation, growth and physiology of Kenaf (*Hibiscus cannabinus*)'', 9th International Phytotechnology Society 2012, Hasselt, Belgium
- R.V. Srikanth Lavu, et al., "In vitro conversion of selenium species by human intestinal microbiota'', European COST Action meeting 2011, Antalya, Turkey
- R.V. Srikanth Lavu, et al., "Bioactive selenium species in selenium-enriched leek'', 11th International Conference on the Biogeochemistry of Trace Elements 2010, Florence, Italy
- R.V. Srikanth Lavu, et al., "Selenium uptake and speciation in selenium-enriched leek'', SETAC Europe 20th Annual Meeting 2010, Seville, Spain

PUBLICATIONS

- Fertilizing soil with Selenium fertilizers: impact on concentration, speciation and bioaccessibility of Selenium in leek (*Allium ampeloprasum*)", Rama V. Srikanth Lavu, Koen Willekens, Bart Vandecasteele, Filip Tack, Gijs Du Laing. Journal of Agricultural and Food Chemistry 2012; 60:10930-5.

- "Use of selenium fertilizers for production of Se-enriched Kenaf (*Hibiscus cannabinus*): Effect on Se concentration and plant productivity". Rama V.Srikanth Lavu, Veerle De Schepper, Kathy Steppe, Filip Tack, Gijs Du Laing. Journal of Plant Nutrition and Soil Science 2013; 176:634-9.

- "Trace metals Accumulation in *Bacopa monnieri* and their Bioaccessibility" Rama V. Srikanth Lavu, Majeti N.V. Prasad, Varalakshmi L. Pratti, Ralph Meißner, Jörg Rinklebe, Tom Van De Wiele, Filip Tack, Gijs Du Laing. Planta Medica 2013; 9:1081-3.

- "Arsenic prone rice cultivars-a study in endemic region". Anirban Biswas, Saroni Biswas, Rama V. Srikanth Lavu, Prakash Chandra Gupta, Subhas Chandra Santra. Paddy and Water Environment 2013; In press.