

**UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”  
FACULDADE DE ENGENHARIA  
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**CHARACTERIZATION OF GENOTYPE VARIATION AND AGRONOMIC  
BIOFORTIFICATION OF COWPEA WITH SELENIUM: IMPACTS ON PHYTIC  
ACID AND NUTRITIONAL QUALITY OF GRAINS**

Ilha Solteira  
2019

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**CHARACTERIZATION OF GENOTYPE VARIATION AND  
AGRONOMIC BIOFORTIFICATION OF COWPEA WITH SELENIUM  
TO OBTAIN GRAINS OF HIGH NUTRITIONAL QUALITY AND LOW  
CONCENTRATION OF PHYTATES**

Dissertation presented to the Engineering  
College of Ilha Solteira – UNESP as part of  
the requirements to obtain the title of Master in  
Agronomy. Specialization: Production  
Systems

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Ilha Solteira  
2019

FICHA CATALOGRÁFICA

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- S586c Silva, Vinicius Martins.  
Characterization of genotype variation and agronomic biofortification of cowpea with selenium: impacts on phytic acid and nutritional quality of grains / Vinicius Martins Silva. -- Ilha Solteira: [s.n.], 2019  
123 f. : il.
- Dissertação (mestrado) - Universidade Estadual Paulista. Faculdade de Engenharia de Ilha Solteira. Área de conhecimento: Sistemas de Produção, 2019
- Orientador: André Rodrigues dos Reis  
Coorientador: Marco Eustáquio de Sá  
Inclui bibliografia
1. Biofortificação agrônômica. 2. Feijão-caupi. 3. Variação genotípica. 4. Selênio.

  
Raiane da Silva Santos

Supervisora: Eliana de Siqueira  
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Diretoria Técnica de Biblioteca e Documentação  
CSUB - 1999



UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Ilha Solteira

**CERTIFICADO DE APROVAÇÃO**

**TÍTULO DA DISSERTAÇÃO:** Characterization of genotype variation and agronomic biofortification of cowpea with selenium: impacts on phytic acid and nutritional quality of grains

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Ilha Solteira, 05 de fevereiro de 2019

Aos meus pais, Eloisa Maria Biglia Martins Silva e Amarildo Aparecido da Silva. Pelo amor incondicional e pelo inesgotável suporte e confiança que sempre me forneceram, privilégios que aprendi a valorizar. Por terem me mostrado como lutar pelos meus sonhos, por terem me ensinado a aproveitar as oportunidades que a vida me oferece.

DEDICO

Aos meus avôs e avós: Elói Cruz Martins, Ana Rita Biglia Martins (em memória), Antônio Aparecido da Silva (em memória) e Maria Madalena da Silva. Aos meus padrinhos Rinaldo Roberto da Silva e Eliane Flavia da Silva, e a todos os meus familiares que acreditaram em mim e me deram apoio de alguma forma.

OFEREÇO

## ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Professor André Rodrigues dos Reis, for all the teachings (which was a LOT) and for the friendship in these years. Thank you for believing in my potential, sometimes, even more than myself. You made me believe that I could do something awesome. You showed me how to deal with my struggles to keep going with ours research.

I wish to thank my girlfriend, Líria Nakamit Novaes, for the love, trust and support in all these years we have been together is this journey. Thank you for choosing me to make you happy, thank you for making me a better person every day.

I would like to thank my co-supervisor Prof. Marco Eustáquio de Sá, to Prof. Martin Roger Broadley, Prof. Scott Young and Prof. Fernando Ferrari Putti. All of you taught me so much in this journey. Professor Marco with his knowledge that helped me to conduct my field experiments, professor Martin and Professor Scott received me and supported me, acting as co-supervisors throughout my internship in the University of Nottingham, Professor Putti gave me the expertise required to analyze the research data. All these steps were necessary to conclude this work.

Thanks to EMBRAPA's researcher Dr. Maurisrael de Moura Rocha for providing the 29 genotypes of cowpea used in this research. To Professor Enes Furlani Junior for allowing the use of his laboratory. To São Paulo State University (UNESP) School of Engineering, Ilha Solteira-Brazil, and School of Sciences and Engineering Tupã – Brazil, for allowing me to use its facilities to conclude my work.

Regarding the financial support, this study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) -Finance Code 001.

I also would like to thank the financial support in my master course provided by the grant #2016/19773-8, São Paulo Research Foundation (FAPESP). And the financial support to realize an internship abroad in the University of Nottingham (UK) provided by the grant #2017/21950-8, São Paulo Research Foundation (FAPESP).

I would like to thank all my friends and co-workers, who helped me to perform the field experiments, the lab work, to write the papers and reports, or even helping me with advices to reach my goals. Thus, I would like to thank Eduardo Marcandalli, Juliana Martins, Flavia Mendes, Flavia Meireles, João Bossolani, Daiene Corsini, Vanessa Britto, Anne Caroline, Rafael Lawandovski, Prof. Regina Castilho, Rodrigo Castilho, Fernando Galindo, José Matheus, Alvino Silva, Amanda Paixão, Geraldo Gouveia, Tauan Tavanti, Matheus

Mateus, Alexandre Ascoli, Bruno Miranda, Ana Júlia Nardeli, Maria Gabriela, Nandhara Angélica, Leandro Souto, Evandro Amaral, Roberto Casetta, Jessica Pigatto, Elcio Santos, Ediu Carlos, Heitor Reis, Matheus Vinícios, Lolita Wilson, Kent Davies, Tom Alcock, Josefina Lozano, Mu Zhang, Jaswant Khokhar, Diriba Kumssa. In a way or another, each one of you helped me in this journey.

Thanks to my good old friends Matheus, Douglas, Guilherme, Marcelo, Marco Tulio, João César and Kauã, whom offered joyful moments that helped me to be a happier person, specially, in hard times. To my brother Vitor Martins Silva, for the partnership and for the help in my experiments.

I wish to express my gratitude to the staff of São Paulo State University (UNESP) and University of Nottingham that was involved in my research in a way or another.

I also would like to thank the Prof. Liliane Santos de Camargos, Prof. Edson Lazarini, Prof. Lavres Junior, Prof. Salatier Buzetti and Prof. Marcelo Carvalho Minhoto Teixeira Filho, whom comprises the group of professors which accepted to took part in my qualification exam or in the defense of my dissertation.

I would like to thank every single person that, in a way or another, helped me to achieve my goal by finishing this dissertation. Thank you, everyone.

“Somewhere, something incredible is waiting to be known”

Sharon Begley - August 15, 1977, Newsweek magazine

## RESUMO

O selênio (Se) é um nutriente para humanos e animais e um elemento benéfico para as plantas, sua baixa concentração nos solos do Brasil pode gerar deficiência nos animais e humanos. O fitato é a principal forma de reserva de fósforo (P) encontrado nas sementes de plantas, sendo considerado um “anti-nutriente” por formar complexos não digeríveis com nutrientes como Fe, Ca e Zn. Desta forma, existe a necessidade de se buscar alternativas para aumentar os teores de Se e reduzir o teor de fitato nas partes comestíveis de cultivares modernos. O objetivo do trabalho foi avaliar doses e fonte ótimas de Se a serem aplicadas em condições brasileiras, bem como a influência do Se na produção e qualidade nutricional em 29 genótipos de feijão-caupi. Para isso, foram desenvolvidos dois experimentos a seguir: Experimento 1: Foi avaliada a eficiência da biofortificação agronômica utilizando 2 fontes de Se (selenato e selenito) e 7 doses de Se (0; 2,5; 5,0; 10,0; 20,0; 40,0 e 60 g ha<sup>-1</sup>) aplicados via solo, foi realizada análise de fitatos nos grãos. Experimento 2: Foi realizado um experimento para caracterizar a absorção e acúmulo de Se, teor de fitatos, açúcares, proteínas de reserva e amino ácidos nos grãos de diferentes genótipos de feijão-caupi, nesse experimento, 29 genótipos foram avaliados na presença e ausência de Se (0 e 25 g ha<sup>-1</sup>), cultivados até o final do ciclo para a obtenção dos grãos. No experimento 1, observou-se que a aplicação de selenato proporciona maiores concentrações de Se nos grãos em comparação com o selenito. A aplicação de Se não teve influencia direta na concentração de fitatos nos grãos. No experimento 2 observou-se que os a aplicação de Se aumentou a concentração do elemento nas raízes, parte aérea e principalmente grãos de todos os genótipos avaliados. A aplicação de Se pode proporcionar aumento, diminuição ou não influenciar o teor de fitatos, açúcares, proteínas de reserva e aminoácidos, dependendo do genótipo.

**Palavras-chave:** Biofortificação agronômica. Feijão-caupi. Variação genotípica. Selênio.

## ABSTRACT

Selenium (Se) is a nutrient for humans and animals, and a beneficial element for plants, its low concentration in Brazilian soils might cause deficiency in animals and humans. Phytate is the main form of phosphorus (P) storage found in plant seeds, being considered an "anti-nutrient" by forming nondigestible complexes with nutrients such as Fe, Ca and Zn. Thus, there is a need to find alternatives to increase Se and decrease phytate concentration in the edible parts of modern cultivars. The objective of this study was to evaluate optimal levels and sources of Se to be applied in Brazilian conditions, as well as the influence of the Se in the production and nutritional quality in 29 genotypes of cowpea. Two experiments were performed: Experiment 1: The efficiency of agronomic biofortification was evaluated using 2 sources of Se (selenate and selenite) and 7 levels of Se (0; 2.5; 5.0; 10.0; 20.0, 40.0 and 60 g ha<sup>-1</sup>) applied via soil, phytate analysis was analyzed. Experiment 2: An experiment was carried out to characterize the uptake and accumulation of Se, phytate, sugars, storage proteins and amino acids in the grains of different genotypes of cowpea. In this experiment, 29 genotypes were evaluated in the presence and absence of Se (0 and 25 g ha<sup>-1</sup>), grown until the end of the cycle to obtain the grains. In experiment 1, it was observed that the application of selenate provides higher concentrations of Se in the grains compared to selenite. Selenium application showed no direct influence on the phytate concentration in the grains. In experiment 2 it was observed that the application of Se increased the concentration of the element in roots, shoot and grains of all evaluated genotypes. Se application may provide increase, decrease or not influence the content of phytates, sugars, reserve proteins and amino acids, depending on the genotype.

**Keywords:** Agronomic biofortification. Cowpea. Genotypic variation. Selenium.

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## INTRODUCTION

Foods as a primary source of nutrients and its demand grows with the increasing of worldwide population. In 1800, the world's population was 1 billion. Currently, with an average growth rate of 1.37%, the worldwide population is 6.9 billion people (RAYMAN, 2012). For 2050, it is estimated that the worldwide population hits 9.4 billion people.

The cereal yield has maintained the same rising rate of the worldwide population. It is estimated that the global demand for food may double in the period between 1990 and 2030, with a two and a half to three-fold increase in developing countries (GARVIN; WELCH; FINLEY, 2006). On the other hand, the malnutrition has increased reaching almost half of the world's population, especially pregnant women, teenagers and children (GRAHAM et al., 2007). This is, in part, due to plant breeding aiming to yield increasing, and thus, presenting an inverse relation with mineral content in grains (GARVIN; WELCH; FINLEY, 2006; MURPHY; REEVES; JONES, 2008; WHITE et al., 2009).

Selenium is considered a scarce element, with irregular distribution in earth's crust, and besides the origin material, its content in soil depends highly on water regime of each region (HAUG et al., 2007). The levels of Se in agricultural food products are strongly dependent on the presence of this element in the soil and also on the regulation in function of plant species/genotype, although due to the few studies carried out, there are doubts about the agronomic significance of the variation/genotypic regulation in their accumulation in cultivated plants (LYONS et al., 2005). The Se when supplied in the source of selenate competes with Sulfur (S) transporters, and when supplied in the source of selenite competes with the phosphate transporters in plants (REIS et al., 2017a).

The phytic acid (or myo-inositol hexaphosphate, or InsP6 or IP6) is the main compound of phosphorus (P) storage in seeds, its found in salt named phytate and comprises between 60 and 90% of all P in cereals, oilseeds and nuts (LOTT et al., 2000; 2001; BOHN; MEYER; RASMUSSEN, 2008). The phytic acid and its derivatives (mainly IP5) bound to metallic ions in pH values similar to duodenum (CARLI et al, 2006). Therefore, phytate is considered an antinutrient, able to form insoluble complexes with minerals, proteins, enzymes and starch, a trait that can impair in the absorption of Calcium (Ca), Magnesium (Mg), Copper (Cu), Fe and Zn, primarily, when the intake of these minerals is inadequate (GRAF; EATON, 1990; PRYNNE et al, 2010). Among the problems caused by phytate, there are severe deficiencies in populations with diets composed primarily by cereals and pulses, mostly in vulnerable groups, such as young, and pregnant women in needy regions that

commonly ingest low quantities of Ca, Fe, Mg e Zn (WEAVER; KANNAN, 2002). Thus, regarding the food security, is important to research for means to reduce the levels of phytate in plant seeds used as food, mostly the ones important in needy regions, as the cowpea.

The agronomic biofortification with Se increases the nutritional quality of food (RAYMAN, 2012) and the potential to reduce phytate in grains turns necessary a better knowledge of the Se accumulation influence in the synthesis of this antinutrient, as well as the influence of Se in the quality of cowpea, that is one of the most important sources of protein in Brazilian northeast, region that presents the greatest rates of malnutrition in the country.

# **1 CHAPTER 1. REVIEW: OVERVIEW ON SELENIUM ROLES IN MINERAL PLANT NUTRITION**

## **ABSTRACT**

Selenium (Se) is a micronutrient for humans and animals, and considered a benefit for plants. Animals acquire Se mostly from plants, and plants taken up Se from soils, which usually have very low concentrations of Se. Thus, studies on Se uptake, transport and metabolism in plants are needed. This review first provides a background about Se importance for human health and the lack of the element in diets. Then is presents strategies to mitigate the lack of Se in human diet by providing edible food enriched with Se. Selenium benefits in plants as an antioxidant, and its concentration in plants, availability in soils and forms of use in agriculture are elucidated. Finally, the mechanisms of uptake, transport and metabolism of Se by plants are reviewed, focusing in selenate and selenite, which are the most common forms of Se absorbed by plants, and also, the most common sources of Se as a fertilizer. Selenate should be the preferable form of Se to fertilize plants, due being easily absorbed and transported from roots to shoots, whereas selenite remains in roots due being rapidly converted in organic Se. The effect of Se as an antioxidant is well known in plants, however, more studies focusing on how exactly Se acts within plant tissue to combat oxidative stress are needed. Selenium share chemical similarities with sulfur (S), and the studies focusing in plant behaviour and uptake mechanisms of S and Se are needed in order to better understand the physiological mechanism during ion transport in the cell.

Keywords: Biofortification. Selenate. Selenite. Uptake. Sulfur.

## 1.1 IMPORTANCE OF SELENIUM AND Se BIOFORTIFICATION

Selenium (Se) is a micronutrient for humans and animals, and considered benefit element for higher plants (ROS et al., 2016; WHITE, 2016). A minimum Se intake of 20  $\mu\text{g day}^{-1}$  is necessary to prevent Keshan disease in humans (ROMAN; JITARU; BARBANTE, 2014; NATASHA et al., 2018), whereas the recommended dietary allowance is set in 55  $\mu\text{g day}^{-1}$  (INSTITUTE OF MEDICINE, 2000; FAIRWEATHER-TAIT et al., 2011). It is estimated that about 1 billion people suffers from Se deficiency around the world (COMBS JUNIOR, 2001; STOFFANELLER; MORSE, 2015), which consequently entails several health problems, such as: hypothyroidism, cardiovascular diseases, viral diseases, male infertility, cognitive decline, weakening of the immune system and increased incidence of various forms of cancer (HAUG et al., 2007, FAIRWEATHER-TAIT et al., 2011; RAYMAN, 2012; FORDYCE, 2013). Usually, Se deficiency in humans is due to agricultural production from soils with low available Se for plants uptake, considering that, most part of Se in human diet is, directly or indirectly, from edible plants (BROADLEY et al., 2006; WHITE; BROADLEY, 2009; CHILIMBA et al., 2011; FAIRWEATHER-TAIT et al., 2011; RAYMAN, 2012; FORDYCE, 2013; JOY et al., 2015).

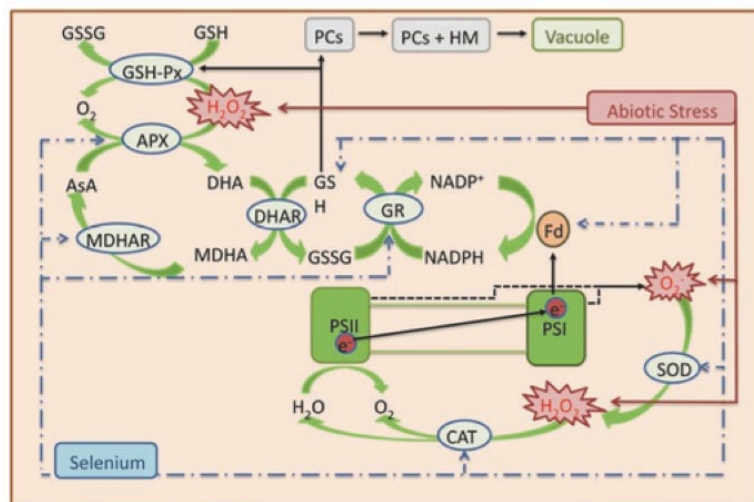
Despite the global importance of Se deficiency has been recognized for decades, strategic interventions to address the micronutrient and reduce its lack are still limited (ROS et al., 2016). The biofortification is based on the use of genotypic variations, in plant breeding, for the purpose of obtaining food products with greater availability of nutrients and vitamins in the edible parts. (WELCH, 2008; BORRILL; CONNORTON; BALK, 2014).

There are two strategies of biofortification aimed at increasing the Se ingested by humans: the first strategy is to directly increase the intake of the nutrient, either by supplementation for animals, by directly fortification of food or by supplementation with Se pills (MILLER; WELCH, 2013). For the second is the strategy, agronomic activities such as plant breeding and fertilization are used to increase Se concentration in edible parts of crops (MALAGOLI et al., 2015). Which strategy is most efficient depends on the natural and socioeconomic properties, on the food systems and on the agro ecological condition of each region (MILLER; WELCH, 2013; MALAGOLI et al., 2015).

There are studies indicating that Se do not provide increase or decrease in crop yield or harvest index (BROADLEY et al., 2010), however there are also studies indicating that fertilization with Se stimulates the growth, the synthesis of antioxidant agents and regulates the transport and assimilation of sulfates (HARTIKAINEN; XUE 1998; XUE;

HARTIKAINEN 2000; TURAKAINEN; HARTIKAINEN; SEPPÄNEN, 2004; VAN HOEWYK et al., 2008; REIS et al., 2018, SILVA et al., 2018). Selenium is not considered an nutrient for plants yet, however, its considered to be a beneficial element, since it can stimulate growth, could confer tolerance to factors inducing oxidative stress, and provide resistance to pathogens and herbivory (QUINN et al., 2007; PILON-SMITS; LEDUC, 2009; WHITE; BROWN, 2010; EL MEHDAWI; PILON-SMITS, 2012; FENG; WEI; TUD, 2013; WHITE, 2016; SCHIAVON; PILON-SMITS, 2017; WHITE, 2018). Selenium act in plants as an enzymatic activator in various enzymes that contribute to decrease the lipid peroxidation and the formation of hydrogen peroxide ( $H_2O_2$ ) in the plant tissue, delaying the senescence and decreasing the oxidative stress (DJANAGUIRAMAN et al., 2005; SAIDI; CHTOUROU; DJEBALI, 2014; SILVA et al., 2018).

**Figure 1.1.** Selenium enhances antioxidant activity which stimulate plant response to oxidative stress increasing plant yield. Abbreviation: ascorbate peroxidase (APX), catalase (CAT), dehydroascorbate reductase (DHAR), glutathione peroxidase (GSH-Px), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), SOD (superoxide dismutase). Metabolites: ascorbate (Asa), reduced glutathione (GSH), phytochelatin (PCs). Reactive Oxygen Species: superoxide radical ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ).



Source: Shiavon et al. (2017).

The reports of Se stimulating plant cell antioxidant stress capacity through the enhancement of the activity of enzymes are related to the following enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), dehydroascorbate reductase (DHAR) and dehydroascorbate reductase (DHAR), monodehydroascorbate

reductase (MDHAR), and ascorbate peroxidase (APX) (SCHIAVON et al., 2017; REIS et al., 2018; SILVA et al., 2018).

Selenium also acts as an antioxidant agent by enhancing the synthesis of non-enzymatic antioxidant molecules, such as reduced form of glutathione (GSH), ascorbate, phytochelatins (PCs), proline, flavonoids, alkaloids (FOYER; NOCTOR, 2012). There are even reports stating that Se may induce the spontaneous dismutation of superoxide radical ( $O_2^{\cdot-}$ ) into  $H_2O_2$  FENG; WEI; TU, 2013).

## 1.2 SELENIUM AVAILABILITY

The average range for total Se in the soil is 0.01-2 mg kg<sup>-1</sup> (KABATA-PEDIAS, 2011; SAHA; FAYIGA; SONON, 2017). Thus, Se-poor soils are generally derived from igneous rocks, found in regions exposed to high erosive rates, and with limited atmospheric deposition of the element, on the other hand, Se-rich soils, usually are originated from sedimentary rocks (CHRISTOPHERSEN et al., 2012).

The distribution of Se around the world is very variable: from practically 0 to 1200 mg kg<sup>-1</sup> in seleniferous soils (OLDFIELD, 2002) which are found in plains of USA, Canada, South America, Australia, India, China and Russia (DHILLON; DHILLON, 2003; FORDYCE, 2013; PILBEAM et al., 2015; JONES et al., 2017), some regions of Canada and USA, even without fertilization, have high bioavailability of Se, providing Se-enriched products (HAUG et al., 2007; FORDYCE et al., 2013). On the other hand, there are some regions in the west and center of Europe, with low Se in soil due to the continuous use of fertilizers that lack in Se (CHRISTOPHERSEN et al., 2012). Soils in which the Se content is less than 0.6 mg kg<sup>-1</sup> are considered deficient (GUPTA; GUPTA, 2000). There are conclusive evidences of Se deficiency in Brazilian soils (REIS et al., 2017a). Thus, application of fertilizer containing Se is required in soils showing low Se availability in order to increase Se concentration in edible part of plants (REIS et al., 2018).

Selenium availability to the plants depends also on the predominant chemical speciation of the nutrient in the soil (REIS et al., 2017a). Selenium exists in different oxidation states, ranging from <sup>+6</sup> to <sup>-2</sup> (FORDYCE et al., 2013). Among the forms of Se in the soil, being: <sup>+6</sup> in selenates, <sup>+4</sup> in selenites, 0 in elemental Se, and <sup>-2</sup> in inorganic and organic selenides, and also, it forms catenated species, such as volatile diselenides (HARTIKAINEN, 2005). The efficiency of Se fertilization depends on the Se form used, the application rate of the fertilizer, the application technology used and the soil properties; the efficiency of uptake

can vary from 1% to 50% (STROUD et al., 2010; KESKINEN; TURAKAINEN; HARTIKAINEN, 2010; CHILIMBA et al., 2011, 2012a; LONGCHAMP et al., 2012; KIKKERT; HALE; BERKELAAR, 2013; JIANG et al., 2018; LIDON et al., 2018)

In an extensive review studying Se application in agriculture, ROS et al (2016) observed that selenate is the most used source of Se, followed by selenite, selenate applications are on average  $14 \text{ g ha}^{-1}$ , while selenite applications are on average  $280 \text{ g ha}^{-1}$ . In addition, uptake efficiency of selenate is higher than that of selenite, this happens because once selenite uptake by roots is rapidly converted into selenide and incorporated into selenocysteine (SeCys) and selenomethionine (SeMet) (SORS; ELLIS; SALT, 2005) which often remain within the root (WHITE, 2016; SHIAVON et al., 2017; WHITE, 2018).

Selenite is strongly adsorbed by aluminum oxides and hydroxides, and to a lesser extent, it also undergoes adsorption by organic matter and clays. On the other hand, selenate is less adsorbed to the soil and relatively more mobile than selenite in the soil solution (FORDYCE, 2013; PILBEAM et al., 2015). Therefore, fertilization with selenate promotes immediate accumulation of Se in plants, whereas application of selenite provides Se that last longer in the soil (BROADLEY et al., 2006; FORDYCE, 2013; PILBEAM et al., 2015).

### 1.3 SELENIUM UPTAKE BY ROOTS

The plant roots are able to uptake Se in the form of selenate ( $\text{SeO}_4^{2-}$ ), selenite ( $\text{SeO}_3^{2-}$ ;  $\text{HSeO}_3^-$ ;  $\text{H}_2\text{SeO}_3$ ) and Se in organic compounds, such as SeCys and SeMet, but cannot uptake elemental Se, or Se in metallic clusters (WHITE; BROADLEY, 2009).

Selenium has a complex relationship with phosphorus (P), and mostly, sulfur (S) in the soil, the Se/S ratio extends from the uptake of the Se through the roots, passes through its transport in the conducting veins and reaches the element metabolism, within the cells (WHITE, 2016). Selenite competes with phosphate, the uptake of  $\text{HSeO}_3^-$ , in rice, is catalysed by the phosphate transporter OsPT2 (table 1.1) (ZHANG et al., 2014), and also, by homologous of the aquaporin channel OsNIP2;1 (table 1.1), which act catalysing the uptake of  $\text{H}_2\text{SeO}_3$  (POMMERENIG; DIEHN; BIENERT, 2015; WHITE, 2018). In plant with lack of P the expression of OsPT2 is higher, resulting in a higher capacity to uptake selenite (ZHANG et al., 2014). Phosphate and selenite are also related by a co-precipitation, given that, when the phosphate precipitation occurs after the fertilizers application, the Se is fixated in the precipitate (CHRISTOPHERSEN et al., 2012). In contrast, phosphate ions bind more strongly to clay minerals in the soil in comparison with selenite, therefore the increase of

phosphate in the soil solution may lead to desorption of selenite from oxides and hydroxides, increasing the availability of the beneficial element (CONSTANTINO et al., 2017; NAKAMARU; TAGAMI; UCHIDA, 2017; 2006). After being uptake by plants, selenite is rapidly converted to Se in organic compounds remaining in the root cells (WHITE et al., 2004; XIMÉNEZ-EMBÚN et al., 2004; LI; MCGRATH; ZHAO, 2008).

**Table 1.1.** List of transporters involved in selenium uptake and translocation in plants.

| Transporter | Species            | Function                             | Reference                         |
|-------------|--------------------|--------------------------------------|-----------------------------------|
| OsPT2       | <i>O. sativa</i>   | uptake of selenite through the roots | ZHANG et al., 2014                |
| OsNIP2;1    | <i>O. sativa</i>   | uptake of selenite through the roots | POMMERRENIG; DIEHN; BIENERT, 2015 |
| AtSULTR1;1  | <i>A. thaliana</i> | uptake of selenate through the roots | EL KASSIS et al., 2007            |
| AtSULTR1;2  | <i>A. thaliana</i> | uptake of selenate through the roots | EL KASSIS et al., 2007            |
| AtSULTR2;1  | <i>A. thaliana</i> | transports selenate by xylem         | GIGOLASHVILI; KOPRIVA, 2014       |
| AtSULTR2;2  | <i>A. thaliana</i> | transports selenate by xylem         | TAKAHASHI et al., 2000            |
| AtSULTR3;5  | <i>A. thaliana</i> | modulates activity of AtSULTR2;1     | KATAOKA et al., 2004              |
| AtALMT12    | <i>A. thaliana</i> | transports selenate by xylem         | MALCHESKA et al., 2017            |

Source: Own authorship

Selenium in the soil as selenate source competes with sulfates. In plant roots, the selenate uptake is catalysed by S transporters, homologous at the transporters AtSULTR1;1 and AtSULTR1;2 (table 1.1) found in *Arabidopsis thaliana* [L.] Heynh. (WHITE et al., 2007; SORS; ELLIS; SALT, 2005; SHINMACHI et al., 2010; GIGOLASHVILI; KOPRIVA, 2014). In studies performed in Arabidopsis, it was observed that the transporter AtSULTR1;1 little contribute to Se uptake in plants with plenty of S, however, the transporter have a higher contribution in Se uptake in plants that lack in S (EL KASSIS et al., 2007; WHITE et al., 2007). In most angiosperms, the genes that encode the transporters SULTR1;1 and SULTR1;2 are highly expressed when the S supply is not satisfactory for the full development of plants (EL KASSIS et al., 2007; SCHIAVON et al., 2015). The increase in Se in foliar tissue also increase these genes expression (ZHANG et al., 2006; ROUACHED et al., 2008; HSU et al., 2011; INOSTROZA-BLANCHETEAU et al., 2013). After being uptake by the plants,

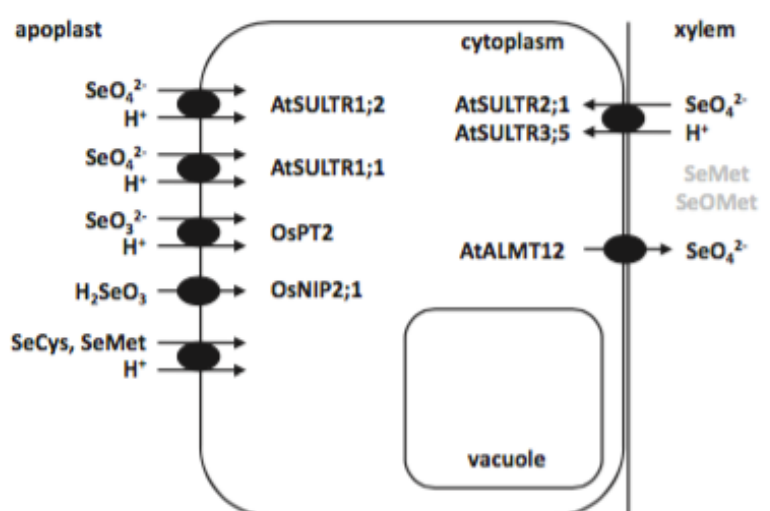
selenate is sent to xylem (WHITE et al., 2004; XIMÉNEZ-EMBÚN et al., 2004; LI; MCGRATH; ZHAO, 2008).

Regarding to Se in organic compounds, transporters that catalyse the uptake and transport of methionine (Met) and cysteine (Cis) within the plant may also catalyse transport and uptake of Se-methionine (SeMet) and Se-cysteine (SeCys) (TEGEDER, 2012).

#### 1.4 TRANSPORT AND METABOLISM OF SELENIUM IN PLANTS

Low affinity sulfate transporters homologous to Arabidopsis: AtSULTR2;1, AtSULTR2;2 and AtSULTR3;5 (table 1.1), have a relation in Se transport as selenate by xylem in plants (TAKAHASHI et al., 2000; GIGOLASHVILI; KOPRIVA, 2014). Members of the AtLMT transporter family, including homologs of Arabidopsis AtALMT12 (table 1.1), are also thought to load selenate into the xylem sap (MALCHESKA et al., 2017; WHITE, 2018) as illustrated in figure 1.2.

**Figure 1.2.** Selenium transport across the root from the apoplast to the xylem.



Source: White (2018).

The organic transport of Se, also occurs, to a very limited extent, as SeMet and selenomethionine Se-oxide (SeOMet) in the xylem (LI et al., 2008). AtSULTR2;1 and AtSULTR2;2 catalyse selenate uptake into cells within the stele, AtSULTR3;5 do not act directly in the transport of Se, but modulates the activity of AtSULTR2;1 (KATAOKA et al., 2004). In other plants, the expression of genes that encode homologous to AtSULTR2;1 and

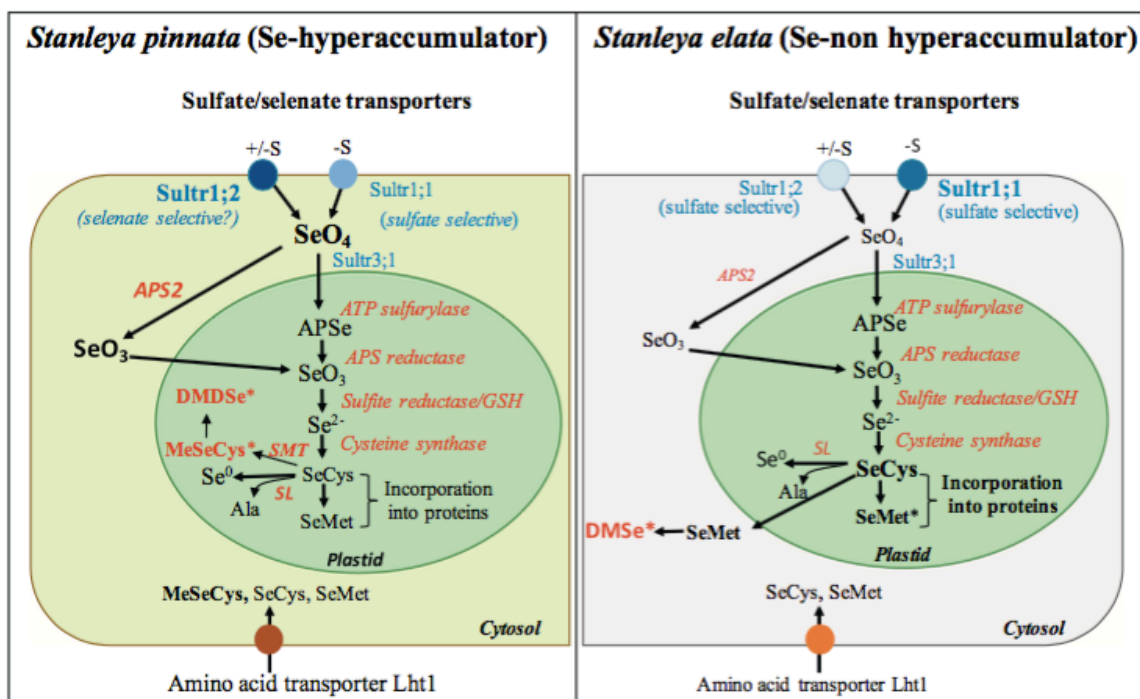
AtSULTR2;2 is stimulated by the lack of S or by the increase of Se availability (TAKAHASHI et al., 2000; GIGOLASHVILI; KOPRIVA, 2014; BOLDRIN et al., 2016; TIAN et al., 2017). Selenate is converted to selenite after undergoing the action of two enzymes: first, the selenate is activated by adenosine triphosphate sulfurylase (ATPS) to form adenosine 5'-phosphoselenate (APSe), which is then reduced to selenite by adenosine 5'-phosphosulfate reductase (APR) using reduced glutathione (GSH) as the electron donor (SCHIAVON et al., 2015; WHITE, 2016). The reduction of selenate into selenite appears to be the rate-limiting step in the assimilation of Se into organic compounds (PILON-SMITS; LEDUC, 2009).

Selenium metabolism in higher plants is illustrated in Figure 1.3 according to LIMA; PILON-SMITS; SCHIAVON, (2018). Selenite is reduced to selenide enzymatically by sulphite reductase (PILON-SMITS, 2012). The selenide along with serine are converted into SeCys by the cysteine synthase enzyme complex (WHITE et al., 2007; OGRA; ANAN, 2012; PILON-SMITS, 2012). SeCys along with O-phosphohomoserine (OPHS) are converted into SeMet after undergoing into the sequential actions of cystathionine  $\gamma$ -synthase (CcS), which produces selenocystathionine (SeCysta), cystathionine  $\beta$ -lyase (CBL), which produces selenohomocysteine (SeHCys), and methionine synthase (WHITE, 2016). SeCys is the most abundant form of Se in garlic without Se application (CAI et al., 1995), SeMet is the most abundant form of Se edible seeds and grains of cereals (SHAO et al., 2014), in Lecythidaceae seeds (NÉMETH et al., 2013) and in potato tubers (GIONFRIDDO et al., 2012).

Selenium toxicity has been attributed to the non-specific replacement of cysteine and methionine in proteins by SeCys and SeMet, which prejudices the formation of disulfide bridge, impairing the formation of proteins (VAN HOEWYK, 2013). This replacement seems to be related to the tissue Se/S quotient, rather than the Se content alone (WHITE et al., 2004; EL KASSIS et al., 2007). Thus, the conversion of SeCys and SeMet to non-toxic or volatile Se metabolites can increase plant Se tolerance, such as, the methylation of SeCys and SeMet into Se-methylselenocysteine (SeMSeCys), and Se-metilselenomethionine (SeMSeMet), which occurs, by the action of selenocysteine methyltransferase (SMT) and S-adenosyl-methionine:methionine methyl transferase (MMT) respectively (PILON-SMITS; LEDUC, 2009; VAN HOEWYK, 2013). Only a gene that encodes MMT was found in Arabidopsis genome (TAGMOUNT; BERKEN; TERRY, 2002). It is believed that there is no genes that encode SMT in plants that are not tolerant to high Se content (VAN HOEWYK, 2013; ZHAO et al., 2015), however, there is evidence of enzymatic methylation of SeCys and SeMet in plants that accumulate Se, such as broccoli, and species of *Astragalus* e *Stanleya* (LYI et al.,

2005, SORS; MARTIN; SALT, 2009; FREEMAN et al., 2010; LIMA; PILON-SMITS; SCHIAVON, 2018).

**Figure 1.3.** Selenate/sulfate uptake and assimilation mechanisms in the model hyperaccumulator *Stanleya pinnata* and non-accumulator *Stanleya elata*. Abbreviations: adenosine phosphoselenate (APSe), adenosine phosphosulfate (APS), reduced glutathione (GSH), selenocysteine (SeCys), selenomethionine (SeMet), selenocysteine methyltransferase (SMT), methylselenocysteine (MeSeCys); dimethylselenide (DMSe), dimethyl diselenide (DMDS<sub>2</sub>).



Source: Lima, Pilon-smits and Schiavon (2018).

## 1.5 CONCLUSIONS AND FUTURE PERSPECTIVES

Selenium is an intriguing element that might act as a benefactor for plants but at high concentrations could be toxic. Thus, agronomic biofortification of crops with Se should be studied to provide new information on specific quantities of Se supplied for edible crops. However, the actual studies led us to believe that selenate, is more readily available to plants, providing more bioavailable Se in edible parts of foods.

The mechanisms of assimilation of Se are studied mostly in situations where Se shares similarities with other elements, for instance, selenite and its similarities with phosphate specifically in the transport from soil to roots. Selenium also share similarities with sulfate

and S, but ins this case, not only on uptake from soil to roots, but also within the plant, in its transport to xylem sap and assimilation in organic forms of SeCys and SeMet.

## 2 CHAPTER 2. REVIEW: PHYSIOLOGICAL TRAITS REGULATING PHYTIC ACID ACCUMULATION IN PLANTS

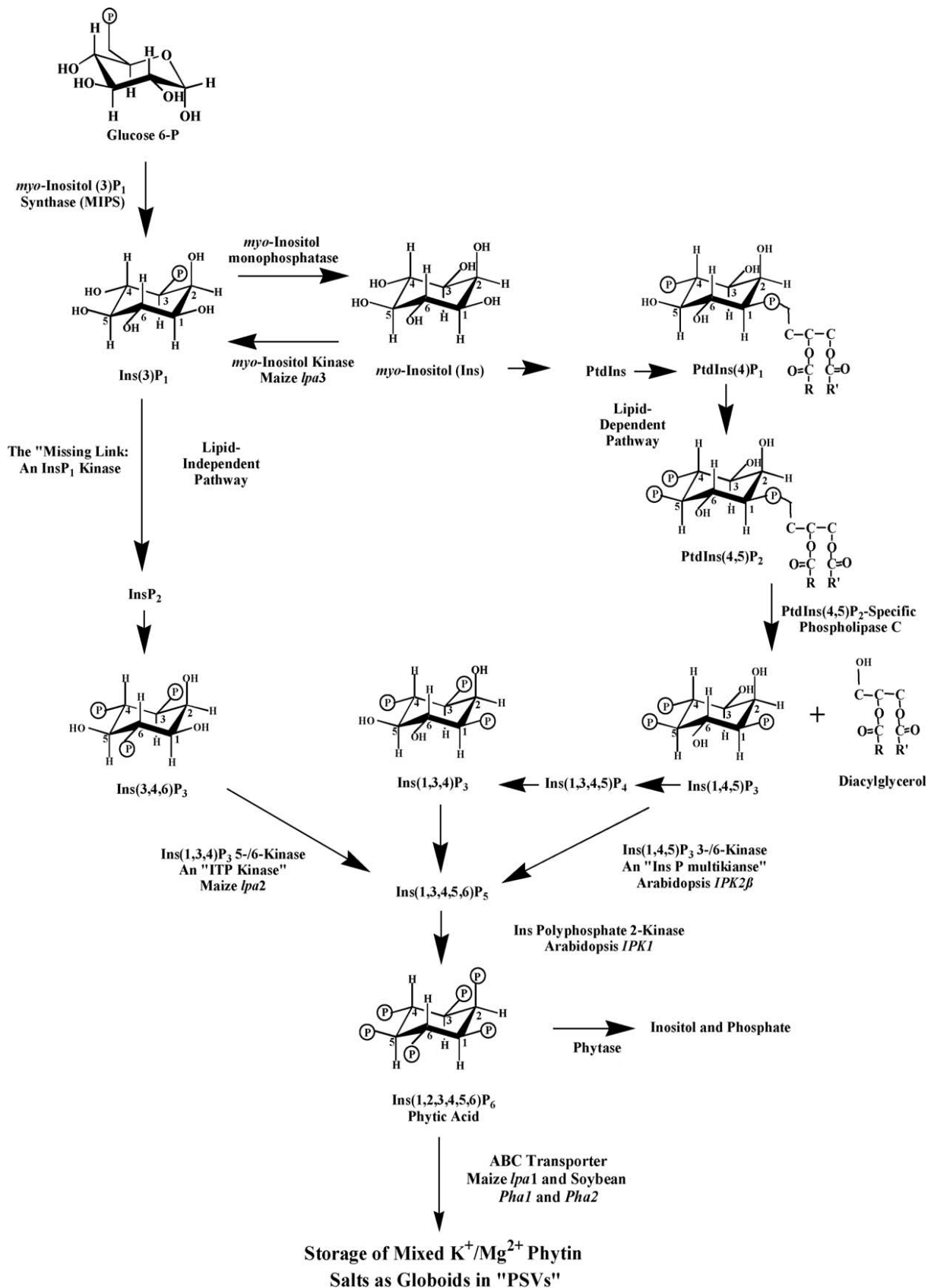
### ABSTRACT

Phytate or phytic acid (PA), is a phosphorus (P) salt generated by the stepwise phosphorylation of *myo*-inositol. It forms complexes with some nutrients, such as Ca, Fe and Zn, compromising their absorption and thus acting as an anti-nutrient in the digestive tract of humans and monogastric animals. Conversely, PA is an important form of P storage in seeds, making up to 90% of total seed P. Phytates also play a role in germination and are related to the synthesis of abscisic acid and gibberellins, the hormones involved in germination. In the present study, we review current knowledge of the genes encoding enzymes that function in different stages of PA synthesis, from the first phosphorylation of *myo*-inositol to PA transport into seed reserve tissues, and the application of this knowledge to reduce grain PA concentrations in edible crops. The studies regarding decrease in PA concentration on seeds in conjunction with no impairment in seed germination should keep going to provide seed material with low PA content, and at the same time, high germination potential.

### 2.1 INTRODUCTION: PHYTIC ACID IN PLANTS, HUMANS AND ANIMALS

Phytic acid (PA) is the main phosphorus (P) storage compound in seeds, contributing 60% to 90% of the total P in cereals, oil seeds and nuts (LOTT et al., 2000, 2001; BOHN; MEYER; RASMUSSEN, 2008). It is generated by the stepwise phosphorylation of *myo*-inositol (Ins), forming several intermediate *myo*-inositol phosphates (InsP) before undergoing the final phosphorylation and conversion into D-*myo*-inositol (1,2,3,4,5,6) hexakisphosphate (InsIP<sub>6</sub>), i.e. phytic acid (RABOY, 2003). Phytic acid can be formed by two different pathways: a lipid-dependent pathway, where phospholipase C catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>) into *myo*-inositol P<sub>3</sub> (InsP<sub>3</sub> or IP<sub>3</sub>), which is then progressively phosphorylated to InsP<sub>6</sub>; and a lipid-independent pathway, where *myo*-inositol(3)P<sub>1</sub> (Ins(3)P<sub>1</sub>) undergoes a series of phosphorylations until PA is formed (RABOY, 2009). The two PA biosynthesis pathways in plants are illustrated in figure. 2.1 and the genes known to be involved in PA biosynthesis are listed in Table 2.1.

**Figure 2.1.** Phytic acid biochemical pathways. Lipid-dependent pathway on the left and lipid-independent pathway on the right.



Source: Raboy (2009).

Phytic acid and its derivatives bind cations between pH 3 and 7 (PERSSON et al., 1998) and are considered to be anti-nutrients capable of forming insoluble complexes with minerals, proteins, enzymes and starch (RABOY, 2009). Thus, PA can interfere with calcium (Ca), magnesium (Mg), copper (Cu), iron (Fe) and zinc (Zn) absorption by the gut, especially when there is inadequate ingestion of these minerals (GRAF; EATON, 1990; PRYNNE et al., 2010). Indeed, PA can magnify nutrient deficiencies in populations with cereal- and legume-based diets, especially in more vulnerable groups such as children and pregnant women in deprived regions, who commonly ingest inadequate amounts of Ca, Fe, Mg and Zn (WEAVER; KANNAN, 2002; KUMSSA et al., 2015). Monogastric animals cannot digest PAs (HUMER; SCHWARZ; SCHEDULE, 2015). Corn- and soybean-based feed possesses high PA concentrations and usually need to be supplemented with phytases or directly with P to meet the needs of livestock (BEAVERS, 2014). Because of the presence of PA in faeces, these animals also release high levels of P into the environment, contributing to water pollution by eutrophication (KNOWLTON et al., 2004; BENTLEY et al., 2015).

Despite the problems derived from their status as anti-nutrients, PAs are important P storage compounds in seeds and play a role in germination and seedling vigour (WHITE; VENEKLAAS, 2012; HUI et al., 2016), in addition to other roles such as antioxidant functions (BOHN; MEYER; RASMUSSEN, 2008). Decreasing plant PA concentrations with the aim of improving human and animal nutrition should therefore be performed while ensuring that germination and seedling vigour is not compromised, which is essential for crop establishment and yield.

**Table 2.1.** Genes involved in phytic acid synthesis and transport in plants (continues)

| Genes          | Proposed EnFunction                            | Plant                    | Tissue                                    | PA alteration | Pi alteration | Reference           |
|----------------|------------------------------------------------|--------------------------|-------------------------------------------|---------------|---------------|---------------------|
| <i>TaITPK1</i> | phosphorylates IP3 to IP4 or IP4 to IP5 in LIP | <i>Triticum aestivum</i> | Roots, Shoot, Leaves, Flag leaf and Seeds | -             | -             | BHATI et al. (2014) |
| <i>TaITPK2</i> | phosphorylates IP3 to IP4 or IP4 to IP5 in LIP | <i>Triticum aestivum</i> | Roots, Shoot, Leaves, Flag leaf and Seeds | -             | -             | BHATI et al. (2014) |
| <i>TaITPK3</i> | phosphorylates IP3 to IP4 or IP4 to IP5 in LIP | <i>Triticum aestivum</i> | Roots, Shoot, Leaves, Flag leaf and Seeds | -             | -             | BHATI et al. (2014) |

Source: Own authorship

**Table 2.1.** Genes involved in phytic acid synthesis and transport in plants (continuation).

| Genes                      | Proposed EnFunction                             | Plant                       | Tissue                                    | PA alteration                     | Pi alteration                     | Reference                                        |
|----------------------------|-------------------------------------------------|-----------------------------|-------------------------------------------|-----------------------------------|-----------------------------------|--------------------------------------------------|
| <i>TaITPK4</i>             | phosphorylates IP3 to IP4 or IP4 to IP5 in LIP  | <i>Triticum aestivum</i>    | Roots, Shoot, Leaves, Flag leaf and Seeds | -                                 | -                                 | BHATI et al. (2014)                              |
| <i>TaIPK2</i>              | phosphorylates IP3 to IP4 or IP4 to IP5 LDP     | <i>Triticum aestivum</i>    | Roots, Shoot, Leaves, Flag leaf and Seeds | -                                 | -                                 | BHATI et al. (2014)                              |
| <i>TaIPK1</i>              | phosphorylates IP5 to IP6                       | <i>Triticum aestivum</i>    | Roots, Shoot, Leaves, Flag leaf and Seeds | -                                 | -                                 | BHATI et al. (2014)                              |
| <i>TaMRP3</i>              | PA transport                                    | <i>Triticum aestivum</i>    | Roots, Shoot, Leaves, Flag leaf and Seeds | -                                 | -                                 | BHATI et al. (2014)                              |
| <i>OsIPK1</i>              | phosphorylates IP5 to IP6                       | <i>Oryza sativa</i>         | Seeds                                     | decreased to about 50% of total P | increased to about 50% of total P | ALI et al. (2013)                                |
| <i>OsLPa1</i>              | ----                                            | <i>Oryza sativa</i>         | Shoot, Root, Panicle                      | decreased (50%)                   | increased                         | LARSON et al. (2000); KIM et al. (2008a)         |
| <i>lpa N15-186 (OsMIK)</i> | phosphorylates Ins to IP1                       | <i>Oryza sativa</i>         | Seeds                                     | decreased (75%)                   | increased                         | KIM et al. (2008b)                               |
| <i>lpa N15-357</i>         | ----                                            | <i>Oryza sativa</i>         | Seeds                                     | decreased (43%)                   | increased                         | KIM et al. (2008b)                               |
| <i>AtIPK1</i>              | phosphorylates IP5 to IP6, and also, IP4 to IP5 | <i>Arabidopsis thaliana</i> | Seeds                                     | decreased (83%)                   | increased                         | STEVENSON-PAULIK et al. (2005); KIM; TAI. (2011) |
| <i>AtIPK2β</i>             | phosphorylates IP3 to IP4 or IP4 to IP5 in LIP  | <i>Arabidopsis thaliana</i> | Seeds                                     | decreased (35%)                   | increased                         | STEVENSON-PAULIK et al. (2005); KIM; TAI. (2011) |
| <i>AtMRP5</i>              | PA transport                                    | <i>Arabidopsis thaliana</i> | Seeds                                     | decreased (80%)                   | increased                         | NAGY et al. (2009); KIM; TAI. (2011)             |
| <i>At5g60760</i>           | ----                                            | <i>Arabidopsis thaliana</i> | Seeds                                     | decreased (40~70%)                | increased                         | KIM; TAI. (2010; 2011)                           |

Source: Own authorship

**Table 2.1.** Genes involved in phytic acid synthesis and transport in plants (continuation).

| Genes                  | Proposed EnFunction                            | Plant                       | Tissue                            | PA alteration       | Pi alteration | Reference                                     |
|------------------------|------------------------------------------------|-----------------------------|-----------------------------------|---------------------|---------------|-----------------------------------------------|
| <i>AtITPK1</i>         | phosphorylates IP3 to IP4 or IP4 to IP5 in LIP | <i>Arabidopsis thaliana</i> | Seeds                             | decreased (40~60%)  | increased     | KIM; TAI. (2011)                              |
| <i>AtITPK4</i>         | phosphorylates IP3 to IP4 or IP4 to IP5 in LIP | <i>Arabidopsis thaliana</i> | Seeds                             | decreased (40~60%)  | increased     | KIM; TAI.. (2011)                             |
| <i>AtMIK/At5g58730</i> | phosphorylates Ins to IP1                      | <i>Arabidopsis thaliana</i> | Seeds                             | decreased (40~60%)  | increased     | Kim & Tai. (2011)                             |
| <i>Hvlp1 (HvST)</i>    | ----                                           | <i>Hordeum vulgare</i>      | Seeds                             | decreased (45%)     | increased     | Larson et al. (1998); Dorsch et al. (2003)    |
| <i>Hvlp2</i>           | ----                                           | <i>Hordeum vulgare</i>      | Seeds                             | decreased (70%)     | increased     | Larson et al. (1998); Dorsch et al. (2003)    |
| <i>OslpaXS110-1</i>    | phosphorylates Ins to IP1                      | <i>Oryza sativa</i>         | Seeds                             | decreased           | increased     | Liu et al. (2007)                             |
| <i>Hvlp3-1</i>         | ----                                           | <i>Hordeum vulgare</i>      | Seeds                             | decreased (75%~90%) | increased     | Dorsch et al. (2003); Roslinsky et al. (2007) |
| <i>ZmLpa2 (ZmIPK)</i>  | phosphorylates IP3 to IP4 or IP4 to IP5 in LIP | <i>Zea mays</i>             | Seeds (embryo)                    | decrease (30%~50%)  | increased     | Raboy et al. (2000); Shi et al. (2003)        |
| <i>ZmLpa3 (ZmMIK)</i>  | phosphorylates Ins to IP1                      | <i>Zea mays</i>             | Seeds (embryo)                    | decrease (50%)      | increased     | Shi et al. (2005)                             |
| <i>ZmLpa1 (ZmMPR4)</i> | *converts glucose to IP1; PA transport         | <i>Zea mays</i>             | Laboratory                        | decreased (60%~66%) | increased     | Shi et al. (2007)                             |
| <i>ZmIPK1(A and B)</i> | phosphorylates IP5 to IP6                      | <i>Zea mays</i>             | Leaves, Silks, Ears, Seeds, Roots | -                   | -             | Sun et al. (2007)                             |
| <i>TaMIPS</i>          | converts glucose to IP1                        | <i>Triticum aestivum</i>    | Roots, Stems, Flag leaf, Seeds    | -                   | -             | Ma et al. (2013)                              |

Source: Own authorship

**Table 2.1.** Genes involved in phytic acid synthesis and transport in plants (conclusion).

| Genes                        | Proposed EnFunction       | Plant              | Tissue | PA alteration                        | Pi alteration | Reference               |
|------------------------------|---------------------------|--------------------|--------|--------------------------------------|---------------|-------------------------|
| <i>GmM153</i>                | ----                      | <i>Glycine max</i> | Seeds  | decreased to about 30~40% of total P | increased     | Wilcox et al. (2000)    |
| <i>GmM766</i>                | ----                      | <i>Glycine max</i> | Seeds  | decreased to about 50% of total P    | increased     | Wilcox et al. (2000)    |
| <i>Gm-lpa-TW-1 (GmMIPS1)</i> | converts glucose to IP1   | <i>Glycine max</i> | Seeds  | decreased (66%~94)                   | increased     | Yuan et al. (2007)      |
| <i>Gm-lpa-ZC-2 (GmIPK1)</i>  | phosphorylates IP5 to IP6 | <i>Glycine max</i> | Seeds  | decreased (43%)                      | increased     | Yuan et al. (2007;2012) |
| <i>GmMRP</i>                 | PA transport              | <i>Glycine max</i> | Seeds  | decreased (37~90%)                   | increased     | Shi et al. (2007)       |

Abbreviation: Ins: *Myo*-inositol; InsP: *Myo*-inositol phosphate intermediates; IP1: *Myo*-inositol monophosphate; IP3: *Myo*-inositol trisphosphate; IP4: *Myo*-inositol tetraphosphate; IP5: *Myo*-inositol pentaphosphate; IP6: *Myo*-inositol hexaphosphate; PA: Phytic Acid; LIP: Lipid independent pathway; LDP: Lipid dependent pathway.

Source: Own authorship

## 2.2 HORMONAL REGULATION OF PA BIOSYNTHESIS PATHWAYS

The plant hormones abscisic acid (ABA) and gibberellin (GA) play important roles during seed development and maturation, and control the biosynthesis of several metabolites (KANG et al., 2015). Because PA accumulation occurs mainly during flowering and seed maturation (LOTT et al., 2000, 2001; BOHN; MEYER; RASMUSSEN, 2008), these two hormones have been investigated in studies of the hormonal regulation of PA biosynthesis.

In rice cells in suspension, ABA addition increases PA production up to 2.5-fold and consequently decreases the inorganic P (Pi) concentration up to 66% (MATSUNO; FUJIMURA, 2014). In rice, ABA increases the expression of RINO1 (MATSUNO; FUJIMURA, 2014), which encodes the enzyme *myo*-inositol-3-P<sub>1</sub> synthase (OsMIPS) (KUWANO; TAKAIWA; YOSHIDA, 2009) responsible for the conversion of glucose-6-phosphate into Ins(3)P<sub>1</sub> (MURTHY, 1996) (figure 2.1, Supplementaty Table 2.1). The application of ABA increases mRNA for MIPS levels within 15 minutes, accompanied by an increase in enzymatic activity that contributes to PA accumulation (SMART; FLEMING, 1993; FLORES ; SMART, 2000).

Phospholipase C (PLC) is an enzyme in the lipid-dependent pathway that catalyses the conversion of PtdIns(4,5)P<sub>2</sub> into InsP<sub>3</sub> (RABOY, 2009). In wheat, PLC has been suggested to be regulated by ABA and GA and by the secondary messengers salicylic acid (SA) and cyclic

adenosine monophosphate (cAMP), with ABA promoting the transcription of gene TaPLC1 in wheat and the other three compounds suppressing this gene (AGGARWAL et al., 2015).

Absciscic acid also regulates inositol tris/tetraphosphate kinase (ITPK) activity by stimulating the expression of TaITPK1, TaITPK3 and TaITPK4 in wheat (AGGARWAL et al., 2015), AtITPK2 in arabidopsis (DU et al., 2011; TANG; TAN; XUE, 2013) and OsITPK4 and OsITPK6 in rice (MATSUNO; FUJIMURA, 2014). By contrast, GA represses the expression of TaITPK1, TaIPK3 and TaITPK4 in wheat (AGGARWAL et al., 2015).

Inositol polyphosphate kinase (IPK2), which phosphorylates the inositol ring at carbons 3 and 6, is also regulated by ABA in wheat. Absciscic acid stimulates the expression of TaIPK2, whereas GA, SA and cAMP repress it (AGGARWAL et al., 2015).

Inositol-pentakisphosphate 2-kinase (IPK1), which phosphorylates InsP3 and InsP4, is also regulated by ABA, which stimulates the expression of OsIPK1 in rice (MATSUNO; FUJIMURA, 2014) and TaIPK1 in wheat (AGGARWAL et al., 2015). In rice, the expression of OsMIK and OsLPA1 is also stimulated by ABA (MATSUNO; FUJIMURA, 2014).

In contrast to the genes described above, ABA reduces the expression of inositol monophosphatase (IMP), which catalyses the dephosphorylation of Ins(3)P<sub>1</sub> to generate free Ins (RABOY, 2009), in wheat whilst GA stimulates the expression of TaIMP (AGGARWAL et al., 2015). However, in chickpea (*Cicer arietinum*), ABA induces IMP production (SAXENA et al., 2013).

Absciscic acid and GA seem to play antagonistic roles in PA synthesis. In general, these hormones play opposite roles in seed maturation, as ABA regulates dormancy, nutrient accumulation and desiccation, whereas GA activates enzymes that act in breaking dormancy breaking and mobilisation of seed reserves (WHITE et al., 2000; PENFIELD, 2004; KUCERA; COHN; LEUBNER-METZGER, 2005; BETHKE, 2006; LIU et al., 2010). However, the antagonism between ABA and GA in PA synthesis is not fully resolved, since GA has been shown to promote the rapid accumulation of InsP<sub>3</sub> in the aleurone layer in barley seeds (VILLASUSO et al., 2003) and ABA alone does not increase the InsP<sub>3</sub> concentration in rice seeds (KASHEM et al., 2000).

## 2.3 STORAGE OF PA IN SEEDS

Phytic acid accumulation occurs in different seed structures depending on the plant species and family. In most monocot seeds, such as barley, millet, rice and wheat, PA accumulates in the aleurone layer (COULIBALY; KOUAKOU; CHEN, 2011). More than

80% of PA has been observed to accumulate in the aleurone layer in wheat and rice (O'DELL et al., 1972), and more than 90% in barley (OCKENDEN et al., 2004). An exception to this is maize, which accumulates approximately 85% of PA in the embryo endosperm (SAKAI et al., 2015).

In dicots, PA is stored in protein bodies in the endosperm or cotyledons (PRATTLEY; STANLEY, 1982; SCHLEMMER et al., 2009). In the endosperm and cotyledons of monocots and dicots, PAs accumulate in protein vacuoles with three compartments: a matrix containing soluble protein reserves, protein crystalloids and spherical inclusions composed of PA and oxalate crystals, called globoids (LOTT, 1980; BOHN; MEYER; RASMUSSEN, 2008; IWAI et al., 2012).

In rice, PA concentrations increase rapidly during seed development, between 10 and 25 days after flowering. In developing and mature seeds, K, Ca and Fe accumulate in the aleurone layer together with P, indicating that they are bound to P in the aleurone layer (IWAI et al., 2012).

Although they show high PA concentrations, globoids also store protein. In wheat, globoids show 46% protein and 40% PA (BOHN et al., 2007). In soybean, globoids are included in cotyledon protein bodies and possess approximately 25% PA. In soybean seeds, there is also a smaller fraction of PA associated with protein outside globoids but inside protein bodies (PRATTLEY; STANLEY, 1982).

During seed development, as globoids fill aleurone layer cells, metals that do not form PA salts gradually flow from the aleurone layer into the endosperm cells (IWAI et al., 2012; SAKAI et al., 2015). Cu and Zn, which have a high affinity for complex formation with PA (BOHN; MEYER; RASMUSSEN, 2008), do not occur at high concentrations in globoids or the aleurone layer (BOHN et al., 2007; LIU; PETERSON; RABOY, 2007). However, lower Cu and Zn concentrations are observed in milling fractions of lpa wheat when compared with the wild type (GUTTIERI et al., 2004), indicating that the low Cu and Zn concentrations observed in globoids may be due to their low natural concentrations in plants.

The higher the PA concentration is, the larger the globoids are (BOHN; MEYER; RASMUSSEN, 2008). A decreased globoid size has been observed in seeds of lpa rice, barley and maize (OCKENDEN et al., 2004; LIN; OCKEDEN; LOTT, 2005; LIU; PETERSON; RABOY, 2007; LIU et al., 2007). Globoids with a diameter up to 4  $\mu\text{m}$  are observed in wheat (ANTOINE et al., 2004) and up to 1  $\mu\text{m}$  in soybean, including in protein bodies (PRATTLEY; STANLEY, 1982).

## 2.4 PHYTIC ACID INTAKE AND THEIR SOURCES IN FOODS

Cereals, legumes and oil seeds and nuts are the main PA food sources (SCHLEMMER et al., 2009; KUMSSA et al., 2015), showing up to 90% of the P in mature seeds in the form of PA (LOTT et al., 2000, 200; BOHN; MEYER; RASMUSSEN, 2008). In the last decade, cereals have made up to 69.5% of food production and 77.3% of PA production, legumes up to 7.6% of food production and 13.0% of PA production, and fruits up to 17.2% of food production and less than 1% of PA production (LOTT et al., 2002).

In cereals, PA concentrations vary from 0.08 to 6% in ground fractions and protein products, 0.03% to 2.4% in breads and other products, 0.05% to 3.29 in ready-to-eat food products and 0.06% to 1.38% in infant cereals (REDDY, 2002). Phytic acid concentrations between 0.4% (KASIM; EDWARDS, 1998) and 1.1% (SCHLEMMER et al., 2009) were observed in barley grain and of 0.7% in millet grain (LESTIENNE et al., 2005). For maize grain, DRAGICEVIC et al. (2010) found PA concentrations of 0.2% for lpa populations and 0.5% for altPA populations, whereas other authors found PA concentrations varying between 0.3% (JOUNG et al., 2004) and 2.2% (SCHLEMMER et al., 2009). In corn gluten meal, 1.4% PA was observed (KASIM; EDWARDS, 1998).

Oat grain PA concentrations were observed between 0.6% (KASIM; EDWARDS, 1998) and 0.8% (SHEN et al., 2005). In rice grain, 1% to 2.5% PA were observed for unpolished grain (RAVINDRAN; RAVINDRAN; SIVALOGAN, 1994; LEE et al., 2015), and 0.25% to 0.6% for polished grain (RAVINDRAN; RAVINDRAN; SIVALOGAN, 1994; REDDY, 2002). For rice grain components, KASIM; EDWARDS (1998) observed 0.6% PA in bran, 0.2% in mills and 0.2% in mill feed polishing, but there are also reports of 8.7% PA for rice bran (LEHRFELD, 1994).

In sorghum, PA concentrations vary between 0.7% (JOUNG et al., 2004) and 3.35% (SCHLEMMER et al., 2009) in wheat grain. In sorghum, KASIM; EDWARDS (1998) observed PA concentrations of 4% in bran, 0.7% in grain, 0.3% in middlings, 0.3% in pollard and 0.2% in shorts. For wheat, 1% PA was observed in grain (SHEN et al., 2005), 0.2 to 0.4% PA in refined flower and 0.6 to 1% in whole flour (FEBLES et al., 2002).

In legumes, PA concentrations vary from 0.17% to 9.15% in whole beans, from 0.58 to 4.20% in bean flours and bean protein products and from 0.05 to 5.20% in legume-based foods (REDDY, 2002). The PA concentration in cowpea beans varies from 0.6% (Lestienne et al., 2005) to 2.9% (SCHLEMMER et al., 2009), in mung beans from 0.2% (LESTIENNE et al., 2005) to 0.9% (RAVINDRAN; RAVINDRAN; SIVALOGAN, 1994) and in common

beans from 0.6% (JOUNG et al., 2004) to 2.4% (SCHLEMMER et al., 2009). In chickpea and lentils, 0.7% PA was observed (RAVINDRAN; RAVINDRAN; SIVALOGAN, 1994).

In soybean, PA concentrations of 0.2% were observed for soybean mill feed, 1.4% for soybean meal (44%) (KASIM; EDWARDS, 1998) and between 0.9% (LESTIENNE et al., 2005) and 4.7% (PANDEY et al., 2016) for soybean seeds. In other oil seeds, 0.5% PA was observed for sunflower meal (38%) (KASIM; EDWARDS, 1998), 1.4% PA for peanut seeds (RAVINDRAN; RAVINDRAN; SIVALOGAN, 1994) and 1.6% (JOUNG et al., 2004) to 3.8% PA for sesame seeds (RAVINDRAN; RAVINDRAN; SIVALOGAN, 1994).

Phytic acid concentrations of up to 0.18% were observed for potato and sweet potato tubers, 0.14% for manioc roots and 0.32% (*Colocasia esculenta*), 0.11% (*Dioscorea esculenta*) and 0.14% (*D. alata*) for different yam bulbs (RAVINDRAN; RAVINDRAN; SIVALOGAN, 1994). HARLAND; SMIKLE-WILLIAMS; OBERLEAS, 2004, analysed foods bought in a market and observed up to 4% PA for walnuts and other high PA concentrations in different nuts such as pecans (1.9%), peanuts (2%), hazelnuts (2.3%) and pistachio nuts (2.8%).

In plants from the family Amaranthaceae, PA concentrations vary from 230 mg kg<sup>-1</sup> in dry leaves of *Aerva lanata* (OMOYENI; ADEYEYE, 2009) to 46,820 mg kg<sup>-1</sup> in blanched leaves of *Amaranthus hybridus* (AKINDAHUNSI; OBOH, 1999).

In Asteraceae, PA concentrations vary from 88.1 mg kg<sup>-1</sup> in dry leaves of *Tithonia diversifolia* (FADIYIMU; FAJEMISIN; ALOKAN, 2011) to 40,430 mg kg<sup>-1</sup> in seeds of *Helianthus annuus* (KASIM; EDWARDS, 1998). In Basellaceae, PA concentrations in blanched leaves of *Basella alba* vary between 600 (ADEGUNWA et al., 2011) and 48,610 mg kg<sup>-1</sup> (AKINDAHUNSI; OBOH, 1999).

In the family Fabaceae, PA concentrations of 72,100, 75,400 and 84,500 mg kg<sup>-1</sup> PA have been detected in dry leaves of *Gliricidia sepium*, *Senna siamea* and *Leucaena leucocephala*, respectively (FADIYIMU; FAJEMISIN; ALOKAN, 2011). An average PA concentration of 34,400 mg kg<sup>-1</sup> was observed in soybean (PANDEY et al., 2016) and of 133 mg kg<sup>-1</sup> in dry leaves of *Indigofera astragalina* (GAFAR et al., 2010).

In the family Poaceae, PA concentrations vary from 2811 mg kg<sup>-1</sup> in maize seeds (JOUNG et al., 2004) to 92,300 mg kg<sup>-1</sup> in sorghum seeds (KASIM; EDWARDS, 1998). Phytic acid concentrations reach 50,500 mg kg<sup>-1</sup> in barley seeds and 59,990 mg kg<sup>-1</sup> in rice seeds (KASIM; EDWARDS, 1998).

The average daily intake of PA per capita in the African population is 2270 mg, of which 68% is obtained from cereals, 17% from pulses and beans and 7% from roots and

tubers (JOY et al., 2014). In Sweden, a value of 369 mg has been determined in people with Western diets and 1146 mg in vegetarians (BRUNE; ROSSANDER; HALLBERG, 1989). In China, this value is 1186 mg, varying between 781 mg in urban residents and 1342 mg in rural residents (MA et al., 2007), and in South Korea, it is 839 mg in men and 752 mg in women (JOUNG et al., 2004).

In Africa, several studies have analysed leaf PA concentrations in many different foods. Some of them have shown very high leaf PA concentrations, such as waterleaf (*Talinum triangulare*), for which 10% PA was observed in leaves (AKINDAHUNSI; OBOH, 1999).

A wide variation in PA concentrations has been observed for plants collected in Nigeria. Amongst the highest PA concentrations, 101,260 mg kg<sup>-1</sup> PA was observed in oven-dried leaves of *Talinum triangulare* and 48,610 mg kg<sup>-1</sup> PA in blanched leaves of *Basella alba* (AKINDAHUNSI; OBOH, 1999). FADIYIMU et al. (2011) examined the dry leaves of material collected in Nigeria, and observed PA concentrations of 57,400 mg kg<sup>-1</sup> in *Ficus exasperata*, 72,100 mg kg<sup>-1</sup> in *Gliricidia sepium*, 75,400 mg kg<sup>-1</sup> in *Senna siamea* and 84,500 mg kg<sup>-1</sup> in *Leucaena leucocephala*. In Sri Lanka, a concentration of 38,700 mg kg<sup>-1</sup> PA was observed in seeds of *Sesamum indicum* (RAVINDRAN; RAVINDRAN; SIVALOGAN, 1994).

There are ways of decreasing the PA concentration in foods, such as cooking, roasting and soaking (JOUNG et al., 2004; LESTIENNE et al., 2005). Phytic acid concentrations can decrease up to 90% in roasted sesame seeds (JOUNG et al., 2004). LESTIENNE et al. (2005) observed decreases in PA concentration of 23%, 21% and 28% in grain from soybean, maize and millet, respectively, after soaking for 24 hours.

## 2.5 CONCLUSIONS AND FUTURE PERSPECTIVES

A large amount of research in agriculture focuses on quantitative improvements, but interest in qualitative improvements of foods has been increasing. Examples of this are the studies reviewed in the present review with the aim of clarifying the relationship between gene expression, PA and its intermediates, especially in model plants such as *A. thaliana*, maize and soybean.

Because PA is a P storage form in seeds and an important compound for seed germination, studies examining the effects of decreased PA concentrations on germination are also extremely relevant. Although decreased PA concentrations may improve the quality of

the consumed food, the importance of P accumulation in seeds and crop germination cannot be overlooked so the high yield standards achieved by the crop plant genotypes and varieties commonly used for food production are maintained.

Investigating the behaviour of PA concentration depending on the plant species, tissue exhibiting accumulation, region of origin and food handling is an important first step in the direction of healthier diets with lower PA intake. Human nutrition depends on many factors, such as geographical location, purchasing power and cultural heritage, resulting in a wide variation in food origin and manner of preparation. The geographical distribution of foods and respective PA concentrations are important indicators of regions of the world where PA may act more frequently and lead to increased challenges related to hidden hunger.

### 3 CHAPTER 3. AGRONOMIC BIOFORTIFICATION OF COWPEA WITH SELENIUM: EFFECT OF SELENATE AND SELENITE APPLICATION ON PHYTATE AND SELENIUM CONCENTRATION IN SEEDS

#### ABSTRACT

Selenium (Se) is an essential element in human and animal nutrition, and is considered beneficial to higher plants. Selenium concentrations are low in most soils, which can result in a lack of Se in plants, and in human and animal diets. Phytic acid (PA) is the main storage form of phosphorus in seeds, and is able to form insoluble complexes with essential minerals in the monogastric gut. Cowpea (*Vigna unguiculata* (L.) Walp.) is an important source of protein in developing countries and is suitable for cultivation in arid regions. This study aimed to establish optimal levels of Se application to cowpea, with the aim of increasing Se concentrations in edible tissue and to reduce PA in seeds. The efficiency of agronomic biofortification was evaluated by the application of seven levels of Se (0; 2.5; 5; 10; 20; 40 and 60 g ha<sup>-1</sup>) from two sources (selenate and selenite) applied via soil under field conditions. Application of Se as selenate led to greater plant Se concentrations than selenite. Selenite application did not increase Se concentration in seeds due to higher selenite adsorption in the soil. Considering a human cowpea consumption of 54.2 g day<sup>-1</sup>, Se application of up to 20 g ha<sup>-1</sup> and 10 g ha<sup>-1</sup> in the first and second year of cultivation (2016 and 2017) applied as selenate, provided a suitable daily intake of Se (between 20 and 55 µg day<sup>-1</sup>), considering minimum and maximum intake recommendations, respectively. Phytic acid showed no direct response to Se application. Cowpea yield, shoot dry weight, pod length, pods per plant and seed per pod were not affected by Se application rates.

**Keywords:** Agronomic biofortification. Cowpea. Selenate. Selenite. Selenium. *Vigna unguiculata*.

### 3.1 INTRODUCTION

In the years between 1960 and 2007, agricultural food production increased almost three-fold, in order to provide food for a growing population and an increase in consumption per person (GREEN 2005; CAZALIS; LOREAU; HEDERSON, 2018). However, over 800 million people worldwide are still estimated to consume insufficient food to meet dietary energy requirements (FAO et al., 2017). It is also expected that the global population will increase further from the 2014 estimate of 7.3 billion (CAZALIS; LOREAU; HEDERSON, 2018), to 9.7 billion people by 2050 (UNITED NATIONS, 2015). In order to adequately nourish this increase in population, whilst increasing dietary energy intakes of those currently undernourished, huge increases in global food production will be required over the next few decades.

The prevalence of human malnutrition, defined as inadequate, unbalanced or excessive consumption of macronutrients and / or micronutrients (FAO et al., 2017) is a noteworthy global issue. Almost half of the world's population is likely to be affected, in particular pregnant women, teenagers and children in developing countries (RAYMAN, 2012, DAS et al., 2018). Malnutrition in human populations is linked to recent, intensive plant breeding strategies, which have focused almost entirely on increasing yield. This has resulted in lower concentrations of essential human and animal mineral nutrients in edible crop tissue due to the dilution effect (GARVIN; WELCH; FINLEY, 2006; MURPHY; REEVES; JONES, 2008; WHITE et al., 2009).

Humans and animals require at least eighteen mineral elements of which eight are macronutrients, being nitrogen (N), phosphorus (P), sulphur (S), calcium (Ca), magnesium (Mg), potassium (K), sodium (Na), chlorine (Cl), and ten are micronutrients, being zinc (Zn), iron (Fe), fluorine (F), manganese (Mn), copper (Cu), iodine (I), selenium (Se), molybdenum (Mo), chromium (Cr), cobalt (Co), arsenic (As), boron (B), nickel (Ni), silicon (Si) and vanadium (V) (WHITE, 2016a). Among these elements, Fe, Zn, Cu, Ca, Mg, Se and I might lack in human diets (WHITE; BROADLEY, 2009).

Selenium is an essential human and animal mineral nutrient. It is involved in thyroid function, proper functioning of the immune system, and also acts as an antioxidant (FAIRWEATHER-TAIT et al., 2011). Selenium deficiency in humans has been linked to an elevated risk of cancer (FRITZ et al., 2011). Selenium is also considered a beneficial trace element for plants, which can promote growth and help resist oxidative, biotic, and abiotic stress (WHITE, 2016b; REIS et al., 2017a, 2017b, SILVA et al., 2018). Selenium deficiency

is estimated to be a problem for more than 1 billion people (COMBS JUNIOR, 2001; SCHIAVON; PILON-SMITS, 2017). Among human micronutrients, it is also associated with the third highest deficiency risk in Africa (RAYMAN, 2012; JOY et al., 2014).

Selenium concentration in agricultural food products depends on availability in the soil (JOY et al., 2015). Increasing concentrations of soil Se through agronomic biofortification (WHITE; BROADLEY, 2005) increases the amount available for plant uptake and subsequently improves nutritional quality of food (WHITE, 2016b). The addition of Se together with NPK fertilisers in agricultural areas seems to be an effective and safe way to reduce Se deficiency in human and animal nutrition, as evident from decades of monitoring programs carried out in Finland (HARTIKAINEN, 2005; HAUG et al., 2007). Different sources of Se such as selenate and selenite present distinct behaviours in soil and plants. For instance, selenate uptake, assimilation and translocation is faster than selenite (GUPTA ; GUPTA, 2017). Selenate is the main Se form in oxygenated soils, and selenite is the main Se source in anaerobic environments, such as paddy soils (MIKKELSEN; PAGE; BINGHAM, 1989; WHITE et al., 2007; FORDYCE, 2013; WHITE, 2016b). Hence, the Se source used must be carefully considered in agronomic biofortification strategies order to have the desired effect. Care must also be taken when aiming to increase Se concentrations of agricultural products, as although Se is essential to humans, the range between deficient and toxic levels of Se is narrow (SCHIAVON; PILON-SMITS, 2017). For instance, a minimum Se intake of 20  $\mu\text{g day}^{-1}$  is necessary to prevent Keshan disease (ROMAN; JITARU; BARBANTE, 2014; NATASHA et al., 2018) but the maximum Se intake recommended for adults is 55  $\mu\text{g day}^{-1}$  (INSTITUTE OF MEDICINE, 2000; FAIRWEATHER-TAIT et al., 2011).

Phytic acid (PA) (or *myo*-inositol hexaphosphate, InsP6 or IP6) is the main compound of phosphorus (P) storage in seeds (LOTT et al., 2000). Phytic acid comprises between 60 and 90% of all P in cereals, oilseeds and nuts (LOTT et al., 2000; 2001; BOHN; MEYER; RASMUSSEN, 2008). Phytic acid and its derivatives bound to metallic ions in pH values similar to duodenum (CARLI et al., 2006), in this condition, the ions bounded to the PA are not assimilated by humans. The bioavailability Fe, Zn, Cu and Mg in human diets is often reduced by the presence of PA (WHITE; BROADLEY, 2009). Phytic acid is considered an antinutrient, as it forms insoluble complexes with human nutrients in the gut which are largely excreted (RABOY 2001). For this reason, there are commonly severe deficiencies in populations with diets composed primarily by cereals, particularly in vulnerable groups such as children and pregnant women from poor regions that commonly ingest low quantities of bioavailable Ca, Fe, Mg and Zn (WEAVER; KANNAN, 2002). Thus, strategies to reduce the

PA levels in edible part of plants are also needed in order to enhance the nutritional quality of foods.

Selenate is taken up from the rhizosphere solution by high-affinity,  $H^+$ /sulphate symporters in the plasma membrane of root cells and selenite is taken up as  $HSeO_3$  by members of the phosphate transporter Pht1 family or as  $H_2SeO_3$  through aquaporins (WHITE, 2018). Thus, increasing Se in the rhizosphere solution could compete with sulphate and phosphate for uptake. A reduction in P uptake can influence the synthesis of PA and, consequently, the concentrations of PA and cations associated with it in the seed.

Leguminous plants provide on average  $2 \mu g$  of Se capita<sup>-1</sup> day<sup>-1</sup> (JOY et al., 2014). Cowpea (*Vigna unguiculata* (L.) Walp.) is a leguminous plant with an important role as a dietary protein and mineral source, complementing cereals, tuberous vegetables, and root vegetables in diets in developing countries in Africa, Latin America, and Asia (PHILLIPS et al., 2003; MANZEKE et al., 2017). Due to cowpea's high nutritional value, adaptability to different production conditions (EHLERS; HALL, 1997), and drought tolerance, it is considered a suitable crop for cultivation in hot, arid regions (HALL, 2012).

The biofortification of edible crops with Se can affect the concentrations of other essential mineral elements, including both macronutrients and micronutrients, and antinutrients, such as PA, in seed, and also, Se biofortification using different Se-fertilisers, such as selenate or selenite, could have contrasting effects on seed nutritional quality. In this chapter the effects of Se biofortification using selenate or selenite on the elemental composition and PA concentrations in seeds of cowpea were studied. A better understanding of factors that affect bioavailable concentrations of Se in cowpea will enable strategies to be employed to safely increase the daily intake of Se by the global population.

## 3.2 MATERIALS AND METHODS

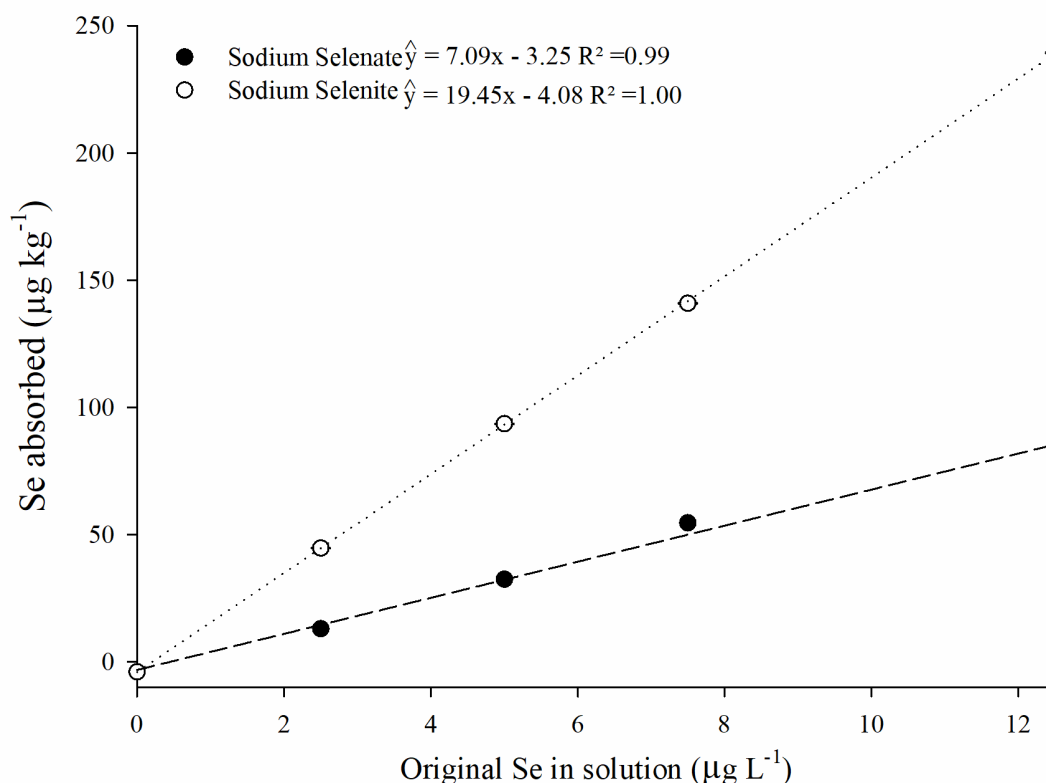
### 3.2.1 Experimental conditions

The experiment was conducted at the Teaching, Research and Extension Farm of São Paulo State University (UNESP) in the municipality of Selviria, Mato Grosso do Sul State, Brazil (20°20'43"S; 51°24'7"W, 355 m above sea level). Daily rainfall and mean maximum and minimum temperatures recorded during the experiment can be found in figure suppl. 3.1. The soil of the experimental area was classified as Rhodic Haplustox according to the SOIL SURVEY STAFF (2014) with the following chemical characteristics: pH (water) 5.5;

phosphorus (resin)  $34 \text{ mg dm}^{-3}$ ; sulfur (calcium phosphate)  $8 \text{ mg dm}^{-3}$ ; potassium (resin)  $2.7 \text{ mmolc dm}^{-3}$ ; calcium (resin)  $14 \text{ mmolc dm}^{-3}$ ; magnesium (resin)  $14 \text{ mmolc dm}^{-3}$ ; H+Al (SMP buffer)  $26 \text{ mmolc dm}^{-3}$ ; cation exchange capacity  $56.7 \text{ mmolc dm}^{-3}$ ; base saturation 54%; boron (hot water)  $0.19 \text{ mg dm}^{-3}$ ; copper (DTPA)  $2.7 \text{ mg dm}^{-3}$ ; iron (DTPA)  $19 \text{ mg dm}^{-3}$ ; manganese (DTPA)  $12.4 \text{ mg dm}^{-3}$ ; zinc (DTPA)  $6.1 \text{ mg dm}^{-3}$ , and organic matter  $18 \text{ g dm}^{-3}$ . These chemical characteristics were determined as described by RAIJ et al. (1997) after the collection of composite soil samples from 0 to 20 cm depth on September 10, 2016. Readily available soil Se concentration was  $3.6 \text{ } \mu\text{g dm}^{-3}$ . To determine Se concentration, 4 g of air dried soil was added to a 50 mL centrifuge tube along with 20 mL of 0.01M  $\text{KNO}_3$ . The solution was shaken in a rotary shaker for 2 hours, then centrifuged for 30 min at 3500 rpm. After centrifugation, 9 mL of the supernatant was pipetted into a  $< 0.22 \text{ } \mu\text{m}$  syringe filter. The supernatant was filtered into a tube containing 10% tetramethylammonium hydroxide (TMAH) and 0.1 M  $\text{KH}_2\text{PO}_4$  for determination of readily available Se using inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany).

An adsorption isotherm for selenate and selenite was performed (figure 3.1). To perform the adsorption, 1 g soil samples were weighed, and 20 mL of 0.01 M  $\text{Ca}(\text{NO}_3)_2$  containing different concentrations of Se: 0 (control), 2.5; 5.0; 7.5 and  $12.5 \text{ } \mu\text{g L}^{-1}$  as sodium selenate or sodium selenite was added to each sample. Soil samples with solutions were stored in 50 mL polyethylene falcon tubes and keep shaking in a rotating shaker at 20 rpm for 48 hours. After shaking, the samples were centrifuged for 15 minutes at 3000 rpm. A 9.6 mL aliquot was collected from each samples and filtered using a  $0.22 \text{ } \mu\text{m}$  filter to avoid contamination. To this, 0.4 mL of 50 % nitric acid ( $\text{HNO}_3$ ) was added. The concentration of Se in each sample was obtained using ICP-MS.

**Figure 3.1.** Sodium selenate and sodium selenite adsorption isotherm in soils from Selviria city, State of Mato Grosso do Sul, Brazil.



Source: Own authorship

### 3.2.2 Growth of plant material and treatment application

Experiments were performed in two agricultural years (2016 and 2017). The soil was prepared with subsoiling, heavy disking, medium disking (twice), and leveling. Sowing was carried out on October 18<sup>th</sup>, 2016 (first year) and March 23<sup>rd</sup>, 2017 (second year) with spacing of 0.45 m between rows and a sowing density of 11.2 seeds m<sup>-1</sup>. Fertilization of the planting furrow consisted of 20 kg ha<sup>-1</sup> K<sub>2</sub>O applied as KCl (33 kg ha<sup>-1</sup>) and 20 kg ha<sup>-1</sup> P<sub>2</sub>O<sub>5</sub> applied as single superphosphate (110 kg ha<sup>-1</sup>). The experiment design was a complete randomized block in a factorial scheme (7 x 2), in which four replicates of seven Se rates (0; 2.5; 5.0; 10; 20; 40 and 60 g ha<sup>-1</sup>) from two sources (sodium selenate and sodium selenite) were applied. Each experimental plot was composed of 5 m long rows in 0.45 m intervals.

Cowpea seeds (*Vigna unguiculata* (L.) Walp.) of the BRS Tumucumaque variety were treated with pyraclostrobin (25 g L<sup>-1</sup> commercial product), thiophanate-methyl (225 g L<sup>-1</sup> commercial product), and fipronil (250 g L<sup>-1</sup> commercial product) at 2 mL product per kg of seeds. After the seeds were dried, they were inoculated with a premium peat inoculum for cowpea (strain SEMIA 6462, product registration number SP 00581-10030-1, 2.0x10<sup>9</sup> colony

forming units  $\text{g}^{-1}$ , BIOMAX, São Joaquim da Barra city, Brazil), at  $8 \text{ g kg}^{-1}$  of seed. The inoculum was dissolved in a sugar solution (1 mL of water per gram of inoculant, 10% sugar) and gradually added and mixed with the seeds in a concrete mixer at a constant speed of 18 rpm for 5 min. Emergence began on October 22<sup>nd</sup>, 2016, four days after sowing (4 DAS), in the first year, and March, 28<sup>th</sup>, 2017, five days after sowing (5 DAS), in the second year.

Application of selenium treatments was performed at 33 days after emergence of seedlings (DAE) in the first year, and 35 DAE in the second year. Total Se required for each treatment across all four replicates was weighed and diluted in 2 L of water, generating a stock solution for each treatment. The stock solution was then subdivided into four portions of 500 mL each. Solution application was via soil; in each plot, 100 mL of solution was applied to each line.

Pest control applications were as follows: in the first year, pest controls were carried out at 12 DAE (abamectin,  $0.50 \text{ L ha}^{-1}$ ), 23 DAE (bentazone,  $0.8 \text{ L ha}^{-1}$ ; abamectin,  $0.5 \text{ L ha}^{-1}$  and imidacloprid,  $0.4 \text{ L ha}^{-1}$ ), and at 33 DAE (haloxyfop-p-methyl,  $0.3 \text{ L ha}^{-1}$ ). In the second year, pest controls were carried out at 10 DAE (clethodim + alquilbenzene  $0.40 \text{ L ha}^{-1}$ , beta-cyfluthrin  $0.15 \text{ L ha}^{-1}$ , abamectin,  $0.50 \text{ L ha}^{-1}$ ), 14 DAE (bentazon  $1.2 \text{ L ha}^{-1}$ ), 15 DAE (deltamethrin  $0.06 \text{ L ha}^{-1}$ , beta-cyfluthrin+imidacloprid  $0.87 \text{ L ha}^{-1}$ ) and 28 DAE (beta-cyfluthrin  $0.15 \text{ L ha}^{-1}$ , abamectin,  $0.50 \text{ L ha}^{-1}$ ).

### 3.2.3 Sampling

Leaf samples were collected for mineral analysis 23 days after soil application of Se in the first year and 24 days after soil application of Se in the second year. The third, trifoliate leaf (counting from the apex) was removed, dried in an oven at  $40^\circ\text{C}$  for 72 hours, until a constant, dry mass, was achieved, and ground in a Wiley mill (Marconi, MA 340, Piracicaba, Brazil) using 1 mm sieve.

Seed harvest was performed at 77 DAE in the first year and 72 DAE in the second year. Harvesting was conducted by the selection of two homogeneous rows from each plot, from which all pods were manually harvested. The following yield components were analysed: yield, shoot dry mass (SDM), pod length (PL), number of pods per plant (NPP) and number of seeds per pod (NSP).

### 3.2.4 Digestion and mineral analysis of leaf, seed and shoot samples

Subsamples of 0.20 g dry weight (DW) of leave and seed material was microwave digested and analysed by ICP-MS as described previously by THOMAS, et al (2016). Subsamples were placed into digestion vessels of perfluoroalkoxy (PFA) liner material and polyethylethylketone (PEEK) pressure jackets (Anton Paar GmbH) with 2 mL 70 % Trace Analysis Grade  $\text{HNO}_3$ , 1 mL Milli-Q water ( $18.2 \text{ M}\Omega \text{ cm}$ ; Fisher Scientific UK Ltd, Loughborough, UK), and 1 mL  $\text{H}_2\text{O}_2$ . Digestion was in a microwave system comprising a Multiwave 3000 platform with a 48-vessel MF50 rotor (Anton Paar GmbH, Graz, Austria). The settings for digestion were: power = 1400 W, temp =  $140^\circ\text{C}$ , pressure = 2 MPa, time = 45 min. In each digestion, two operational blanks were included, and two replicates of certified reference material (CRM) were included every three runs. The CRM used for shoot and leaf sample digestions was tomato leaves (SRM 1573a, NIST, Gaithersburg, MD, USA). For seed digestions, wheat flour CRM was used (SRM 1567b, NIST, Gaithersburg, MD, USA). After digestion, 11 mL of milli-Q water was added to each tube (final volume of 15 mL) and the digestates was transferred to a 25 mL universal tube (Sarstedt Ltd., Nümbrecht, Germany) and stored at room temperature.

Prior to elemental analysis, digestates were diluted 1-in-10 using milli-Q water. The concentrations of 31 elements were obtained using inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany); Ag, Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, Tl, U, V and Zn. Operational modes included: (i) a helium collision-cell (He-cell) with kinetic energy discrimination to remove polyatomic interferences, (ii) standard mode (STD) in which the collision cell was evacuated, and (iii) a hydrogen collision-cell ( $\text{H}_2$ -cell). Samples were introduced from an autosampler incorporating an ASXpress™ rapid uptake module (Cetac ASX-520, Teledyne Technologies Inc., Omaha, NE, USA) through a PEEK nebulizer (Burgener Mira Mist, Mississauga, Burgener Research Inc., Canada). Internal standards were introduced to the sample stream on a separate line via the ASXpress unit and included Sc ( $20 \mu\text{g L}^{-1}$ ), Rh ( $10 \mu\text{g L}^{-1}$ ), Ge ( $10 \mu\text{g L}^{-1}$ ) and Ir ( $5 \mu\text{g L}^{-1}$ ) in 2 % trace analysis grade  $\text{HNO}_3$  (Fisher Scientific UK Ltd). External multi-element calibration standards (Claritas-PPT grade CLMS-2; SPEX Certiprep Inc., Metuchen, NJ, USA) included Ag, Al, As, B, Ba, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti (semi-quant), U, V and Zn, in the range  $0\text{--}100 \mu\text{g L}^{-1}$  ( $0, 20, 40, 100 \mu\text{g L}^{-1}$ ). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, Courtaboeuf, France) was used

to create Ca, K, Mg and Na standards in the range 0–30 mg L<sup>-1</sup>. Boron, P and S calibration utilized in-house standard solutions (KH<sub>2</sub>PO<sub>4</sub>, K<sub>2</sub>SO<sub>4</sub> and H<sub>3</sub>BO<sub>3</sub>). In-sample switching was used to measure B and P in STD mode, Se in H<sub>2</sub>-cell mode and all other elements in He-cell mode. Sample processing was undertaken using Qtegra™ software (Thermo Fisher Scientific) with external cross-calibration between pulse-counting and analogue detector modes when required.

### *3.2.5 Extraction and phytic acid analysis of seeds*

Phytic acid (PA) concentration of seed samples was determined. Approximately 1.0 g DW seed subsamples were weighed and placed into 50 mL conical tubes (SARSTEDT). To each tube, 20 mL of 0.66 M HCl was added, and tubes were put in a rotary shaker to shake overnight at 25 rpm. After this, 1 mL of the homogenized solution was transferred to 2 mL safe lock microtubes (Eppendorf, Hamburg, Germany) and centrifuged at 13,000 rpm for 10 minutes. After centrifugation, 0.5 mL of the supernatant was transferred to a new 2 mL safe lock microtubes and neutralized with 0.5 mL of 0.75 M NaOH. This extract was used to perform an enzymatic dephosphorylation reaction, in order to estimate PA concentration. This was by analysing the difference between total phosphorus and free phosphorus using the Phytic Acid (Total Phosphorus) Assay Kit (Megazyme) composed of: Solution 1 (5.5 pH buffer and sodium azide 0.02 % w/v), suspension 2 (phytase suspension), solution 3 (10.4 pH buffer, plus MgCl<sub>2</sub>, ZnSO<sub>4</sub> and sodium azide 0.02 % w/v) suspension 4 (alkaline phosphatase suspension), standard solution 5 (phosphorus standard solution and sodium azide 0.02 % w/v) and bottle 6 (oat flour control powder).

For total phosphorus dephosphorylation, 0.05 mL of neutralized extract was added to a new 2 mL microtube with 0.60 mL milli-Q water, 0.20 mL solution 1 and 0.02 mL suspension 2., The microtube solution was mixed using a tube mixer. After 10 minutes of incubation in a water bath set to 40 °C, 0.20 mL solution 3 and 0.02 mL suspension were added and the microtube solution was mixed again. After 15 minutes of incubation in a second water bath set to 40°C, 0.30 mL of trichloroacetic acid (50% w/v) was added to stop the reaction. The microtubes were centrifuged at 13,000 rpm for 10 minutes. For free phosphorus quantification, the previous steps were followed exchanging the enzymatic suspensions (suspension 2 and 4) for the same volume of milli-Q water.

After centrifugation, 1 mL of supernatant was transferred to a 1.5 mL microtube, to which 0.5 mL of a colorimetric solution composed of five parts of 10 % ascorbic acid (w/v) in

1 M H<sub>2</sub>SO<sub>4</sub> and one part of 5 % w/v) ammonium molybdate was added. After one hour of incubation in a water bath set to 40 °C, the absorbance was read in 96 well plate readers (Thermo Scientific, Waltham, MA, USA) at 650 nm in an EL808 absorbance reader (BIOTEK). One operational blank (with ultrapure replacing the supernatant) and one oat flour control (bottle 6) were included in each set dephosphorylation reaction. In each analysis in the 96 well plate reader, one phosphorus calibration curve was run (0, 0.5, 2.5, 5 and 7.5 µg mL<sup>-1</sup>), using standard solution 5. To determine the P-phytic acid and PA concentration, the MegaCalc Excel spreadsheet suggested by the Megazyme kit protocol was used. This provided values expressed in g 100 g<sup>-1</sup>, which were subsequently converted to mg kg<sup>-1</sup>.

### 3.2.6 Statistical analysis

The results were first submitted to Anderson-Darling normality tests; homogeneity was evaluated with Leven's test and, finally, with a variance analysis (F test). Differences between treatments were compared using a Tukey test at 5 % probability or a polynomial regression, depending on the data set. Analyses were conducted using Agroestat and Minitab softwares, and graphs were prepared using SigmaPlot 12.5.

## 3.3 RESULTS

There was no influence of sodium selenate and sodium selenite application in cowpea yield in the first and in the second year (figure 3.2a-b). In the first year, average yield was 1770 kg ha<sup>-1</sup>, and in the second year, average yield was 1220 kg ha<sup>-1</sup>. The application of sodium selenite and sodium selenate showed no effect on shoot dry weight in both years of cultivation (figure 3.2c-d). Similarly, pod length (PL), number of pods per plant (NPP) and number of seeds per pod (NSP) were not affected by application of sodium selenate and sodium selenite in both years of cultivation (figure 3.3).

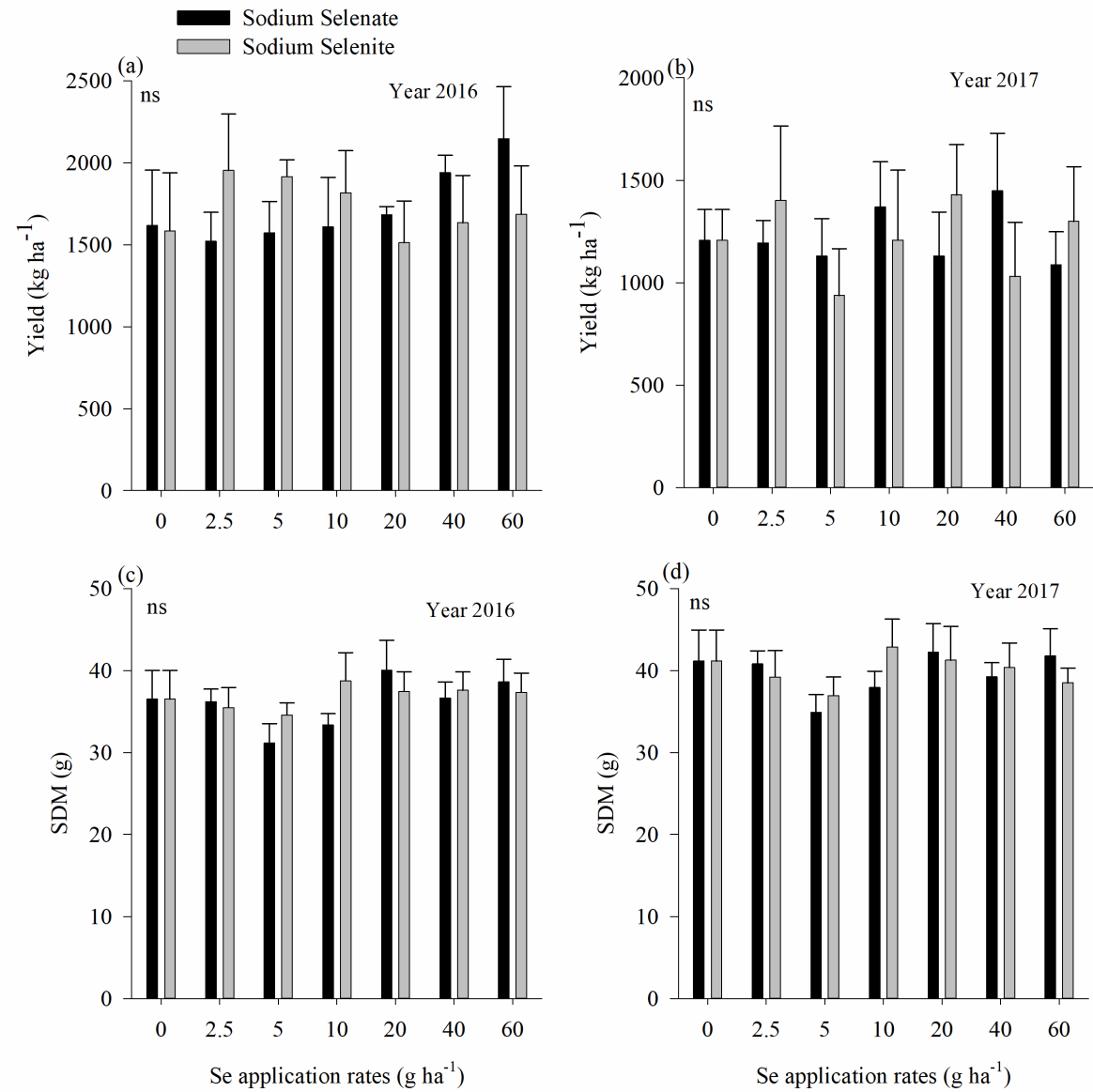
An interaction was observed between sources and concentrations of Se in both the first and second experimental years (figure 3.4). In the first year, leaf Se concentration increased with increased selenate and selenite applications. The applications of selenate provided higher leaf Se concentration than selenite at application rates of 5, 10 and 40 g ha<sup>-1</sup> (figure 3.4a) and Se concentration ranged from 0.06 to 0.52 mg kg<sup>-1</sup>. Similar effects were observed in the second year; leaf Se concentration once again increased in response to elevated selenate and selenite application rates. The applications of selenate provided higher Se concentration than

selenite at application rates of 10, 20, 40 and 60 g ha<sup>-1</sup> (figure 3.4a) and Se concentration ranged from 0.2 to 2.66 mg kg<sup>-1</sup>. Selenium application reduced S leaf concentration in the second year (figure suppl. 3.2), in application rates of 2.5, 10, 40 and 60 g ha<sup>-1</sup>, regardless of the Se source.

Seed Se concentration was affected by sources and concentrations of Se applied in both the first and second experimental years (figure 3.5). In the first year, the Se concentration increased in response to increased selenate and selenite application rates, and Se concentration ranged from 0.05 to 0.83 mg kg<sup>-1</sup>. The applications of selenate provided higher Se concentration than selenite at application rates of 10, 20, 40 and 60 g ha<sup>-1</sup> (figure 3.5a). In the second year, the Se concentration increased with increased selenate applications, and Se concentration ranged from 0.04 to 2 mg kg<sup>-1</sup>. The applications of selenate provided higher Se concentration than selenite at application rates of 20, 40 and 60 g ha<sup>-1</sup> (figure 3.5b).

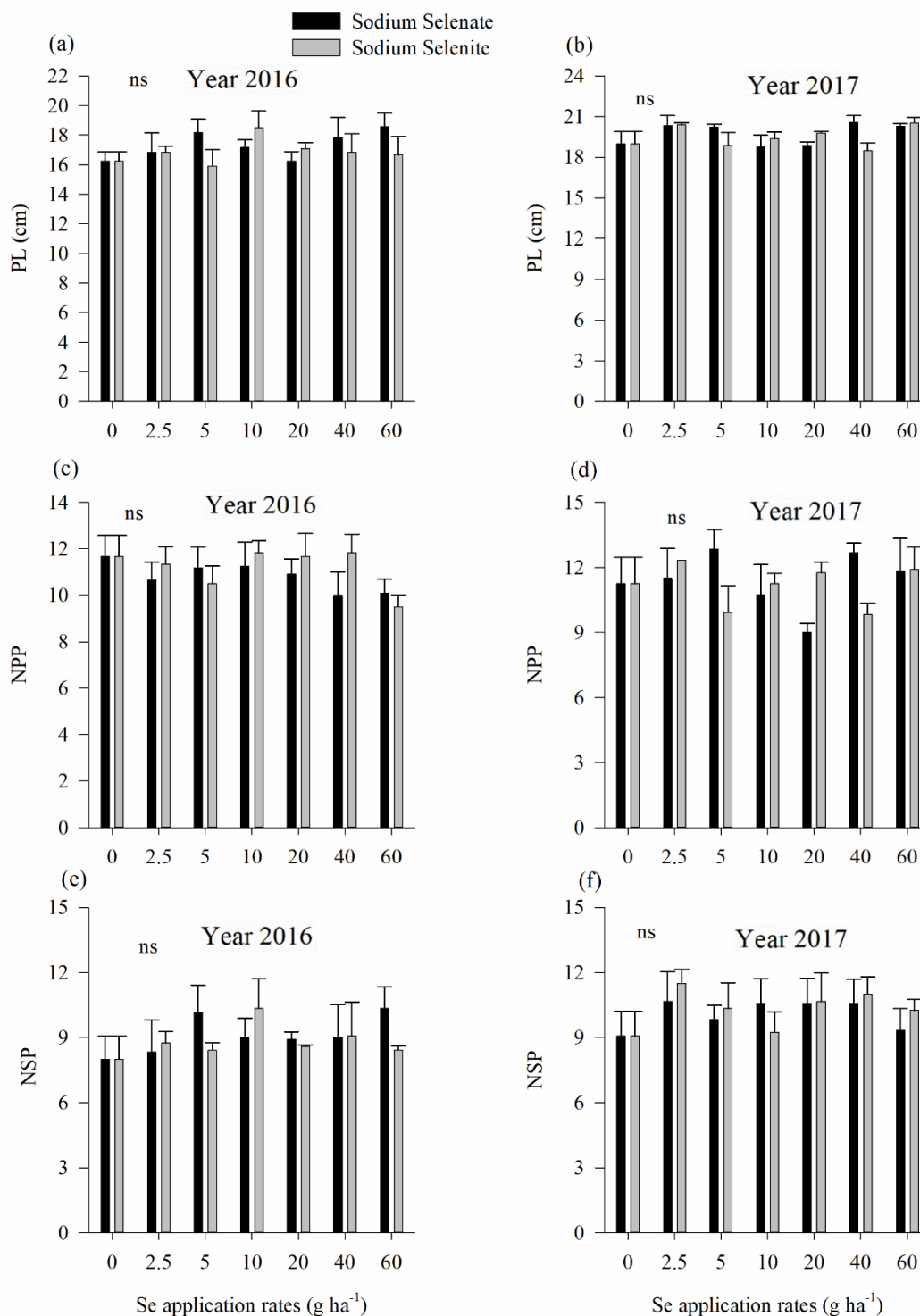
Seed PA concentration was affected by the different sources and levels of Se applied in the first and second experiments years (figure 3.6). In the first year, the application of selenate led to a greater PA concentration than selenite at application rates of 5, 40 and 60 g ha<sup>-1</sup>, and the application of selenite led to a higher seed PA concentration than selenate at application rates of 2.5 and 10 g ha<sup>-1</sup> (figure 3.6a). In the second year, the application of selenate provided a higher seed PA concentration than selenite at application rates of 2.5 and 40 g ha<sup>-1</sup> (figure 3.6b).

**Figure 3.2.** Yield in cowpea plants in the first (a) and second (b) year and shoot dry weight in first (c) and second (d) year of cultivation in response to levels of sodium selenate sodium selenite application. Error bars indicates the standard error of mean (n=4). ns = not significant. CV (%) = 23.82 (a); 34.06 (b); 15.13 (c) and 15.10 (d).



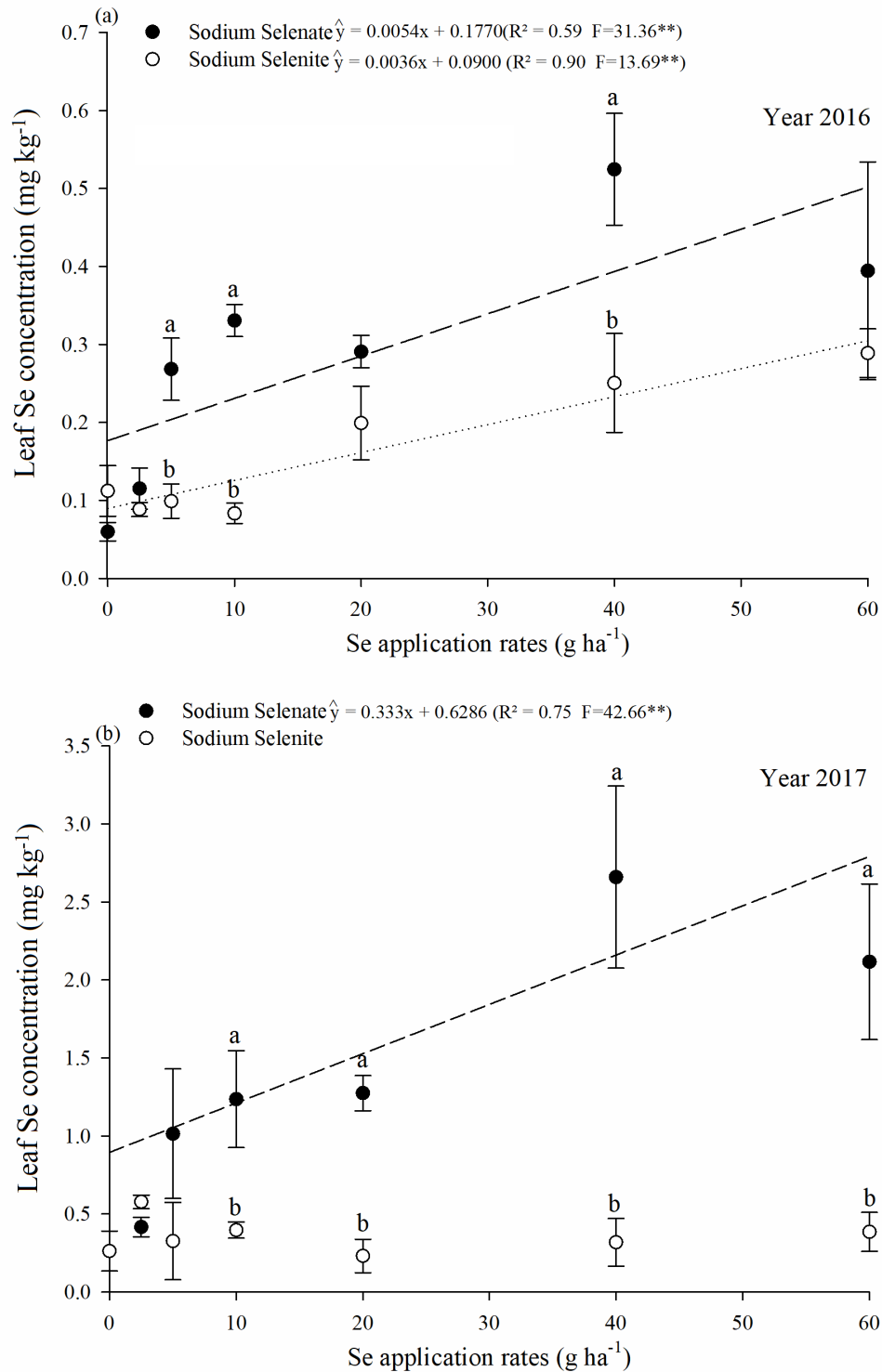
Source: Own authorship

**Figure 3.3.** Pod length in first (a) and second (b) year, number of pods per plant in first (c) and second (d) year and number of seeds per pod in first (e) and second (f) year of cowpea cultivation in response to levels of sodium selenate sodium selenite application. Error bars indicates the standard error of mean (n=4). ns = not significant according to tukey test ( $p \leq 0.05$ ). CV (%) = 11.18 (a); 6.03 (b); 15.07(c); 16.98(d); 23.33(e) and 19.33 (f).



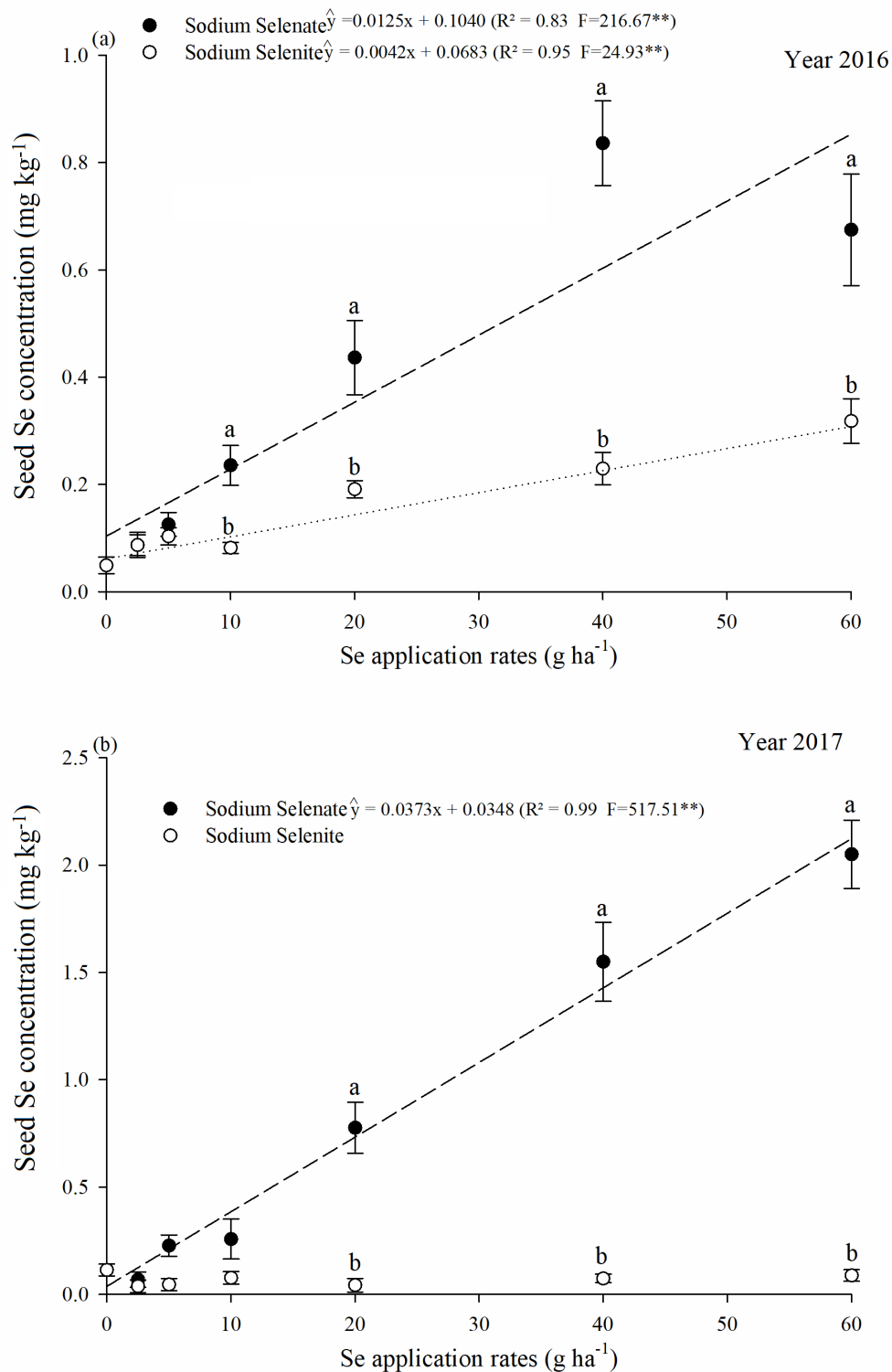
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**Figure 3.4.** Leaf Se concentration in the first (a) and second (b) year of cowpea cultivation under different doses of sodium selenate and sodium selenite. Error bars indicates the standard error of mean (n=4). Different letters indicate difference between means according to tukey test ( $p \leq 0.05$ ). CV (%) = 48.03 (a); 68.47 (b).



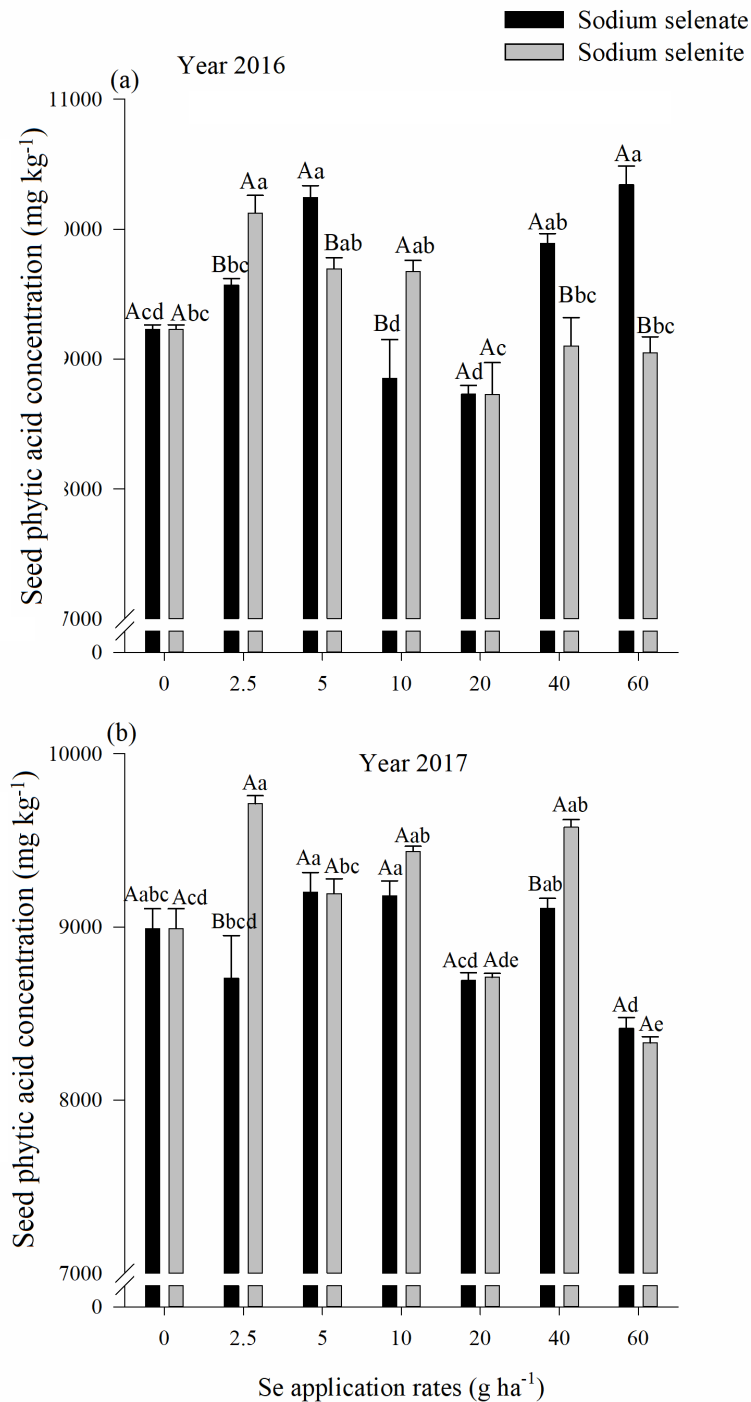
Source: Own authorship

**Figure 3.5.** Seed Se concentration in the first (a) and second (b) year of cowpea cultivation under different doses of sodium selenate and sodium selenite. Error bars indicates the standard error of mean (n=4). Different letters indicate difference between means according to tukey test ( $p \leq 0.05$ ). CV (%) = 37.29 (a); 42.68 (b).



Source: Own authorship

**Figure 3.6.** Seed phytic acid concentration in plants of the first (a) and second (b) year of cowpea cultivation in response to levels of sodium selenate sodium selenite application. Error bars indicates the standard error of mean (n=4). Different letters indicate difference between means according to tukey test ( $p \leq 0.05$ ). Uppercase letters correspond to Se sources, and lowercase letters correspond to Se doses. CV (%) = 2.68 (a); 1.74 (b).



Source: Own authorship

### 3.4 DISCUSSION

The application of Se resulted in a substantial increase in Se concentrations in leaves and seed of cowpea plants (figures 3.4-3.5). However, yield, shoot dry mass, pod length, number of pods per plant and numbers of seeds per pod were not affected by Se application (figures 3.2-3.3). A beneficial effect of Se on biomass, yield and other agronomic parameters is occasionally observed in plant subject to abiotic stress. For example, the application of Se has been shown to increase growth and productivity in salt stressed cowpea plants (MANAF, 2016). In other species, such as mungbean (*Vigna radiata* L. Wilczek), the application of Se to salt stressed plants has been shown to improve reproductive function (KAUR; NAYYAR, 2015). Salt stressed dill plants (*Anethum graveolens*) had greater shoot and seedling dry weight when Se was applied (SHEKARI; ABBASI, MUSTAFAVI, 2017), and the application of Se to wheat plants exposed to ultraviolet-B radiation resulted in increased spike length, weight per spike and yield (YAO et al., 2013). The beneficial effect of Se to stressed plants might be a consequence of its role in mitigating oxidative stress (WHITE, 2016b). In the present study, as plants were not exposed to stress, neither sodium selenate nor sodium selenite influenced the yield or biomass of cowpea.

In the present study, the application of Se led to greater Se concentrations in leaves and seed of cowpea than the application of selenite at the same rate (figures 3.4-3.5). This is consistent with many previous studies reporting that the application of selenate leads to greater tissue Se concentrations than the application of selenite at an equivalent rate, for example in shoots of Indian mustard, broccoli, pak choi (*Brassica chinensis* L.), sugarbeet, ryegrass and rice plants (ZAYED; LYTLE; TERRY, 1998; CARTES; GIANFREDI; MORA, 2005; EICHE et al., 2015; LI et al., 2015; ZAYED et al., 1998), in roots, stems, leaves and grain of wheat (ALI et al., 2017; EICHE et al., 2015) and in seeds of lentils (EKANAYAKE et al., 2015) and rapeseed (LIU et al., 2017).

In plants, the uptake, assimilation and translocation of selenate is faster than selenite (GUPTA; GUPTA, 2017). Indeed, only a minor fraction of the selenite taken up by roots is translocated from roots to shoots since it is readily converted into organic forms (LI et al., 2008; KIKKERT; BERKELAAR, 2013). The conversion of selenate into organic Se forms is slower, and this allows greater mobility in plants (LI; MCGRATH; ZHAO, 2008; NATASHA et al., 2018). Thus, the greater Se concentrations in leaves and seeds of cowpea plants receiving selenate rather than an equal application of selenite is likely to be explained, in part, by the greater translocation efficiency of selenate than selenite. The behavior of selenate and

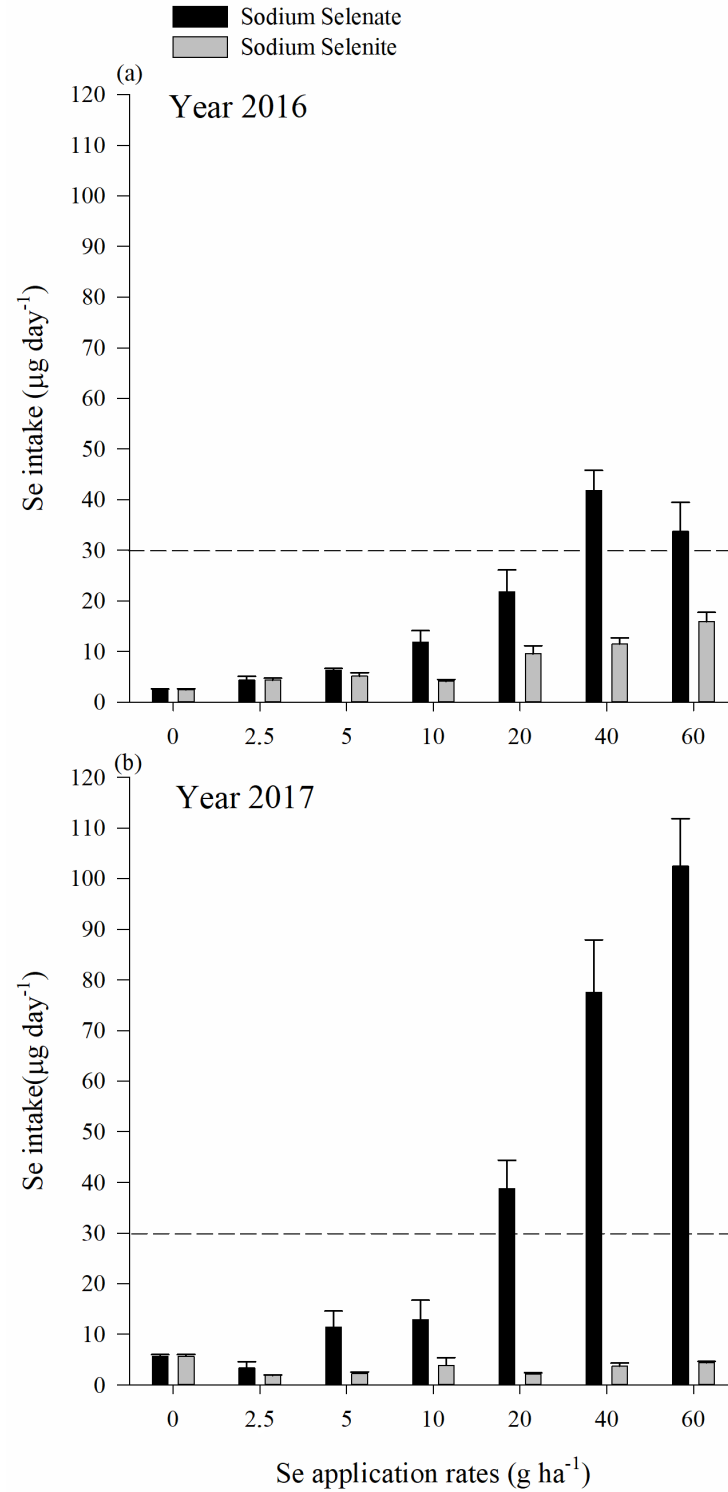
selenite in the soil might also contribute to the differences in tissue Se concentrations when equivalent amounts of selenate and selenite were applied. Selenite was more strongly adsorbed by the soil than selenate (figure 3.1) and is, therefore, less phytoavailable.

Wide variation in seed Se concentration was observed among the Se treatments and the two cultivation years, ranging from approximately 0.5 to 2 mg kg<sup>-1</sup> (figure 3.4). The recommended dietary allowance for adult humans is 55 µg day<sup>-1</sup> and the tolerable upper intake level is 400 µg day<sup>-1</sup> (INSTITUTE OF MEDICINE, 2000; FAIRWEATHER-TAIT et al., 2011). There is no daily intake recommendation set for cowpea, although some recommendations for legume intake have been made, ranging from 50 to 81 g day<sup>-1</sup> (Venter et al., 2013).

Brazilian eating habits vary widely due to social, economic and geographical differences, but foods such rice, cow milk and eggs are commonly consumed throughout the country. The mean Se concentrations in Brazilian rice, cow milk and eggs are 0.31, 0.08 and 0.21 µg g<sup>-1</sup>, respectively (LEMIRE et al., 2010; SANTOS et al., 2017). The daily intake of rice, cow milk and eggs in the northeastern region of Brazil are 142, 33 and 16 g, respectively (IBGE, 2009). Multiplying Se concentration by the daily consumption of each of these foods, the total Se daily intake from them is about 25 µg day<sup>-1</sup> per capita. Subtracting this value from the recommended dietary allowance of 55 µg Se d<sup>-1</sup>, it is possible to establish a guide for the daily Se intake provided by cowpea seeds of 30 µg d<sup>-1</sup>.

The consumption of cowpea beans varies widely. In African countries, consumption varies from 1.5 kg per capita per year in Senegal to 18 kg per capita per year in Nigeria (GÓMEZ; MEJÍA, 2004). In the northeastern region of Brazil, Se consumption per capita per year is 19.79 kg (FREIRE FILHO et al., 2017). which corresponds to 54.22 g of cowpea beans per day. The daily intake of Se from cowpea can be calculated by multiplying the daily cowpea consumption by the Se concentration in each treatment (figure 3.7).

**Figure 3.7.** Daily intake of Se that would be provided by a 54.2 g portion of cowpea seeds biofortified using sodium selenate or sodium selenite in the first (a) and second (b) year of trials. Dashed black line indicates the maximum intake recommendation range.



Source: Own authorship

Application rates of 40 and 60 g Se ha<sup>-1</sup> of sodium selenate in the first year, and 20, 40 and 60 g Se ha<sup>-1</sup> of sodium selenate in the second year would have provided a greater Se intake than the guide value of 30 µg Se d<sup>-1</sup> from consuming 54.20 g d<sup>-1</sup> cowpea seed.

Although phosphate and selenite can compete for uptake by plant roots (HOPPER; PARKER, 1999; LI et al., 2008b; SCHIAVION; PILON SMITHS, 2017; Zhang et al., 2014) the application of neither selenate nor selenite influenced P concentration in seeds (figure suppl. 3.3). Similarly, there was no obvious relationship between the PA concentration in seeds and the application of Se as either selenate or selenite (Figure 3.6). Furthermore, the concentrations in seeds of most elements to which PA might bind were unaffected by the application of selenate or selenite (figures suppl. 3.4 and 3.5). Thus, Se biofortification of cowpea seeds would not appear to impact negatively on the bioavailability of other human and animal nutrients.

### 3.5 CONCLUSION

The uptake of applied Se in cowpea plants is tightly dependent on Se source (selenate or selenite). Selenate showed lower soil adsorption than selenite in Brazilian soils, thus providing more available Se for plant uptake. Application of 10 g ha<sup>-1</sup> of Se as sodium selenate provided safe and beneficial seed Se concentrations, which have the capability to increase daily human Se intake to between 12.8 and 13.9 µg day<sup>-1</sup>. Phytic acid concentration in seeds showed no relation to Se application rate and source. Cowpea yield, and biomass variables were not affected by Se application rates. Considering the tight range of recommended Se intake levels in humans from 20 to 55 µg day<sup>-1</sup>, the present study provides relevant information concerning suitable agronomic biofortification practices for increasing cowpea seed Se concentrations at safe levels.

#### 4 CHAPTER 4. AGRONOMIC BIOFORTIFICATION AND GENOTYPIC VARIATION OF COWPEA WITH SELENIUM AND IMPACTS ON GRAIN NUTRITIONAL QUALITY

##### ABSTRACT

Selenium (Se) is a micronutrient to humans and animals. Agronomic biofortification of edible crops using fertiliser containing Se can be a good strategy to reduce Se deficiency worldwide. Cowpea (*Vigna unguiculata* (L.) Walp.) is an important protein source in developing countries. Therefore, studies regarding increasing Se concentration in edible parts of modern cultivars are required. This study aimed to evaluate the genotypic variation of cowpea genotypes to Se fertilisation and assess the impacts on phytic acid (PA), total sugars, sucrose, storage proteins and amino acids concentration in grains. A pot experiment was performed using 29 genotypes of cowpea plants in absence (control) and presence of Se (25 g ha<sup>-1</sup>) to evaluate the capacity to accumulate Se and their response to Se in terms of primary metabolism and nutritional quality. Sodium selenate application increased the Se concentration in roots, shoots and grains from all genotypes tested. At least twofold changes in total Se concentration in grains between genotypes with low and high Se concentration was found. The change in biological nitrogen fixation analyzed by ureide (allantoic acid), plant growth and grain storage proteins changed between genotypes with low and high Se. Selenium application increased the shoot ureide concentration in 9 genotypes, in grains, Se supply decreased PA concentration in 11 genotypes, and increased total sugar concentration in 2 genotypes, sucrose in 4 genotypes and amino acids in 10 genotypes. Regarding the storage protein in grains, Se application increased the albumin in 5 genotypes, globulin in 12 genotypes, prolamin in 6 genotypes and glutelin in 14 genotypes. This study provides valuable information for the effect of Se on plant growth and metabolism of cowpea genotypes. This information can be used in future breeding programs to select better genotypes for Se genetic biofortification in cowpea plants.

**Keywords:** *Vigna unguiculata* (L.) Walp. Ureides. Phytic acid. Storage protein. Sodium selenate.

## 4.1 INTRODUCTION

Foods is a primary source of nutrients and its demand grows with the increasing of worldwide population, the world population in 2014 was estimated at 7.3 billion (CAZALIS; LOREAU; HEDERSON; 2018) to provide food for a growing population with increase in consumption per person, the agricultural area (crops, arable land and permanent pastures) has expanded from 35.0 to 38.6% between 1960 and 2007, and in the same time, agricultural production increased almost three times (TILMAN et al., 2011; HOOKE; MARTÍN-DUQUE; PEDRAZA, 2012; ALEXANDRATOS; BRUINSMA, 2012; FAOSTAT, 2017; ; et al., 2018).

On the other hand, over 800 million people worldwide are still estimated to consume insufficient food to meet dietary energy requirements, suffering from malnutrition (FAO et al., 2017). About half of the world's population might be affected by malnutrition, mostly, teenagers, pregnant women and children in developing countries (RAYMAN, 2012, DAS et al., 2018). This happens in part due to plant breeding aiming to yield increasing, and thus, presenting an inverse relation with mineral concentration in seeds (GARVIN; WELCH; FINLEY, 2006; MURPHY; REEVES; JONES, 2008; WHITE et al., 2009). Selenium (Se) have a high risk of deficiency in humans, raking third micronutrient with most risks in Africa (JOY et al., 2014).

Selenium deficiency is estimated to be a problem for more than 1 billion people (COMBS, 2001; SCHIAVON; PILON-SMITS, 2017). To humans and other animals, Se is essential to metabolism (RAYMAN, 2012). Selenium is involved in thyroid function, the immune system and is also an antioxidant (FAIRWEATHER-TAIT et al., 2011). Selenium at low concentration is considered a beneficial trace element for plants, that can promote growth and help resist oxidative, biotic, and abiotic stress (WHITE, 2016; REIS et al., 2017a, 2017b). The concentrations of Se in agricultural food products depend on the Se availability in the soil (JOY et al., 2015) and also on the regulation in function of plant species/genotype. Although due to the few studies carried out, there are doubts about the agronomic significance of the variation/genotypic regulation in their accumulation in cultivated plants (LYONS et al., 2005). Selenium when supplied as selenate competes with sulphur (S) transporters, and when supplied as selenite competes with phosphate transporters in plants (REIS et al., 2017a).

Legume plants provide 2 µg of Se capita<sup>-1</sup> day<sup>-1</sup> (JOY et al., 2014), and cowpea (*Vigna unguiculata* (L.) Walp.) it's a legume plant with a role as a dietary protein and mineral source, complementing cereals, tuberous vegetables, and root vegetables in diets in developing

countries in Africa, Latin America, and Asia (PHILLIPS et al., 2003; MANZEKE et al., 2017). Due to its high nutritional value, adaptability, and versatility to different production conditions (EHLERS; HALL, 1997). And regarding the drought tolerance of the crop, it is suitable to hot arid regions (HALL, 2012).

Cowpea breeding programs usually focus on precocity, resistance to pests and diseases, storage, yields and grain physiological traits (ROCHA et al., 2007). Recently, breeders observed the necessity to include, in the breeding programs, genotypes with potential to increase the micronutrients concentration in cowpea, aiming to benefit human health by combating malnutrition (CARVALHO et al., 2012)

This study aimed to evaluate the potential of 29 cowpea genotypes in Se assimilation, as well as the influence of Se in the quality of cowpea grains, which is one of the most important sources of protein in Brazilian northeast region that shows the greatest rates of malnutrition in the country.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Experimental design

The experiment was conducted in a greenhouse at São Paulo State university (UNESP) in the municipality of TUPÃ-Brazil. The experiment was performed with 29 genotypes of cowpea (Table 4.1) cultivated in absence and (25 g Se ha<sup>-1</sup>). The experiment setup was completely randomized design, with three replicates, in a total of 174 pots.

The soil was collected and sieved to fill the pots with 5 dm<sup>3</sup> in December 2016. Soil chemical characteristics are as follows: pH (CaCl<sub>2</sub> 0.01 M) 4.6; phosphorus (resin) 6 mg dm<sup>-3</sup>; sulfur (calcium phosphate) 3 mg dm<sup>-3</sup>; potassium (resin) 0.9 mmolc dm<sup>-3</sup>; calcium (resin) 5 mmolc dm<sup>-3</sup>; magnesium (resin) 3 mmolc dm<sup>-3</sup>; H+Al (SMP buffer) 16 mmolc dm<sup>-3</sup>; cation exchange capacity 24.9 mmolc dm<sup>-3</sup>; base saturation 36%; boron (hot water) 0.07 mg dm<sup>-3</sup>; copper (DTPA) 0.5 mg dm<sup>-3</sup>; iron (DTPA) 11 mg dm<sup>-3</sup>; manganese (DTPA) 12 mg dm<sup>-3</sup>; zinc (DTPA) 0.2 mg dm<sup>-3</sup>. These chemical characteristics were determined as described by RAIJ et al. (1997) after the collection of composite sample of the soil used in the experiment. Natural Se concentration in soil was 45 µg dm<sup>-3</sup>. To determine Se concentration, 4 g of air-dried soil was added to a 50 mL centrifuge tube along with 20 mL of 0.01M KNO<sub>3</sub>. The solution was shaken in a rotary shaker for 2 hours, then centrifuged for 30 min at 3500 rpm. After centrifugation, 9 mL of the supernatant was pipetted into a < 0.22 µm

syringe filter. The supernatant was filtered into a tube containing 10% tetramethylammonium hydroxide (TMAH) and 0.1 M  $\text{KH}_2\text{PO}_4$  for determination of readily available Se using inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany).

**Table 4.1.** Cowpea genotypes used in this study.

| Genotype number | Genotype name     |
|-----------------|-------------------|
| 1               | BR17 Gurgeia      |
| 2               | BRS Tracuateua    |
| 3               | BRS Arace         |
| 4               | BRS Cauame        |
| 5               | BRS Guariba       |
| 6               | BRS Itaim         |
| 7               | BRS Juruá         |
| 8               | BRS Maratada      |
| 9               | BRS Milênio       |
| 10              | BRS Nova Era      |
| 11              | BRS PAGEU         |
| 12              | BRS Potengi       |
| 13              | BRS Rouxinol      |
| 14              | BRS Tumueumaque   |
| 15              | BRS Urubuquara    |
| 16              | BRS Xique xique   |
| 17              | California CBS    |
| 18              | Inhuma            |
| 19              | MNCO1 631 F 20 5  |
| 20              | MNCO4 769 F 62    |
| 21              | MNCO4 782 F 108   |
| 22              | MNCO4 792 F 143   |
| 23              | MNCO4 792F 146    |
| 24              | MNCO4 795F 158    |
| 25              | Patativa          |
| 26              | Paulistinha       |
| 27              | Pingo de Ouro 1 2 |
| 28              | Pingo de Ouro 2   |
| 29              | Pretinho          |

Source: Own authorship

Prior to sowing the cowpea seeds, each pot received 1,25 of lime aiming to pH correction, and application of urea ( $0.10 \text{ g pot}^{-1}$ ), KCl ( $0.17 \text{ g pot}^{-1}$ ), and single superphosphate ( $0.46 \text{ g pot}^{-1}$ ). The soil was incubated 30 days prior sown the seeds. The seeds were inoculated with a premium peat inoculum for cowpea (strain SEMIA 6462, product registration number SP 00581-10030-1,  $2.0 \times 10^9$  colony forming units  $\text{g}^{-1}$ , BIOMAX, São Joaquim da Barra city, Brazil), at  $8 \text{ g kg}^{-1}$  of seed. The inoculum was dissolved in a sugar solution (1 mL of water

per gram of inoculant, 10% sugar) and then added and mixed with the seeds. The sowing was performed on December, 29, 2016. Emergence began on January 5, 2017, 5 days after sowing (DAS). The pots were irrigated by a computerized irrigation system, each pot was irrigated five times daily for one minute each time, at a flow rate of 100 mL min<sup>-1</sup> via a low density polyethylene (LDPE) pipes.

#### *4.2.2 Procedure and sampling*

According to recommendation for cowpea cultivation, 29 days after emergence (DAE), each pot received 20 mL of a solution with urea (0.22 g pot<sup>-1</sup>), KCl (0.17 g pot<sup>-1</sup>), and single superphosphate (0.45 g pot<sup>-1</sup>). The same fertilization was made at 36 DAE and 61 DAE. At 44 DAE the treatments of 25 g ha<sup>-1</sup> of Se were applied in the pots.

The harvest was performed in different days for each genotype according to the pods maturity. The material was separated in grains, shoot and roots. Root material was washed with deionized water. The number of grains, pods and pod length in each experimental plot was also registered. Each part of cowpea plants was dried in oven at 40 °C to a constant mass to estimate the grains dry weight, shoot dry weight and roots dry weight. After dry mass measurement, the sample material was grounded in a Wiley mill for chemical analysis.

#### *4.2.3 Digestion and nutritional analysis of roots, shoots and grains*

All samples (root, shoot and grain) materials were weighted at approximately 0.20 g dry weight (DW) and digested in digestion tubes with 2 mL 70 % Trace Analysis Grade HNO<sub>3</sub>, 1 mL Milli-Q water (18.2 MΩ cm; Fisher Scientific UK Ltd, Loughborough, UK), and 1 mL H<sub>2</sub>O<sub>2</sub>. The digestion occurred in a microwave system comprising a Multiwave 3000 platform with a 48-vessel MF50 rotor (Anton Paar GmbH, Graz, Austria); digestion vessels were perfluoroalkoxy (PFA) liner material and polyethylethylketone (PEEK) pressure jackets (Anton Paar GmbH). The settings for digestion were: power = 1400 W, temp = 140 °C, pressure = 2 MPa, time = 45 min. In each digestion, two operational blanks were included, and two replicates of certified reference material (CRM) were included every three runs, there CRM used for roots, shoots end leaves runs was tomato leaves (SRM 1573a, NIST, Gaithersburg, MD, USA), for grains runs, the CRM used was wheat flour (SRM 1567b, NIST, Gaithersburg, MD, USA). After digestion, in each tube was added 11 mL of milli-Q

water (final volume of 15 mL), the digestates was transferred to a 25 mL universal tube (Sarstedt Ltd., Nümbrecht, Germany) and stored at room temperature.

Prior to elemental analysis, the digestates were diluted 1-in-10 using milli-Q water. The concentrations of 31 elements were obtained using inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany); Ag, Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, Tl, U, V and Zn. Operational modes included: (i) a helium collision-cell (He-cell) with kinetic energy discrimination to remove polyatomic interferences, (ii) standard mode (STD) in which the collision cell was evacuated, and (iii) a hydrogen collision-cell (H<sub>2</sub>-cell). Samples were introduced from an autosampler incorporating an ASXpress™ rapid uptake module (Cetac ASX-520, Teledyne Technologies Inc., Omaha, NE, USA) through a PEEK nebulizer (Burgener Mira Mist, Mississauga, Burgener Research Inc., Canada). Internal standards were introduced to the sample stream on a separate line via the ASXpress unit and included Sc (20 µg L<sup>-1</sup>), Rh (10 µg L<sup>-1</sup>), Ge (10 µg L<sup>-1</sup>) and Ir (5 µg L<sup>-1</sup>) in 2 % trace analysis grade HNO<sub>3</sub> (Fisher Scientific UK Ltd). External multi-element calibration standards (Claritas-PPT grade CLMS-2; SPEX Certiprep Inc., Metuchen, NJ, USA) included Ag, Al, As, B, Ba, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti (semi-quant), U, V and Zn, in the range 0–100 µg L<sup>-1</sup> (0, 20, 40, 100 µg L<sup>-1</sup>). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, Courtaboeuf, France) was used to create Ca, K, Mg and Na standards in the range 0–30 mg L<sup>-1</sup>. Boron, P and S calibration utilized in-house standard solutions (KH<sub>2</sub>PO<sub>4</sub>, K<sub>2</sub>SO<sub>4</sub> and H<sub>3</sub>BO<sub>3</sub>). In-sample switching was used to measure B and P in STD mode, Se in H<sub>2</sub>-cell mode and all other elements in He-cell mode. Sample processing was undertaken using Qtegra™ software (Thermo Fisher Scientific) with external cross-calibration between pulse-counting and analogue detector modes when required.

#### *4.2.4 Extraction and phytic acid analysis of grains*

To determine phytic acid (PA) concentrations of seed samples, approximately 1.0 g DW seed subsamples were weighed and placed into 50 mL conical tubes (SARSTEDT). To each tube, 20 mL of 0.66 M HCl was added, and tubes were put in a rotary shaker to shake overnight at 25 rpm. After this, 1 mL of the homogenized solution was transferred to 2 mL safe lock microtubes (Eppendorf, Hamburg, Germany) and centrifuged at 13,000 rpm for 10 minutes. After centrifugation, 0.5 mL of the supernatant was transferred to a new 2 mL safe

lock microtubes and neutralized with 0.5 mL of 0.75 M NaOH. This extract was used to perform an enzymatic dephosphorylation reaction, in order to estimate PA concentration from the difference between total phosphorus and free phosphorus assayed using the Phytic Acid (Total Phosphorus) Assay Kit (Megazyme) following the manufacturer's instructions.

The absorbance of phosphomolybdate was read in 96 well plate readers (Thermo Scientific, Waltham, MA, USA) at 650 nm in an EL808 absorbance reader (BIOTEK). One operational blank (ultrapure water), one oat flour control and one phosphorus calibration curve (0, 0.5, 2.5, 5 and 7.5  $\mu\text{g mL}^{-1}$ ), were included in each plate.

#### *4.2.5 Total sugar and sucrose concentration*

The total sugar, sucrose and total amino acids concentrations were determined from 0.5 g of milled grain samples extracted in 10 mL of MCW solution (60% methanol, 25% chloroform, and 15% water) and prepared for analyses as described by BIELESKI; TURNER (1966). In a 15 mL conical bottom polystyrene tube (Len<sup>TM</sup>), the material was homogenized by vortexing and then centrifuged in a refrigerated centrifuge at 10,000 rpm for 10 minutes at 4 °C. To another tube was added 5 mL of MCW extract supernatant, 1.25 mL of chloroform and 1.875 mL of water. After a waiting period of 48 hours for phases separation, aliquots from the hydrophilic portion of the extract were used to total sugar, sucrose, and amino acid determination.

Total sugar was quantified according to the protocols in DUBOIS et al. (1956), 20  $\mu\text{L}$  of hydrophilic portion of MCW extract, 500  $\mu\text{L}$  of 5% phenol and 2 mL of concentrated sulphuric acid were added to a glass tube. The mixture was homogenized by vortexing, and after cooling to room temperature, the absorbance at 490 nm was read using a spectrophotometer (SP-220, biospectro<sup>TM</sup>). The results were expressed as  $\text{mg g}^{-1}$  DW.

Sucrose was quantified according to VAN HANDEL (1968), 20  $\mu\text{L}$  of extract, 500  $\mu\text{L}$  of 30% KOH and 2 mL of concentrated  $\text{H}_2\text{SO}_4$  were added to a glass tube. The mixture was homogenized by vortexing and oven-dried at 100 °C for 10 minutes. After cooling to room temperature, the absorbance at 490 nm was read using a spectrophotometer (SP-220, biospectro<sup>TM</sup>). The results for sucrose were expressed as  $\text{mg g}^{-1}$  DW. To quantify both sugar and sucrose, a standard sucrose curve was used.

#### 4.2.6 Amino acids concentration

Amino acids were quantified according to YEMM; COCKING; RICKETTS (1955), 250  $\mu$ L extract aliquot was added to 500  $\mu$ L sodium citrate 0.2M, 200  $\mu$ L ninhydrin 5% in ethylene glycol and 1 mL KCN 0.0002M. The glass tubes were mixed by vortexing and heat at 100 °C for 15 min. Afterwards, the tubes were cooled at tap water for 10 min. Finally, 1 mL of 60% ethanol was added into glass tubes, mixed by vortexing, the absorbance at 570 nm was read using a spectrophotometer (SP-220, biospectro™). A methionine standard curve was used to calculate the concentration of grain amino acids and the results were expressed as g g<sup>-1</sup> DW.

#### 4.2.7 Storage proteins concentration

The storage proteins were analysed in four different fractions: albumin, globulin, prolamin and glutelin. The quantification of storage protein in each extract was performed according to BRADFORD (1976). From 0.25 g of grain samples, was performed an extraction with 5 ml of deionized H<sub>2</sub>O in a 15 mL conical bottom polystyrene tube (Len™), the material was homogenized by vortexing and then centrifuged in a refrigerated centrifuge at 10,000 rpm for 5 minutes at 4 °C. After centrifugation, 20  $\mu$ L of extract was added to 1 ml of Bradford solution, in 2 ml safe lock microcentrifuge tubes (Len™). The microtubes were mixed by vortexing, and then, the absorbance at 595 nm was read using a spectrophotometer (SP-220, biospectro™) for determination of albumin concentration.

The H<sub>2</sub>O extract was disposed, but the grains sample within the tube was kept, in which 5 mL of NaCl 5% was added, the material was homogenized by vortexing and then centrifuged in a refrigerated centrifuge at 10,000 rpm for 5 minutes at 4 °C. After centrifugation, 20  $\mu$ L of extract was added to 1 ml of Bradford solution, in 2 ml safe lock microcentrifuge tubes (Len™). The microtubes were mixed by vortexing, and then, the absorbance at 595 nm was read using a spectrophotometer (SP-220, biospectro™) for determination of globulin concentration.

The NaCl 5 % extract was disposed, but the grains sample within the tube was kept, in which 2.5 mL of ethanol 60% was added, the material was homogenized by vortexing and then centrifuged in a refrigerated centrifuge at 10,000 rpm for 5 minutes at 4 °C. After centrifugation, 40  $\mu$ L of extract was added to 1 ml of Bradford solution, in 2 ml safe lock microcentrifuge tubes (Len™). The microtubes were mixed by vortexing, and then, the

absorbance at 595 nm was read using a spectrophotometer (SP-220, biospectro™) for determination of prolamin concentration.

The ethanol 60% extract was disposed, and the precipitate was resuspended in 5 mL of NaOH 0.4%. The tube was homogenized by vortexing and then centrifuged in a refrigerated centrifuge at 10,000 rpm for 5 minutes at 4 °C. After centrifugation, 20 µL of extract was added to 1 ml of Bradford solution, in 2 ml safe lock microcentrifuge tubes (Len™). The microtubes were mixed by vortexing, and then, the absorbance at 595 nm was read using a spectrophotometer (SP-220, biospectro™) for determination of glutelin concentration.

A bovine serum albumin standard curve was used to calculate the concentration of grains storage proteins and the results were expressed as g g<sup>-1</sup> DW.

#### *4.2.8 Ureide concentration*

For ureides determination, 0.3 g of shoot samples was extracted in 10 mL of MCW solution (60% methanol, 25% chloroform, and 15% water). In a 15 mL conical bottom polystyrene tube (Len™), the material was homogenized by vortexing and then centrifuged in a refrigerated centrifuge at 10,000 rpm for 10 minutes at 4 °C. To another tube was added 4 mL of MCW extract supernatant, 1 mL of chloroform and 1.5 mL of water. After a waiting period of 48 hours for phases separation, aliquots from the hydrophilic portion of the extract were used to perform quantification of ureides according to VOGELS; VAN DER DRIFT (1970). To determinate ureides (allantoic acid), 250 µL of extract, 250 µL of NaOH 0.5M and 20 µL of 0.33% phenylhydrazine were added to a glass tube. The mixture was homogenized by vortexing and oven-dried at 100 °C for 8 minutes. After cooling to room temperature, it was added 250 µL of HCl 0.65 N, the mixture was again homogenized by vortexing and oven-dried at 100 °C for 4 minutes. After another cooling to room temperature, it was added 250 µL of phosphate buffer solution 0.4 M, pH 7.0 and 250 µL of 0.33% phenylhydrazine. The mixture was homogenized and kept at room temperature by 5 minutes, then cooled in ice by 5 more minutes. Then, it was added 1.25 mL of 37% HCl and 250 µL of 1.65% K<sub>3</sub>Fe(CN)<sub>6</sub>. The mixture was homogenized by vortexing. Finally, after 15 minutes waiting at room temperature, ureides were determined by reading in spectrophotometer (SP-220, biospectro™) at absorbance at 535. An allantoin standard curve was used to calculate the concentration of shoot ureides, and the results were expressed as nmol g<sup>-1</sup> DW.

#### 4.2.9 Statistical analysis

The results were first submitted to Anderson-Darling normality tests; homogeneity was evaluated with Leven's test and with a variance analysis (F test). Differences between treatments were compared using a Scott-Knott test at 5% probability. Analyses were conducted using Agroestat and Minitab softwares, and graphs were prepared using SigmaPlot 12.5.

### 4.3 RESULTS

Regarding the root dry weight (figure 4.1, table suppl. 4.1) it was observed that, in genotype 4, Se application increased the root dry weigh, and in genotypes 8 and 19, Se application decreased the root dry weigh. Root dry weigh changes were also observed between genotypes with low and high Se, which genotypes 13, 5, 20, 1, 2, 21, 6, 29, 8, 4 and 7 presented higher root dry weigh. For shoot dry weight, grains dry weight, grains number, pod number and pod length, no effect of Se was observed (figure 4.1 and suppl. 4.2), although, genotypes variations occurred. Genotypes 12, 26, 17, 21, 19, 24, 6, 23, 8, 25 and 4 presented higher shoot dry weight (figure 4.2, table suppl. 4.1); genotypes 15, 6, 24, 12, 26, 10, 27, 19, 4, 25 and 23 presented higher grain dry weight (figure 4.3, table suppl. 4.1). The higher grain number were observed in genotypes 16, 26, 29, 21, 3, 6, 8, 24, 4, 19, 25 and 23 (figure 4.4, table suppl. 4.2), in genotypes 24, 4, 15, 25, 23, 10, 6 and 17, higher pod numbers were observed (figure 4.5, table suppl. 4.2), and genotypes 19, 24, 21, 4, 10, 6, 25, 23 and 17 presented the higher pod length (figure 4.6, table suppl. 4.2).

Selenium application increased Se concentration in roots, shoots and grain of all cowpea genotypes tested (Figures 4.7, 4.8 and 4.9), respectively. Selenium concentration in root increase range was from 0.3 up to 1.3 mg Se kg<sup>-1</sup>. Genotype 18 presented the lowest Se increase in roots (0.3 mg kg<sup>-1</sup>), and genotype 28, the highest difference (1.3 mg kg<sup>-1</sup>) between genotypes with low and high Se. Shoot Se concentration increase range was from 0.3 up to 1.2 mg Se kg<sup>-1</sup>. The lowest shoot Se increase (0.3 mg kg<sup>-1</sup>) was observed in genotype 19. The highest Se concentration in shoots was observed in genotype 5, showing an increase of 1.2 mg Se kg<sup>-1</sup> compared to the untreated genotype. Selenium concentration range in grains was from 0.5 up to 2.4 mg Se kg<sup>-1</sup>. The lowest increase in Se concentration was observed in genotype 16 (0.5 mg kg<sup>-1</sup>), the highest Se concentration in grains was observed in genotype 5, with an increase of 2.4 mg kg<sup>-1</sup> (figure 4.9). There was no difference on Se concentration among all

genotypes untreated with Se. On the other hand, a great genotypic variation was observed to Se concentration in roots, shoots and grains from all genotypes biofortified with Se (Figures 4.7, 4.8 and 4.9), respectively.

Regarding Se concentration in roots from biofortified genotypes, three groups were observed according to scott knott test (figure 4.7, table suppl. 4.3). Group a, comprised by genotypes 13, 24 and 28, which presented mean Se concentration in roots equal or higher than  $1.44 \text{ mg kg}^{-1}$ . Genotypes 15, 6, 25, 11, 14, 12 and 17 comprises group b and presented mean Se concentration in roots between  $1.06$  and  $1.33 \text{ mg kg}^{-1}$ . The other genotypes comprise group c, in which, mean Se concentration ranged from  $0.55$  up to  $0.95 \text{ mg kg}^{-1}$  (figure 4.7).

Four groups were observed according to scott knott test (figure 4.8, table suppl. 4.3), regarding Se concentration in shoots of cowpea plants biofortified with Se. Group a, formed by genotypes 12 and 5 showing Se concentration in shoots equal or higher than  $1.18 \text{ mg kg}^{-1}$ . Group b, showing shoot Se concentration ranging from  $0.68$  up to  $0.89 \text{ mg kg}^{-1}$  and was comprised by genotypes 13, 17, 1, 3, 28, 24, 14 and 4. Group c, comprised by genotypes 10, 29, 7, 25, 26, 2, 18, 15, 23 and 11, in which, mean Se concentration ranged from  $0.47$  up to  $0.64 \text{ mg kg}^{-1}$ . Finally, group d showing Se concentration in shoots ranging from  $0.20$  up to  $0.39 \text{ mg kg}^{-1}$  and being comprised by genotypes 27, 21, 9, 22, 8, 19, 16, 6 and 20 (figure 4.8).

For Se concentration in grains of cowpea plants biofortified with Se, five groups were observed according to scott knott test (figure 4.9, table suppl. 4.3). Presenting a mean Se concentration in grains of  $2.64 \text{ mg kg}^{-1}$  only genotype 5 belongs to group a. Group b was comprised by genotypes 11, 12 and 24, showing a mean Se concentration in grains ranging from  $1.84$  up to  $1.92 \text{ mg kg}^{-1}$ . Group c was comprised by genotypes 2, 28, 15, 26, 3, 25, 1, 23 and 14, presenting a mean Se concentration in grains ranging from  $1.29$  up to  $1.70 \text{ mg kg}^{-1}$ . Genotypes 8, 17, 6, 18, 13, 29 and 4 belongs to group d, and presented a mean Se concentration in grains ranging from  $0.95$  up to  $1.16 \text{ mg kg}^{-1}$ . Group e, which is comprised by genotypes 16, 9, 21, 22, 27, 20, 10, 19, and 7 presented a mean Se concentration in grains ranging from  $0.55$  up to  $0.82 \text{ mg kg}^{-1}$  (figure 4.9).

The highest PA concentration in grains from untreated cowpea plants was observed in genotypes 7 and 15, whereas genotypes 20, 19, 17 and 18 presented the lowest PA concentration. In biofortified plants, genotypes 16, 5, 29, 23, 7 and 15 showed the highest PA concentration. On the other hand, genotype 9 showed the lowest PA concentration in biofortified plants (figure 4.10, table suppl. 4.4). Selenium application decreased PA concentration in grains of genotypes 1, 3, 4, 7, 8, 9, 13, 15, 21, 24, and 27; and increased PA concentration in genotypes 2, 10, 11, 12, 16, 17, 19, 23 and 26 (table suppl. 4.4).

The highest concentration of total sugar in grains from untreated plants was observed in genotypes 24, 16, 5, 1, 17, 10, 21, 3 and 6. Unlikely, genotype 1 showed the highest, and genotypes 9, 23, and 25 presented the lowest PA concentration in biofortified plants (figure 4.11, table suppl. 4.4). Se application increased total sugar concentration in grains in genotypes 1 and 2, and decreased it in grains of genotypes 3, 6, 9, 11, 12, 17, 22, 23, 25 and 29 (table suppl. 4.4).

For sucrose concentration in grains, the highest concentration in control plants were observed in genotype 8, and the lowest, in genotypes 12, 25, 22, 18, 6 and 11. Among biofortified plants, genotype 10 showed the highest sucrose concentration, and genotypes 17, 22, 26, 25, 23, 27, 18, 12 and 13 the lowest concentration in grains (figure 4.12, table suppl. 4.4). Selenium application increase sucrose concentration in grains of genotypes 1, 9, 10 and 15, and decreased sucrose concentration in genotypes 3, 8, 13, 17, 23, 26, 27 (table suppl. 4.4).

Albumin concentration in grains of untreated cowpea plants was higher in genotype 25, and the lowest in genotypes 4, 19 and 16. For biofortified plants, genotype 2, 7, 21, 26 and 25 presented the highest and genotypes 13 and 20 the lowest albumin concentration (figure 4.13, table suppl. 4.5). Selenium application increased albumin concentration in grains of genotypes 2, 4, 16, 19, 24 and 29 and decreased albumin concentration in genotypes 1, 3, 5, 8, 11, 12, 13, 14, 17, 20, 22, 25, 27 and 28 (table suppl. 4.5).

The highest globulin concentration in grains from untreated cowpea plants was observed in genotype 21, and the lowest, in genotypes 1 and 6. For biofortified plants, genotype 5 showed higher and genotype 11 lower globulin concentration (figure 4.14, table suppl. 5). Selenium application increased globulin concentration in genotypes 1, 2, 4, 5, 6, 8, 10, 12, 13, 18, 20, 22, 24, 28 and 28, and decreased globulin concentration in genotypes 7, 9, 11, 16, 17, 21, 23 and 27 (table suppl. 4.5).

Prolamin concentration in grains from untreated cowpea plants were higher in genotype 16, 9, 7, 25, 18, 3, 15, 1 and 28, and lower in genotypes 23 and 21. For biofortified cowpea plants, genotype 28 showed higher, and genotypes 24, 21, 15, 8, 19 and 6 showed lower prolamin concentration (figure 4.15, table suppl. 4.5). Selenium application increased prolamin concentration in genotypes 2, 12, 13, 20, 22, 23, 24, 28 and 29, and decreased prolamin concentration in genotypes 1, 7, 9, 14, 15 and 18 (table suppl. 4.5).

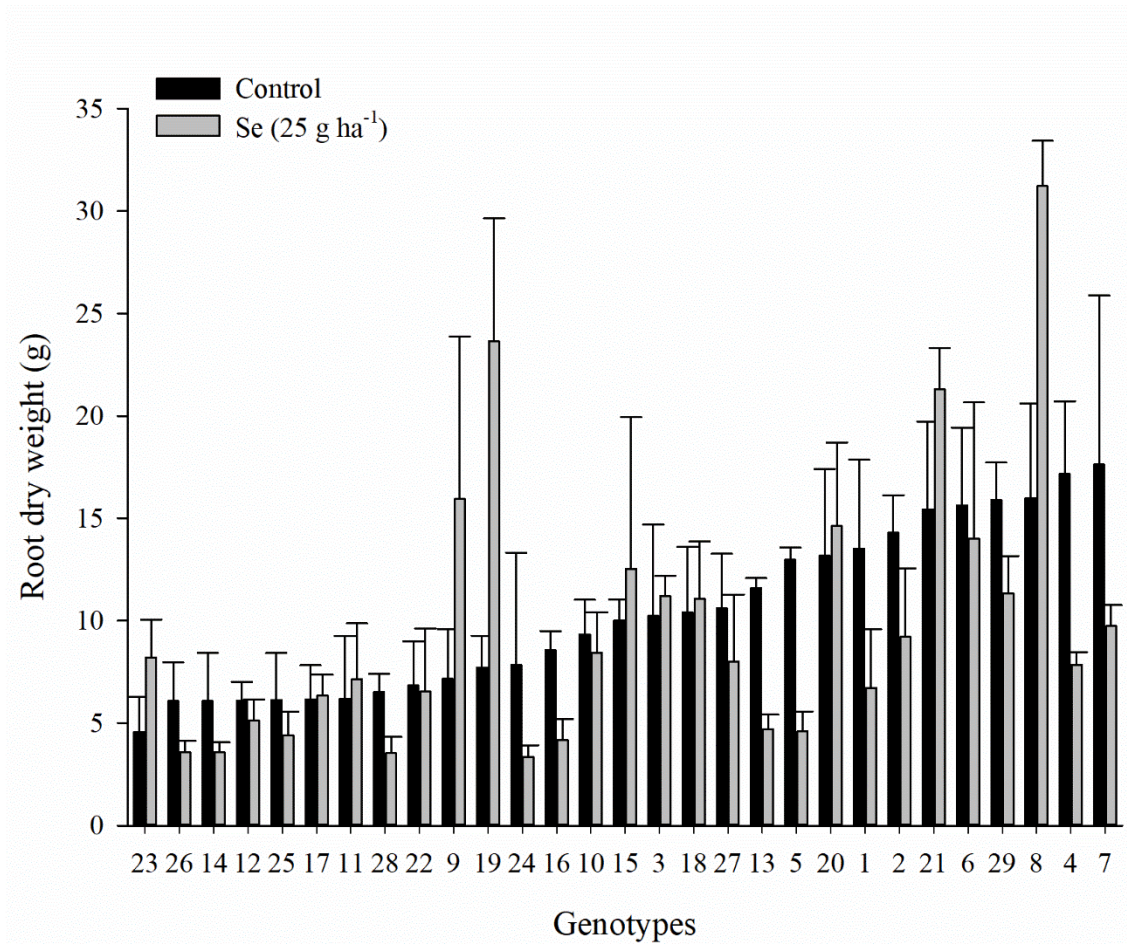
Among the untreated cowpea plants, genotype 7 showed the highest glutelin concentration in grains, and genotypes 17, 29, 9, 2, 4, 13, 5, 21, 23 and 25 showed lower concentration. For biofortified plants, genotypes 14 and 20 showed the highest glutelin

concentration, and genotypes 8, 16, 10, 23, 25, 19, 17, 18, 15, 24, 26, 2 and 6 showed lower concentration (figure 4.16, table suppl. 4.5). Selenium application increased gutelin concentration in grains from genotypes 3, 4, 5, 9, 12, 13, 14, 20, 21, 22, 27 and 29, and decreased glutelin concentration in grains from genotypes 7, 8, 11, 16, 18, 19 and 24 (table suppl. 4.5).

Among untreated cowpea plants, genotype 14 showed higher and genotypes 3, 7, 1, 22, 6, 23, 2, 5, 17, 25 showed lower total amino acids concentration in grains. However, for biofortified plants, genotypes 9 and 26 showed higher and genotypes 15, 10, 3, 7, 2, 6, 11 showed lower amino acids concentration in grains (figure 4.17, table suppl. 4.6). Selenium application increased amino acids concentration in grains of genotypes 1, 9, 12, 16, 19, 22, 23, 24, 27 and 28, and decreased amino acid concentration in genotypes 10, 11, 14, 15, 18, 21 and 26 (table suppl. 4.6).

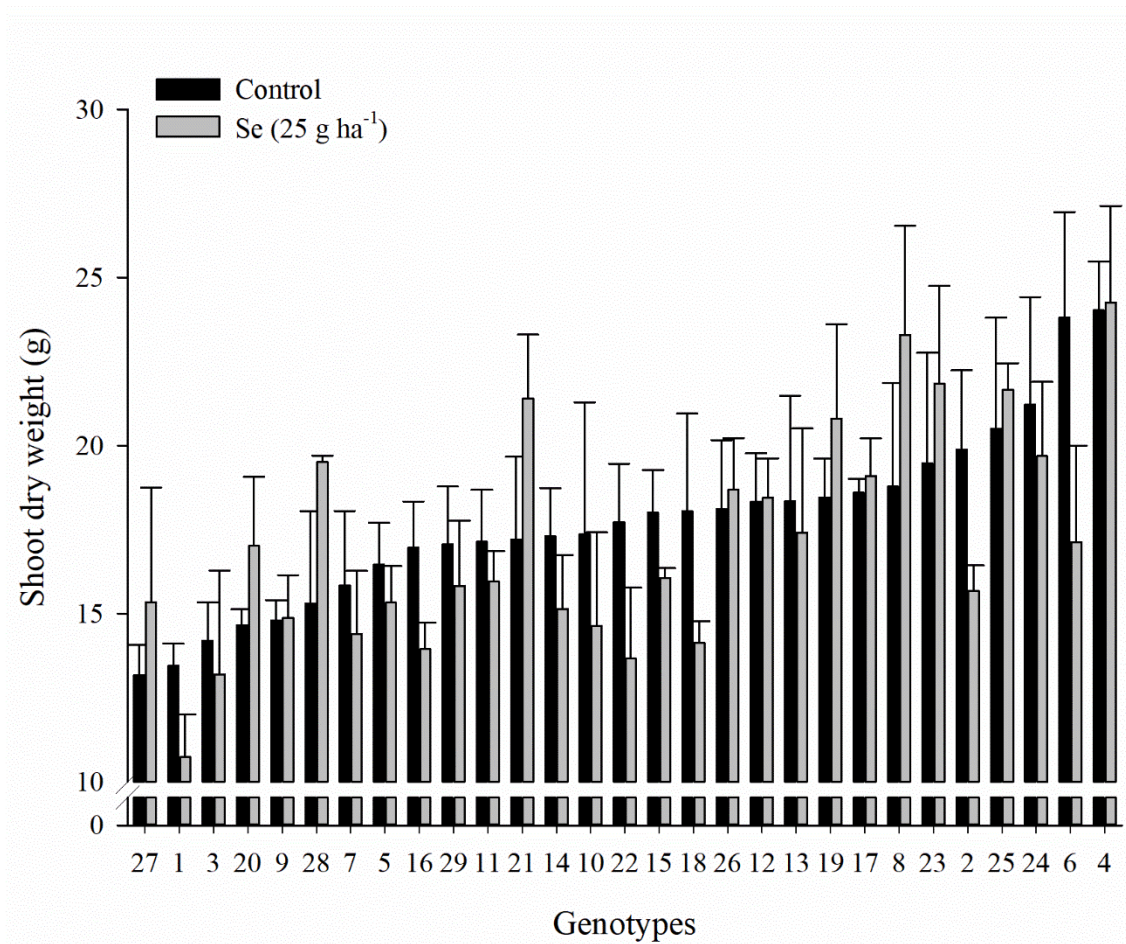
Total ureide concentration in shoots from untreated cowpea plants was higher in genotypes 1 and 21, and lower in genotypes 11, 3, 15, 4, 29, 28, 18, 24 and 17. Among the biofortified plants, genotype 21 showed higher and genotypes 29, 6, 28, 16, 18, 23, 4, 24, 19, 17, 3 and 15 showed lower shoot ureide concentration (figure 4.18, table suppl. 4.6). Se application increased ureide concentration in shoots of genotypes 2, 3, 8, 10, 11, 12, 15, 21 and 27, and decreased ureide concentration in shoots of genotypes, 1, 9, 13 and 20 (table suppl. 4.6).

**Figure 4.1.** Root dry weight of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 55.28



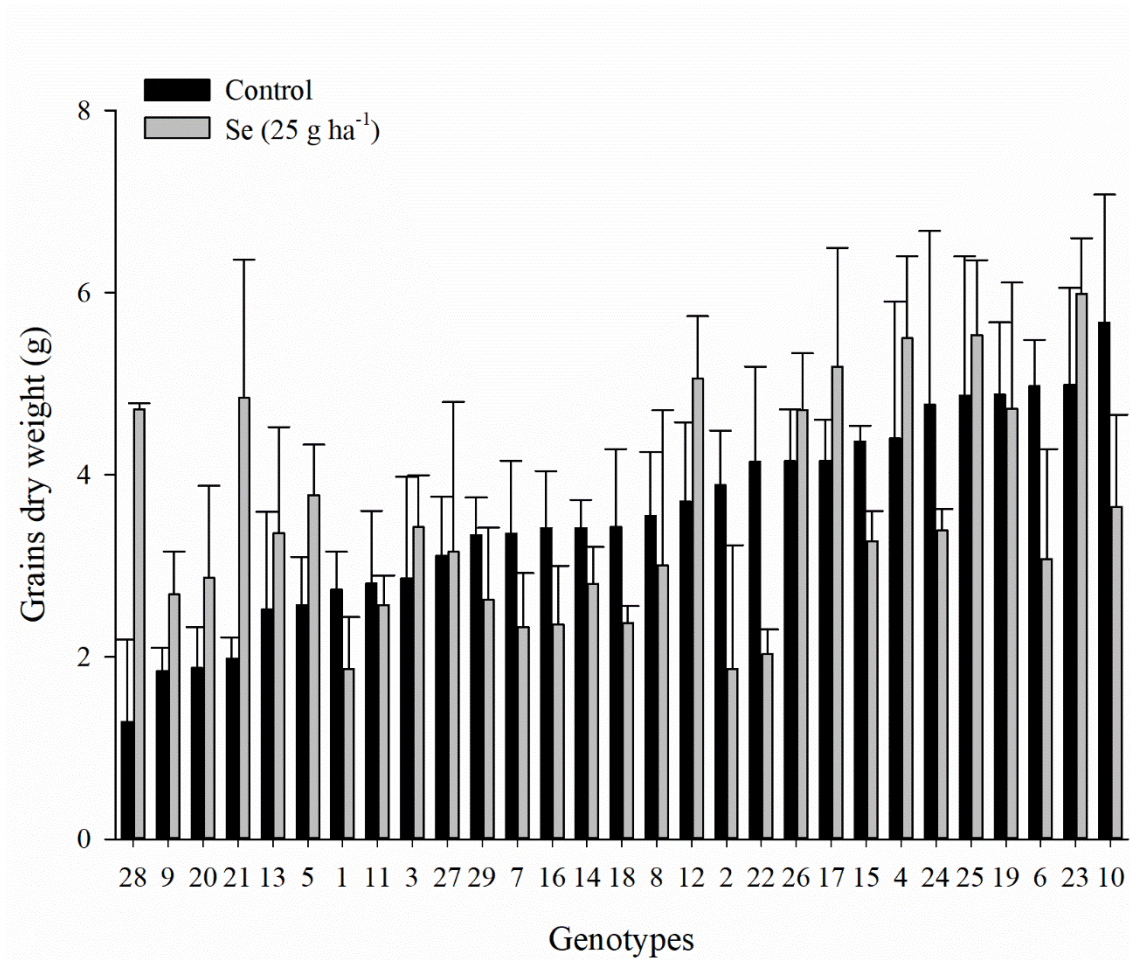
Source: Own authorship

**Figure 4.2.** Shoot dry weight of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 20.97



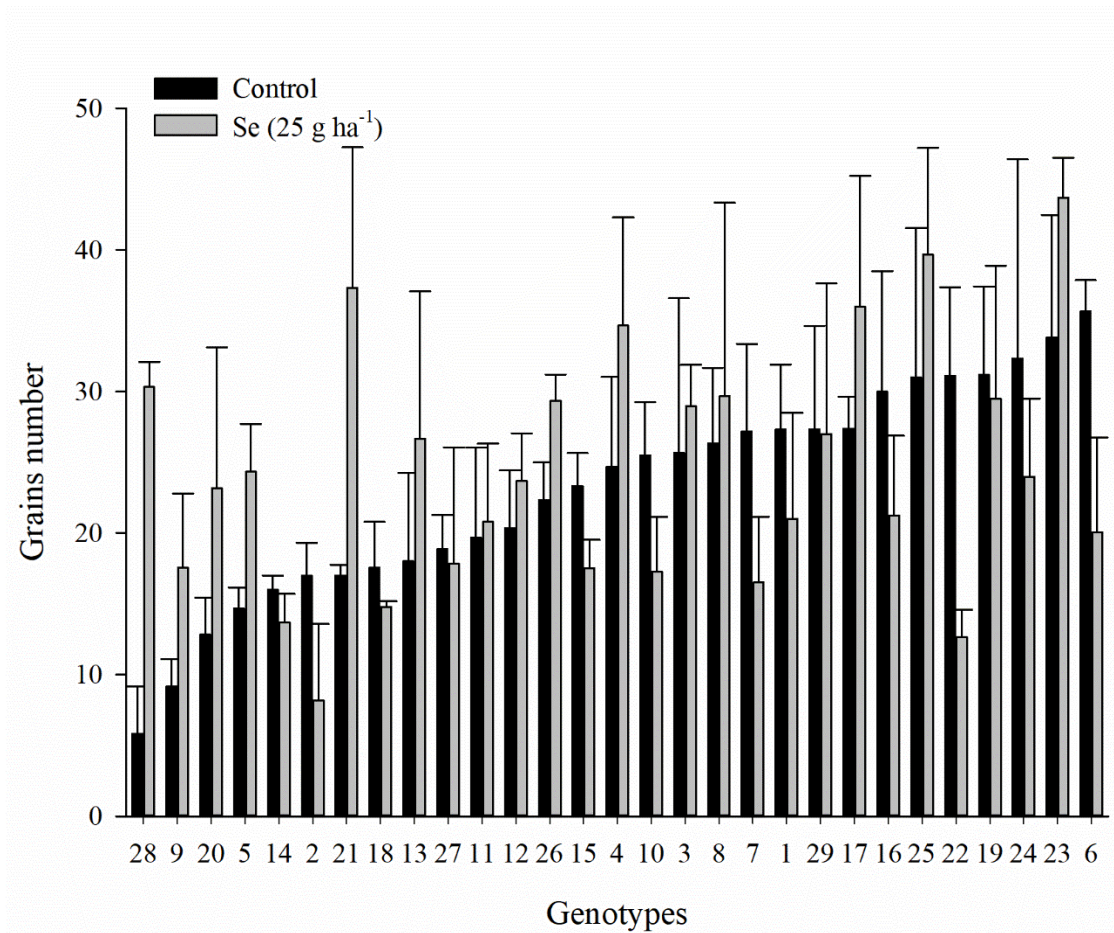
Source: Own authorship

**Figure 4.3** Grains dry weight of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 43.75



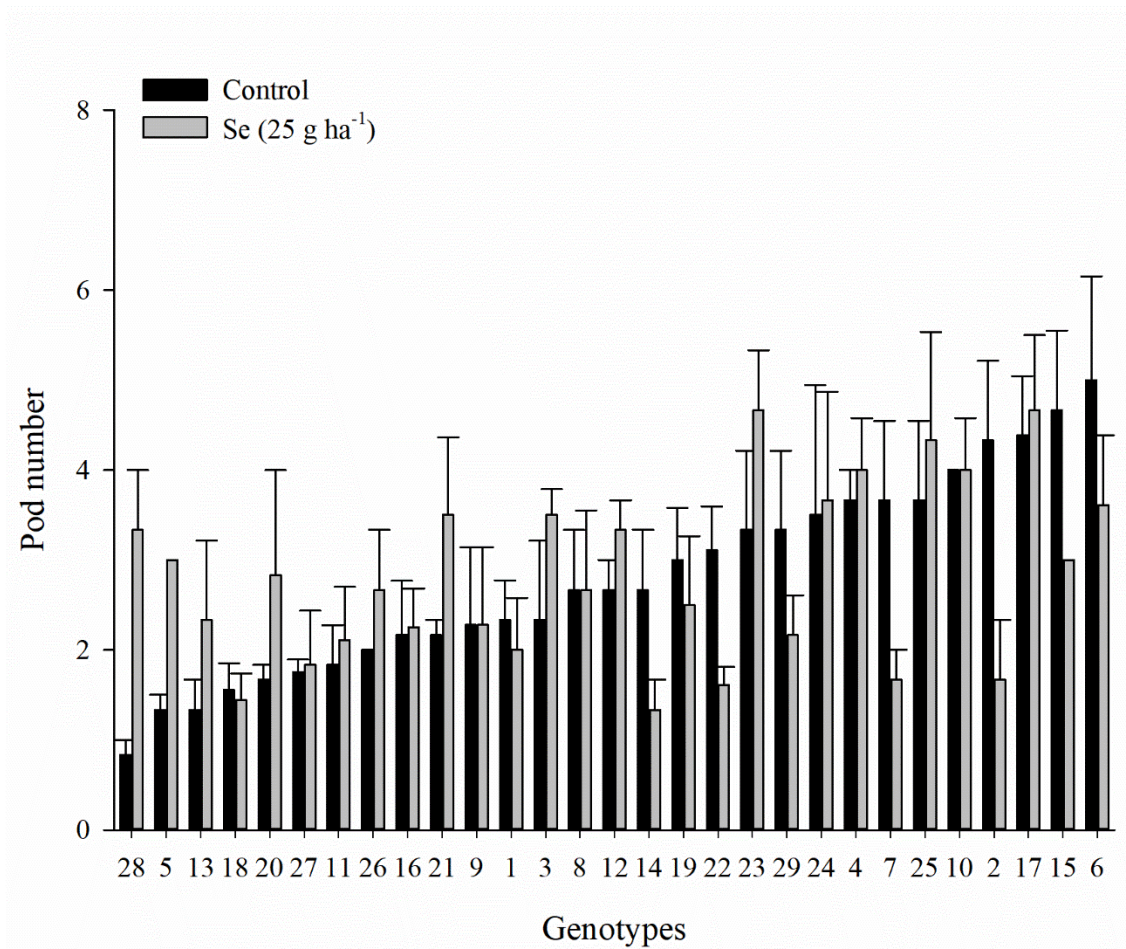
Source: Own authorship

**Figure 4.4.** Grains number of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 46.19



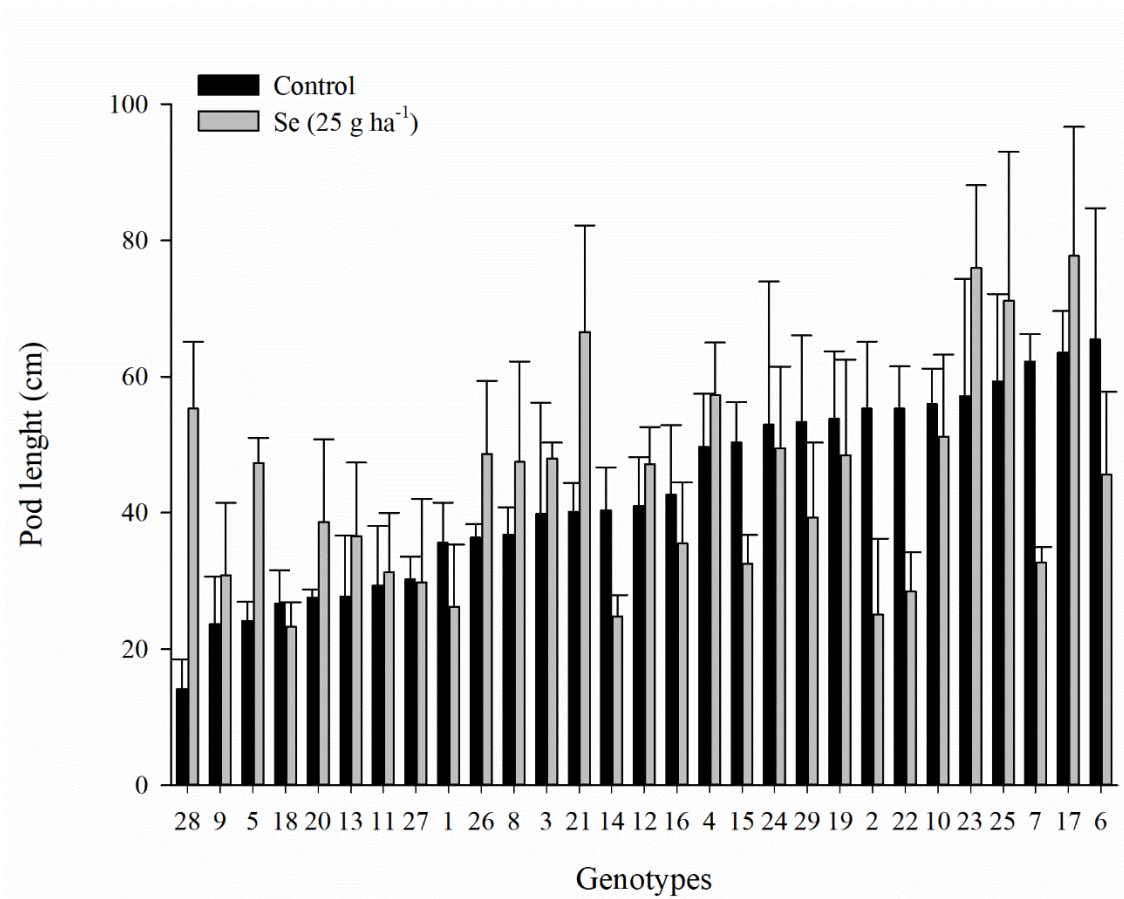
Source: Own authorship

**Figure 4.5.** Pod number of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 41.50



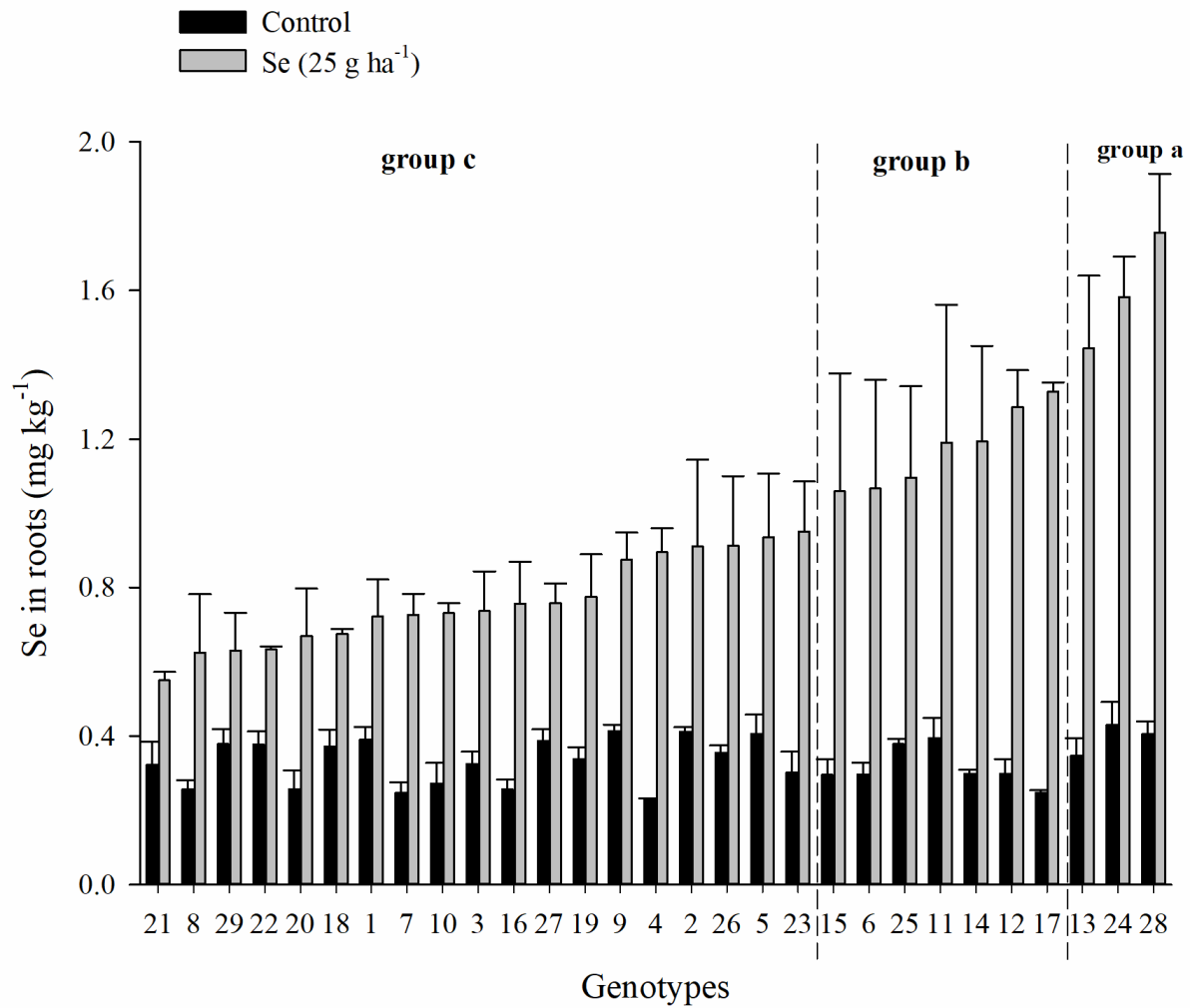
Source: Own authorship

**Figure 4.6.** Pod length of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 40.93



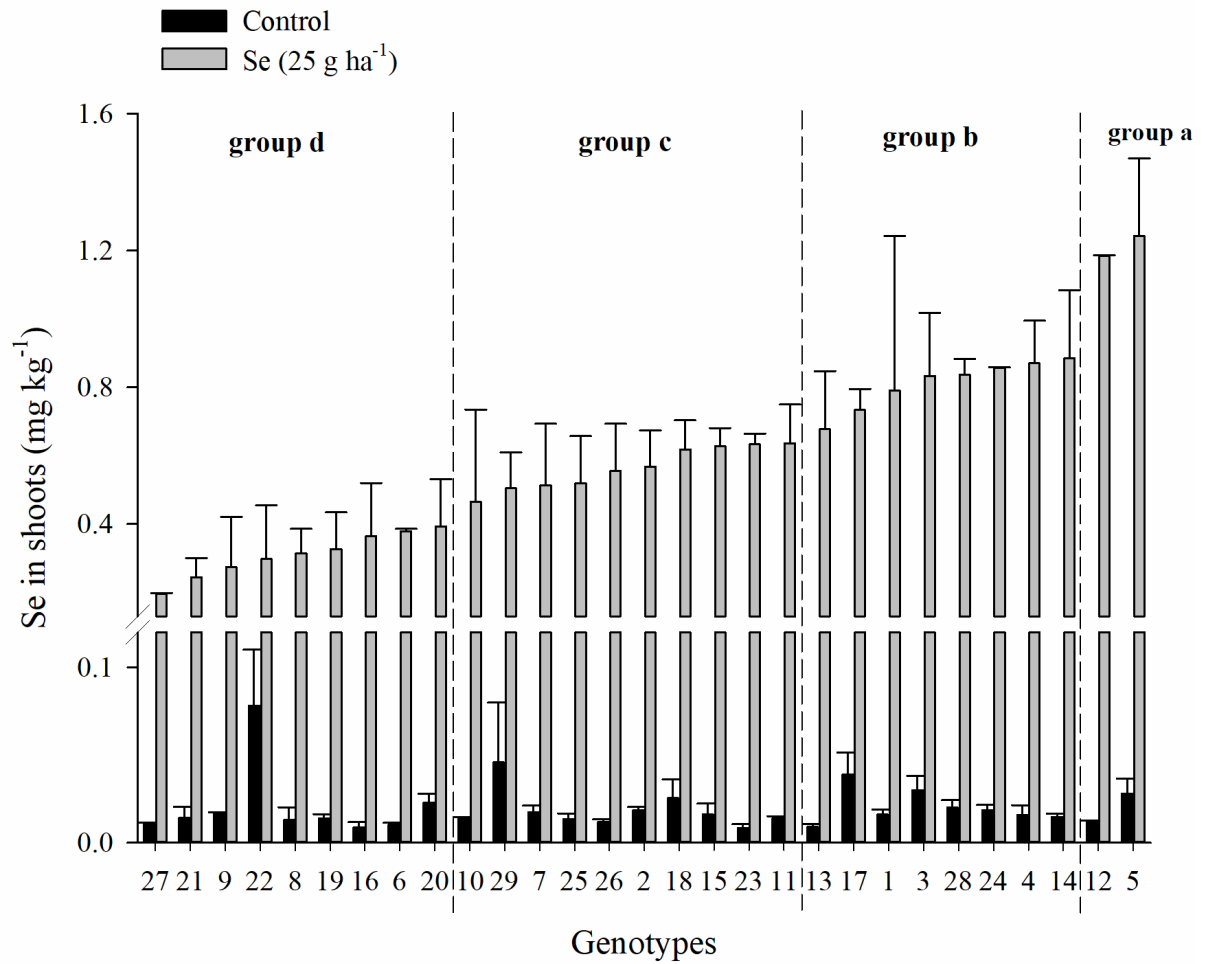
Source: Own authorship

**Figure 4.7.** Root Se concentration cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=4). CV (%) = 36.07.



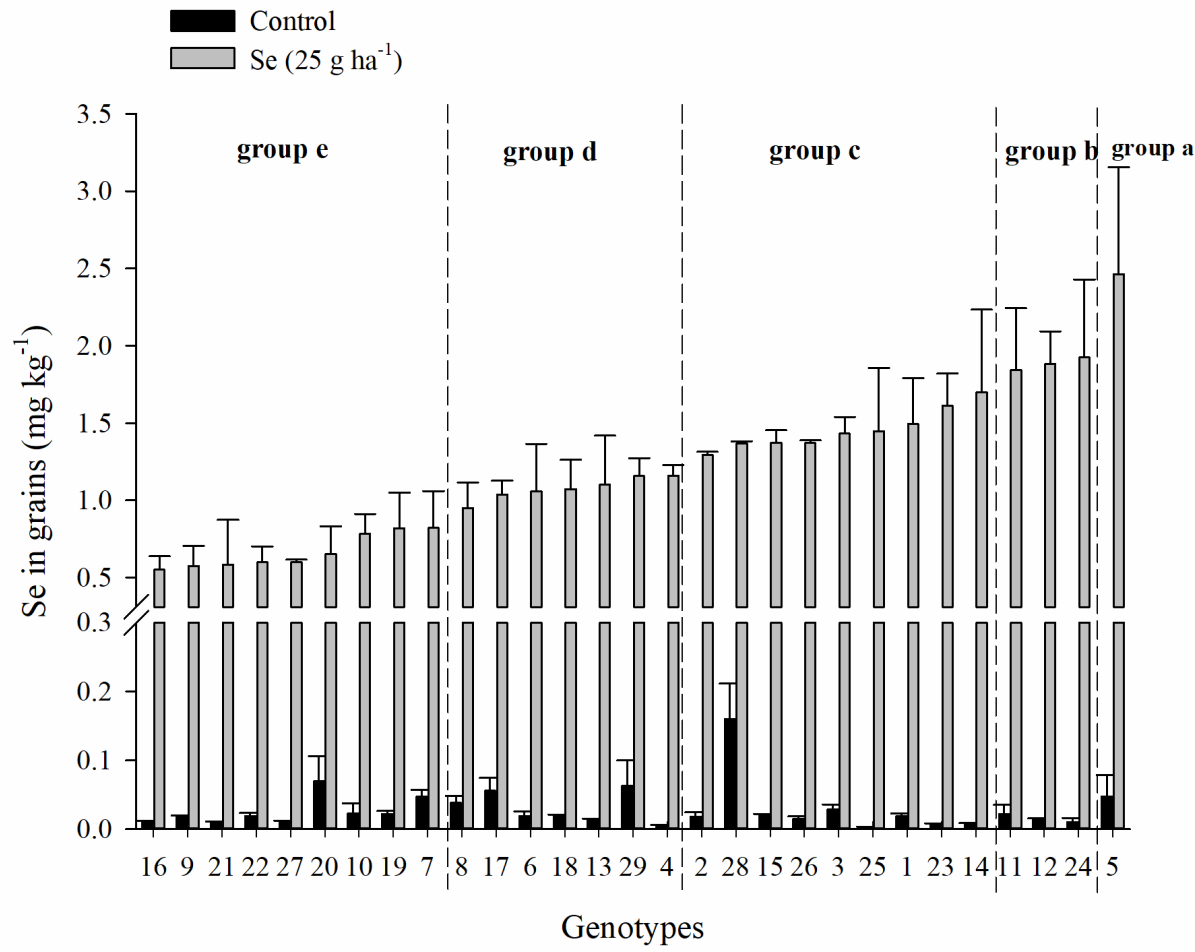
Source: Own authorship

**Figure 4.8.** Shoot Se concentration cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=4). CV (%) = 57.24.



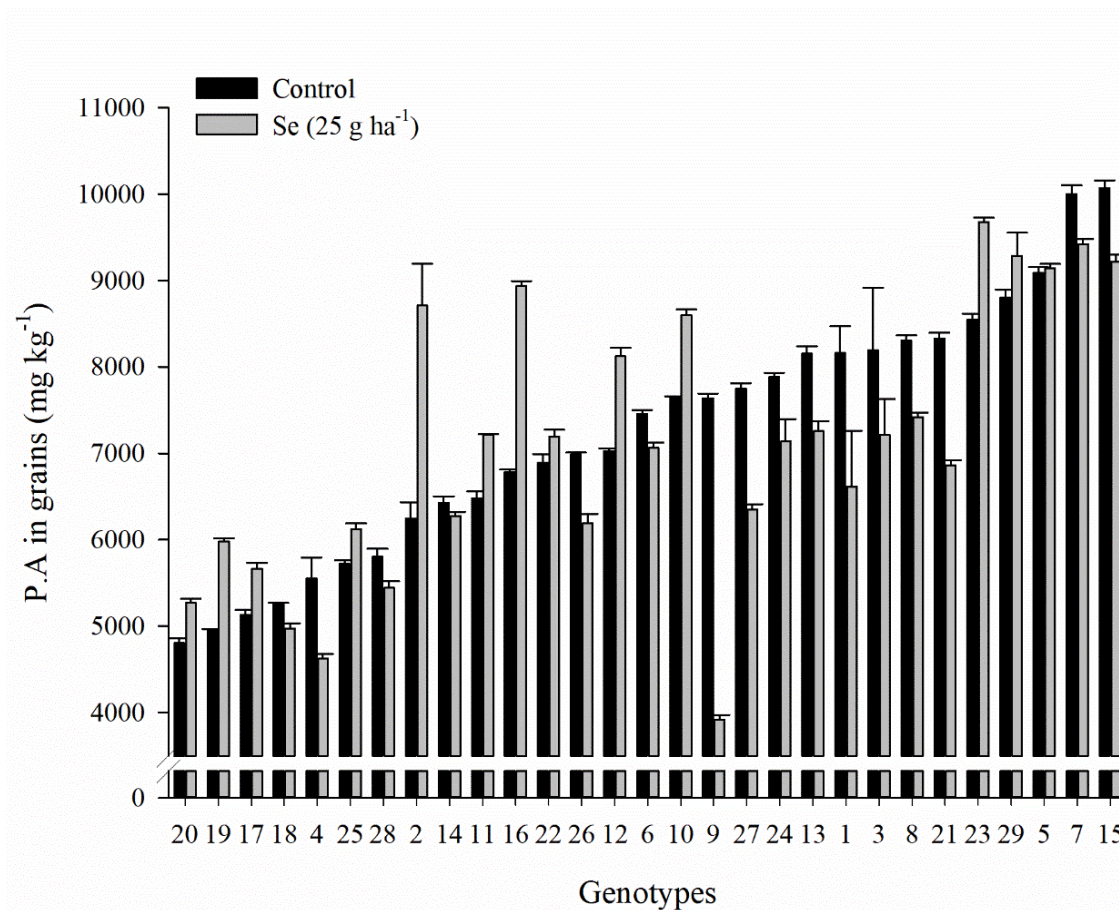
Source: Own authorship

**Figure 4.9.** Grain Se concentration cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=4). CV (%) = 66.85.



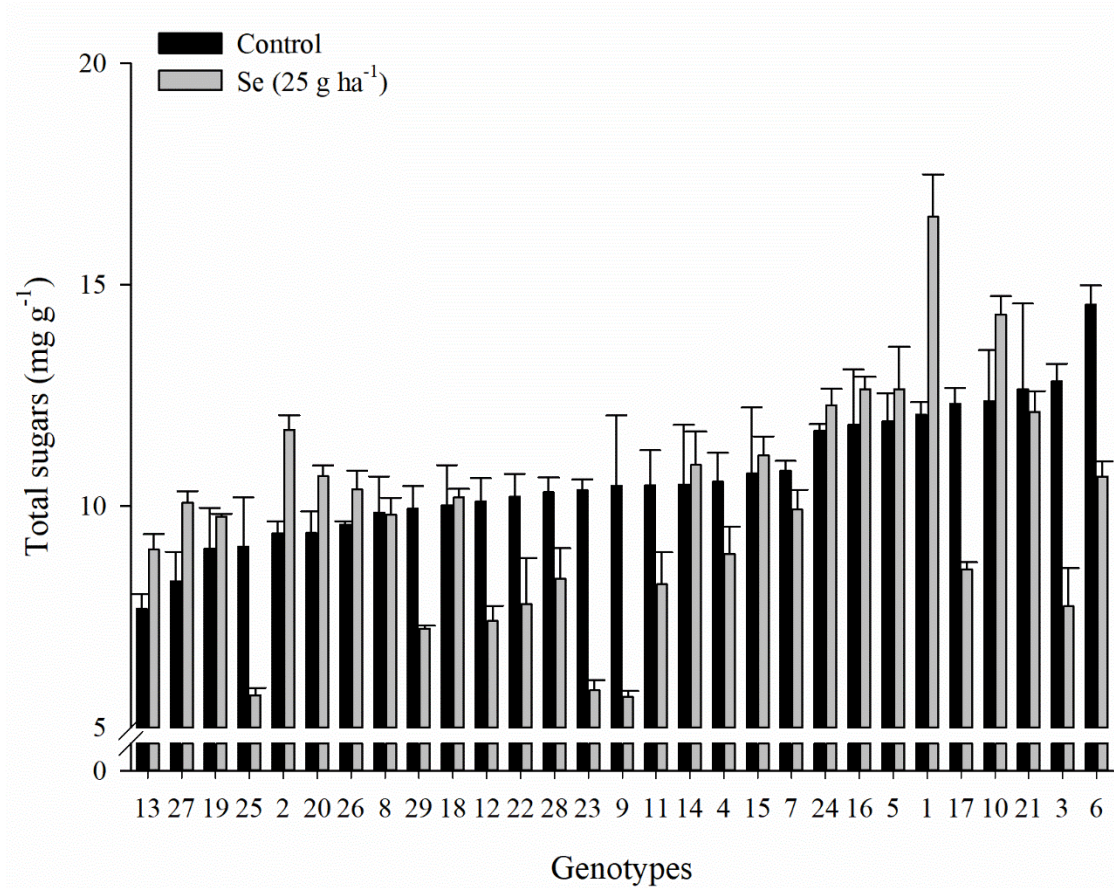
Source: Own authorship

**Figure 4.10.** Phytic acid concentration in grains of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 4.35.



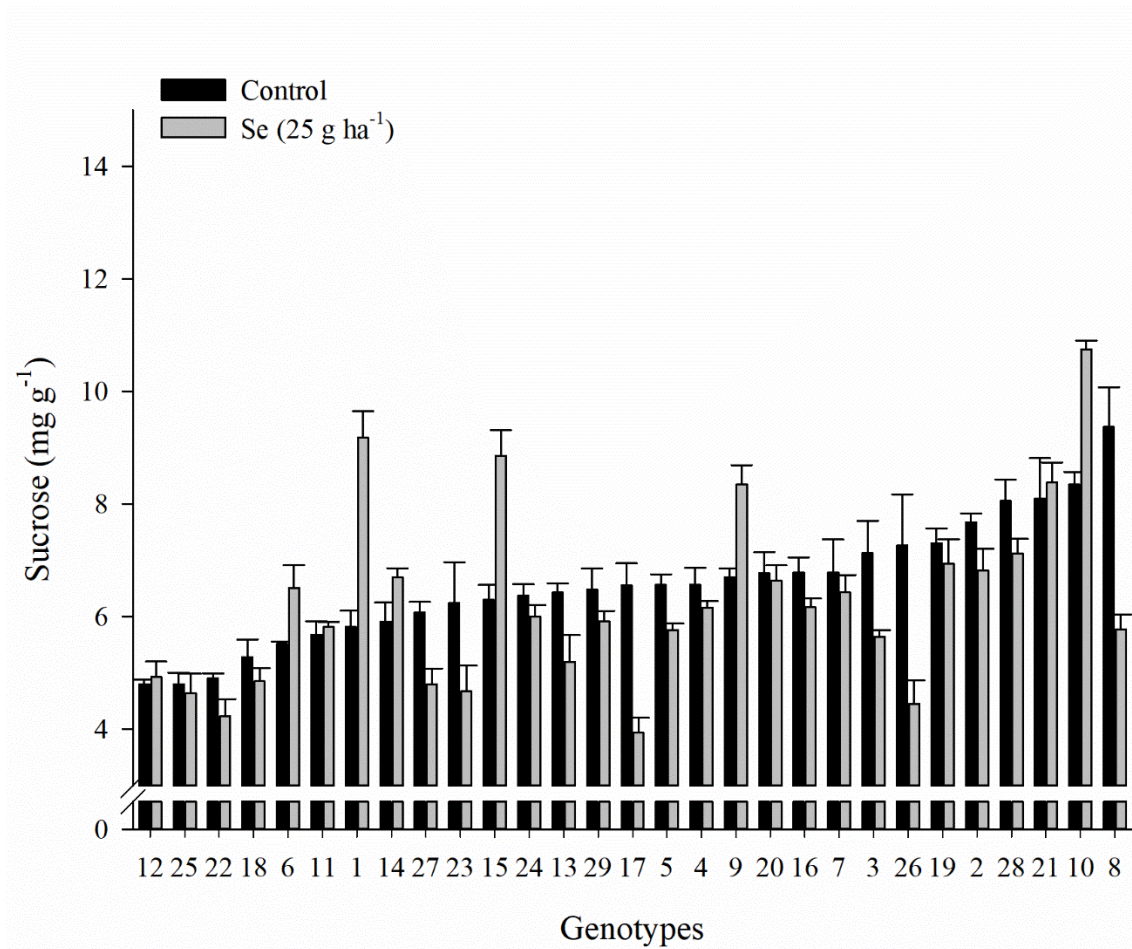
Source: Own authorship

**Figure 4.11.** Total sugar concentration in grains of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 11.80



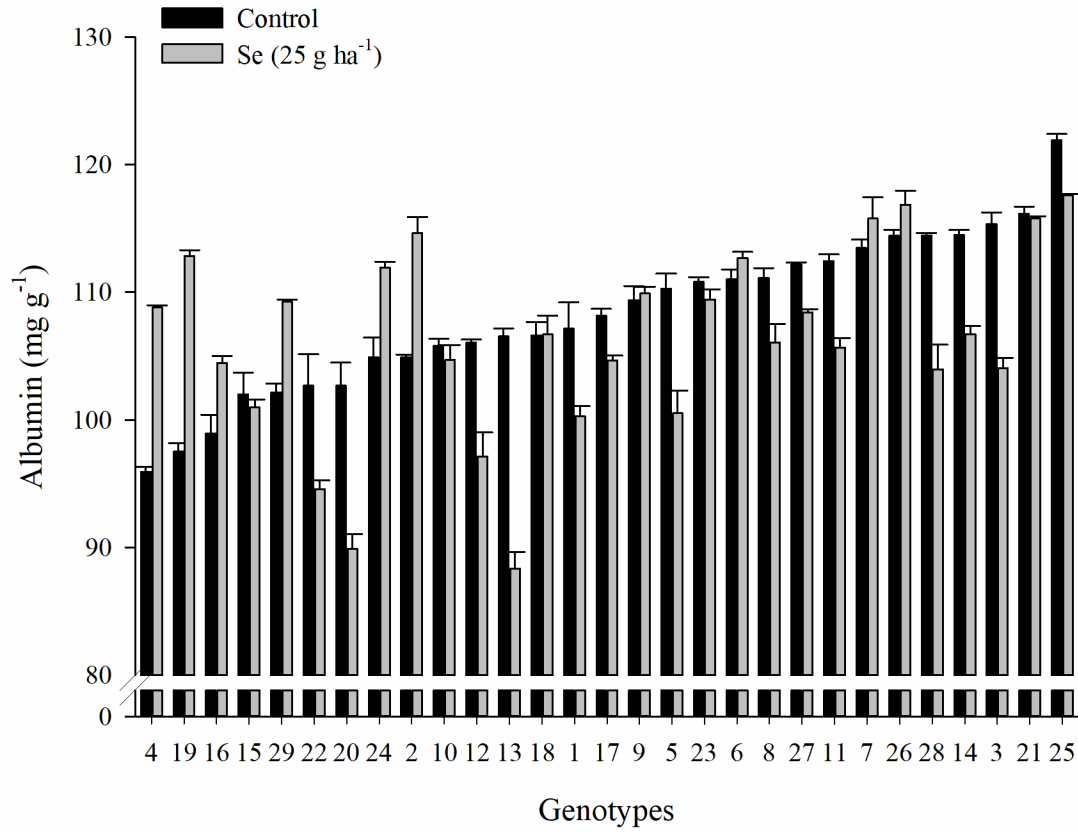
Source: Own authorship

**Figure 4.12.** Sucrose concentration in grains of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 9.62



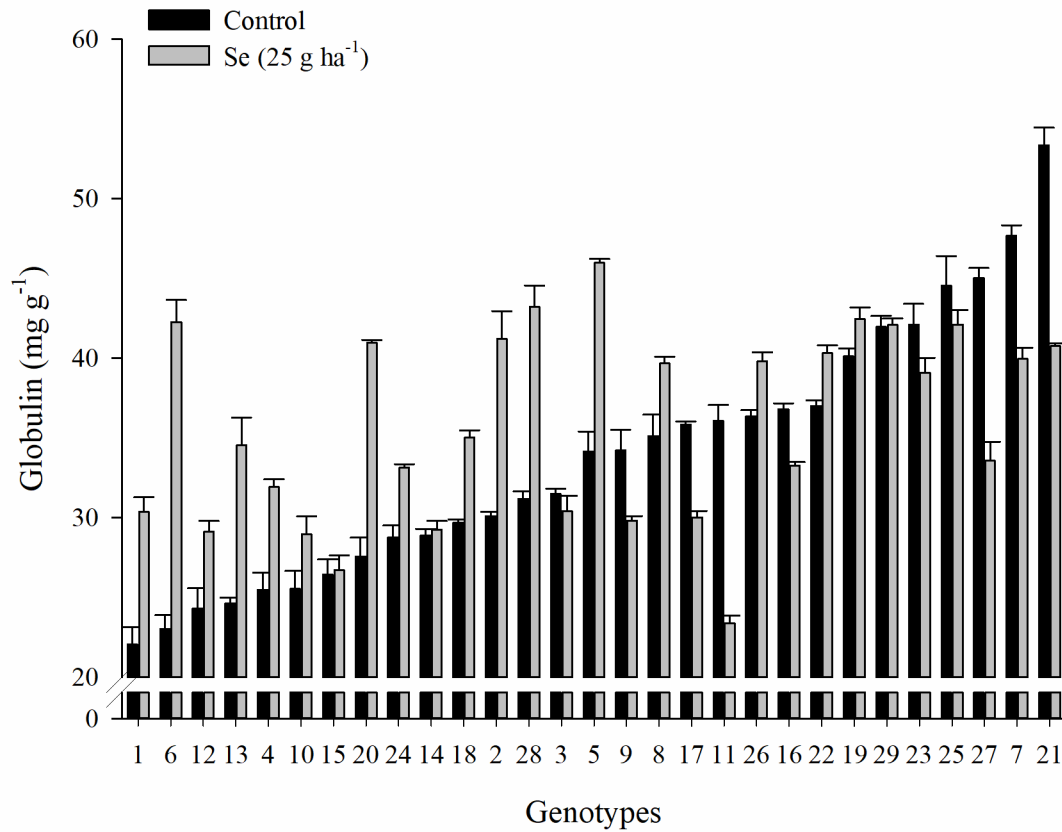
Source: Own authorship

**Figure 4.13.** Albumin concentration in grains of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 1.65



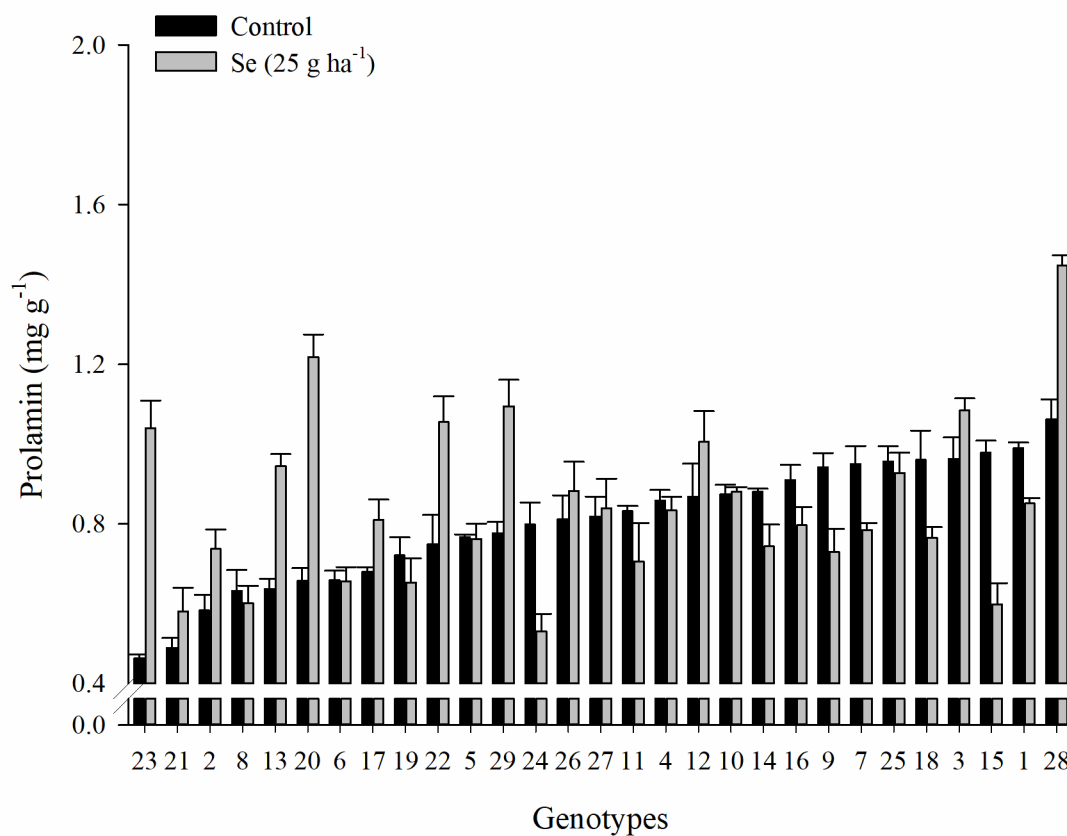
Source: Own authorship

**Figure 4.14.** Globulin concentration in grains of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 4.36



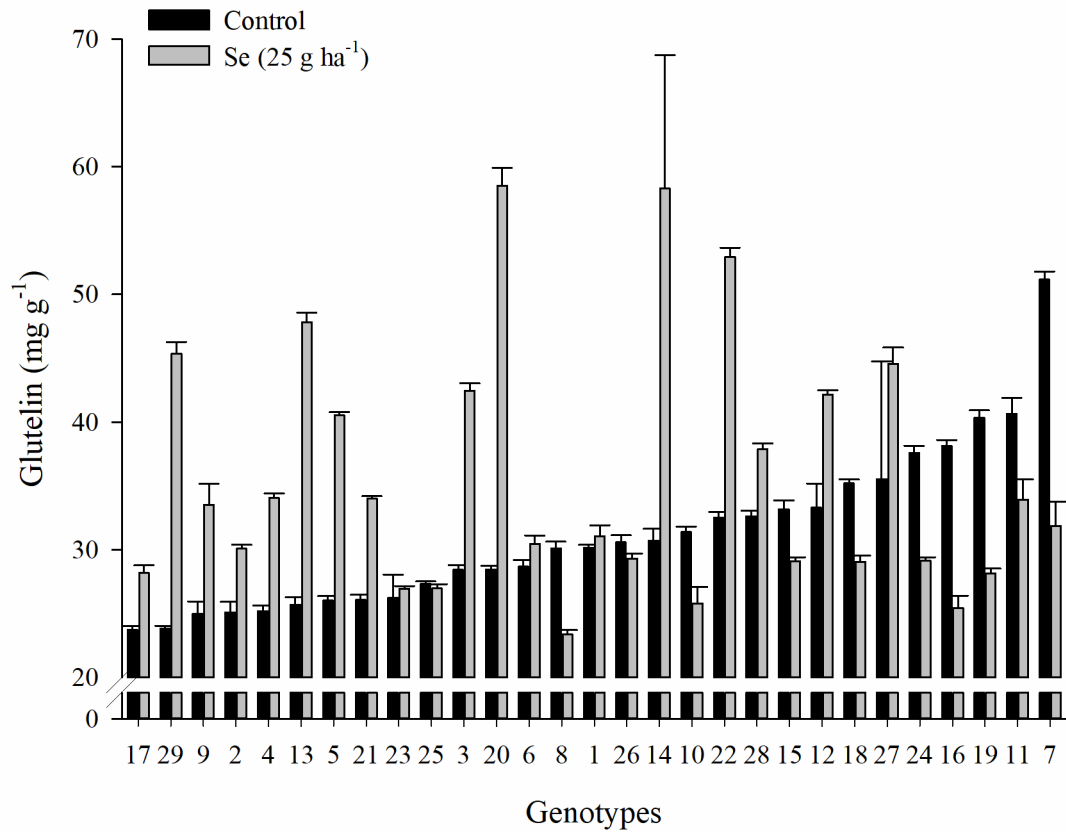
Source: Own authorship

**Figure 4.15.** Prolamin concentration in grains of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 9.98



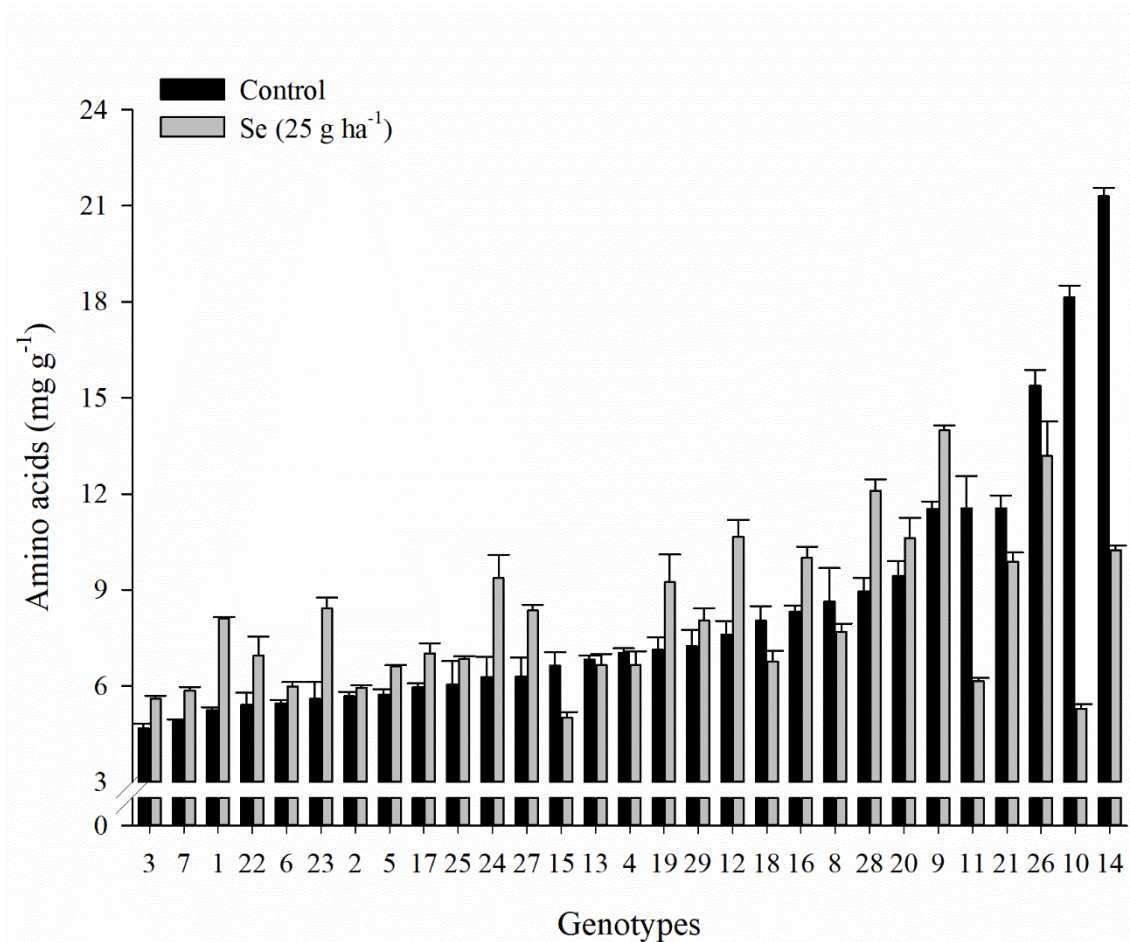
Source: Own authorship

**Figure 4.16.** Glutelin concentration in grains of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 10.36



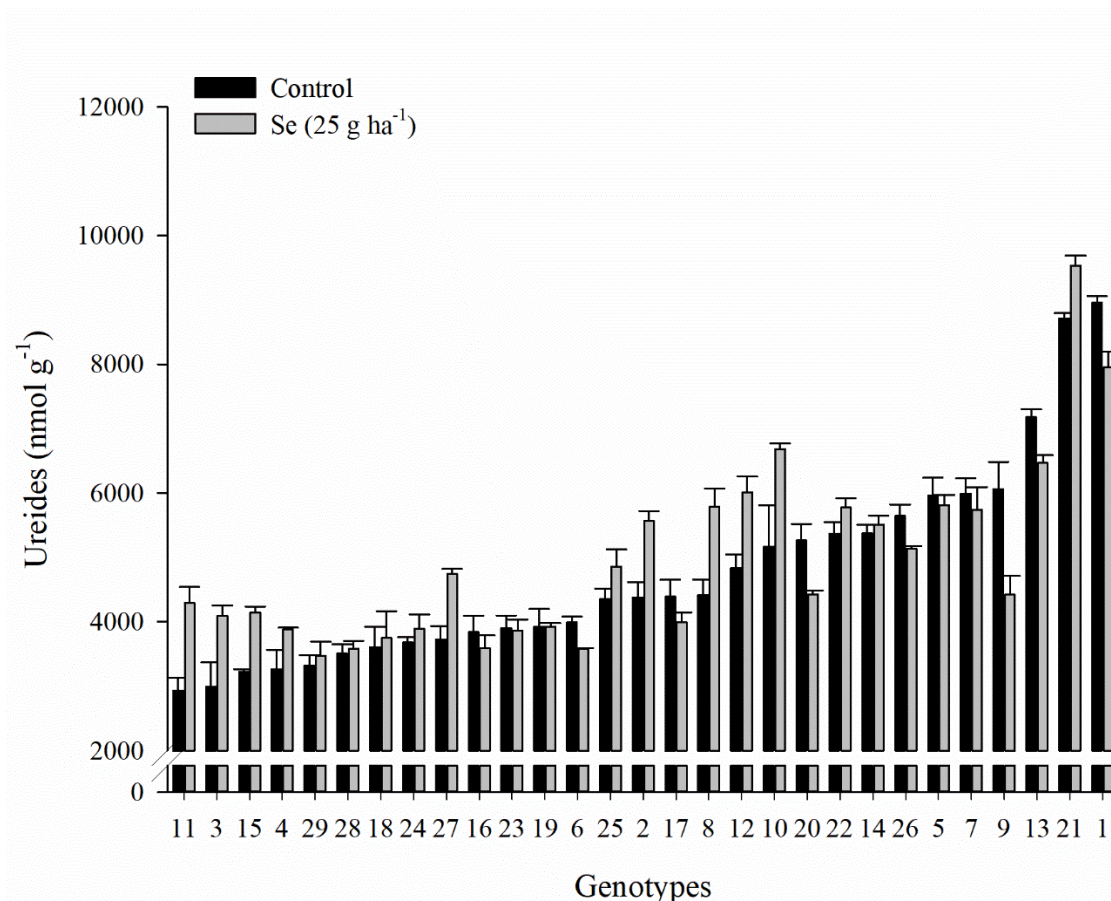
Source: Own authorship

**Figure 4.17.** Amino acids concentration in grains of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 9.07



Source: Own authorship

**Figure 4.18.** Ureides concentration in shoots of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 9.07



Source: Own authorship

#### 4.4 DISCUSSION

Regarding the growth parameters, the application of Se did not affected shoot dry weight, grain dry weight, grain number, pod number and pod length, 3 of the 29 genotypes showed increase in root dry weight. As observed in this study, increase and growth and yield due to Se application are uncommonly observed in genotypic variation experiments for non-legume plants. This study is the first report showing the positive effect of Se on growth and yield of cowpea genotypes. Application of Se at low concentration can promote plant growth and stimulate the yield increase of crops (REIS et al., 2018). On the other hand, application of high Se concentration can negatively affect the plant growth (SILVA et al., 2018). Studying the effect of Se application in lettuce accessions, Ramos et al (2011) observed that selenate

increased shoot biomass in only 5 out of 30 accessions observed. Observations in common bean genotypes under Se application showed that the element did not increase seeds fresh and dry weight (FIGUEIREDO et al., 2017). In a study with 20 accession of wheat under effect of Se and zinc, Souza et al (2014) observed that Se application increased grain, shoot and root dry weight of 3 out of 20 accessions and 100 grains dry weight of 2 out of 20 accessions. These studies were performed in hydroponic solution using relatively high concentration of Se in nutrient solution.

The natural Se concentration in grains observed in this study ranged from 0.004 in genotype 25 up to 0.16 mg Se kg<sup>-1</sup> in genotype 28 (figure 4.9). Similar result was found in monocotyledon by Lyons et al (2005) that observed a natural variation in Se concentration in grains of wheat ranging from 0.009 up to 0.244 mg kg<sup>-1</sup>. However, in an extensive study regarding natural Se grain content in 191 lentils accessions from 12 different countries, Thavarajah et al, (2017) stated Se grain concentration in lentils of most accessions fell into the 0.25 to 0.75 mg Se kg<sup>-1</sup> range, with some accession reaching more than 2 mg kg<sup>-1</sup>. Compared the values of these pulses, the natural Se concentration in cowpea genotypes used in this study is relatively low. Studies on Se concentration in cowpea genotypes, or even pulses in general are scarce, and the information of these cowpea genotypes Se concentration is important for select better genotypes for breeding programs aiming increase the seed density of micronutrients lacking in human health.

Although the natural grain Se concentration observed was low in cowpea seeds, the Se concentration increased substantially in all genotypes observed, ranging from 0.55 up to 2.5 mg Se kg<sup>-1</sup>. Selenium concentration in grains ranged from 0.5 up to 2.4 mg Se kg<sup>-1</sup>, shoots and roots Se concentration ranged from 0.3 up to 1.2 mg Se kg<sup>-1</sup>, and 0.3 up to 1.3 mg Se kg<sup>-1</sup>, respectively. Applied Se was assimilated in grains in greater amounts than in shoots and roots. This might be explained by Se metabolism in plants, when applied as selenate, Se uptake by root cells occurs by sulphate transporters (CABANNES; BUCHNER; HAWKESFORD, 2012; NATASHA et al.; 2018), and then Se is readily absorbed by xylem and transported via xylem sap from root to shoot (ZHU et al., 2009; WILKEL et al., 2015; NATASHA et al.; 2018), and then to reproductive organs by phloem (BROWN; SHRIFT, 1982; NATASHA 2018).

The limit range between beneficial and toxic effects of Se is narrow, and both effects induce alteration in plant growth and their physiology (HAWRYLAK-NOWAK; MATRASZEK; POGORZELEC, 2015). This study showed varied effect of Se on cowpea growth, increasing the growth, yield, biochemical compounds in some genotypes and

decreasing in another. The effect of Se on PA, total sugar, sucrose, storage proteins and amino acids concentration in grains, and the effect of Se in ureides in shoots is variable, reducing the concentration of these compounds in some genotypes and increasing in others (table c. d and e). Phytic acid synthesis occurs by the consecutive phosphorylations of *myo*-inositol (RABOY, 2003), and *myo*-inositol is a compound originated from the phosphorylated simple sugar d-glucose-6-phosphate (SONG et al., 2016). In this study, genotypic variation affected total sugar and sucrose concentration in grains of cowpea, as already stated in other group of cowpea genotypes (NASSOUROU et al., 2017).

The variable effect of Se in total sugar and sucrose content in different genotypes, increasing in some cases, decreasing in others, might indicates that, the element interaction with cowpea depends on each genotype particularities. Previous studies already demonstrated that Se might increase, but and also, decrease sugar compounds in plants (RIBEIRO et al., 2016; ZHU et al., 2018).

Selenium increases soluble sugar in young potato leaves (TURAKAINEN; HARTIKAINEN; SEPPÄNEN, 2004), sucrose and glucose content in tomato fruits (ZHU et al., 2018) and total soluble sugar in leaves of cowpea (MANAF, 2016). Selenium increases activity of amylases and invertases (MALIK et al., 2011), which might increase the total sugars and sucrose concentration, respectively. On the other hand, evidence that soluble sugars can be reduced by Se has also been observed. Ribeiro et al., (2016) observed lower starch degradation at nightfall in *Arabidopsis thaliana*, and conclude that under increased Se conditions, Se might inhibit starch degradation.

The Se effect on total sugar and sucrose, and the different sugar concentration within each genotype indicates an indirect relation between Se and sugar compounds. Which led to a possible indirect relation between PA synthesis and Se application, and further researches on Se effect on PA pathway and sugar metabolism are needed.

The concentration of storage proteins and amino acids in grains from cowpea indicates that Se does not directly affect storage protein and amino acids concentration. Total storage protein in the studied genotypes varied from a minimum of 147 mg g<sup>-1</sup> in control of genotype 4, up to a maximum of 213 mg g<sup>-1</sup> in control of genotype 7. Which represents a total of storage protein varying from about 15 to 21%.

Ureides are the major forms of fixed nitrogen transported in the xylem of nitrogen-fixing plants (BARAL; SILVA; IZAGUIRRE-MAYORAL, 2016). Selenium can improve biological nitrogen fixation, since it plays a structural role in hydrogenases (BALTAZAR et al., 2011), and the effect of Se improving nitrogen fixation was already observed in lentils

(EKANAYAKE et al., 2017). This might explain the increase in ureides concentration in genotypes 2, 3, 8, 10, 11, 12, 15, 21 and 27. The role of Se on ureide metabolism is still not understood and further studies are needed to evaluate the genetic and metabolic pathway impacted by Se in legume plants.

#### **4.5 CONCLUSION**

Sodium selenate application increased the Se concentration in roots, shoots and grains from all genotypes tested. At least twofold changes in total Se concentration in grains between genotypes with low and high Se concentration was found.

The change in biological nitrogen fixation analyzed by ureide (allantoic acid), plant growth and grain storage proteins changed between genotypes with low and high Se. Selenium application increased the shoot ureide concentration in 9 genotypes, grain weight in 13 genotypes, number of grain per plant in 11 genotypes, pod number in 7 genotypes.

Selenium application increased the albumin in 5 genotypes, globulin in 12 genotypes, prolamin in 6 genotypes and glutelin in 14 genotypes. This study provides valuable information for the effect of Se on plant growth and metabolism of cowpea genotypes. This information can be used in future breeding programs to select better genotypes for Se genetic biofortification in cowpea plants.

## CONCLUSION

Agronomic biofortification of crops with Se should be studied to provide new information on specific quantities of Se supplied for edible crops. The mechanisms of assimilation of Se are studied mostly in situations where Se shares similarities with other elements, for instance, selenite and its similarities with phosphate specifically in the transport from soil to roots. Selenium also share similarities with sulfate and S, but ins this case, not only on uptake from soil to roots, but also within the plant, in its transport to xylem sap and assimilation in organic forms of SeCys and SeMet.

Because PA is a P storage form in seeds and an important compound for seed germination, studies examining the effects of decreased PA concentrations on germination are also extremely relevant. Although decreased PA concentrations may improve the quality of the consumed food, the importance of P accumulation in seeds and crop germination cannot be overlooked so the high yield standards achieved by the crop plant genotypes and varieties commonly used for food production are maintained.

The uptake of applied Se in cowpea plants is tightly dependent on Se source (selenate or selenite). Application of 10 g ha<sup>-1</sup> of Se as sodium selenate provided safe and beneficial seed Se concentrations, which have the capability to increase daily human Se intake to between 12.8 and 13.9 µg day<sup>-1</sup>. Phytic acid concentration in seeds showed no relation to Se application rate and source.

Sodium selenate application increased the Se concentration in roots, shoots and grains from all genotypes tested. At least twofold changes in total Se concentration in grains between genotypes with low and high Se concentration was found, this study provides valuable information for the effect of Se on plant growth and metabolism of cowpea genotypes. This information can be used in future breeding programs to select better genotypes for Se genetic biofortification in cowpea plants.

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## SUPPLEMENTARY MATERIAL - TABLES

**Table suppl. 4.1.** Mean comparison of roots, shoots and grains dry weight of cowpea genotypes with and without application of Se. Different letters indicate difference between means according to Scott Knott test ( $p \leq 0.05$ ). Uppercase letters correspond to absence or presence of Se application, lowercase letters correspond to genotypes.

| Treatment   | Root dry weight |    | Shoot dry weight | Grain dry weight |
|-------------|-----------------|----|------------------|------------------|
|             | Control         | Se |                  |                  |
| Genotype 1  | a               | c  | b                | b                |
| Genotype 2  | a               | c  | b                | b                |
| Genotype 3  | b               | c  | b                | b                |
| Genotype 4  | aA              | cB | a                | a                |
| Genotype 5  | a               | c  | b                | b                |
| Genotype 6  | a               | c  | a                | a                |
| Genotype 7  | a               | c  | b                | b                |
| Genotype 8  | aB              | aA | a                | b                |
| Genotype 9  | b               | b  | b                | b                |
| Genotype 10 | b               | c  | b                | a                |
| Genotype 11 | b               | c  | b                | b                |
| Genotype 12 | b               | c  | a                | a                |
| Genotype 13 | a               | c  | b                | b                |
| Genotype 14 | b               | c  | b                | b                |
| Genotype 15 | b               | c  | b                | a                |
| Genotype 16 | b               | c  | b                | b                |
| Genotype 17 | b               | c  | a                | a                |
| Genotype 18 | b               | c  | b                | b                |
| Genotype 19 | bB              | bA | a                | a                |
| Genotype 20 | a               | c  | b                | b                |
| Genotype 21 | a               | b  | a                | b                |
| Genotype 22 | b               | c  | b                | b                |
| Genotype 23 | b               | c  | a                | a                |
| Genotype 24 | b               | c  | a                | a                |
| Genotype 25 | b               | c  | a                | a                |
| Genotype 26 | b               | c  | a                | a                |
| Genotype 27 | b               | c  | b                | b                |
| Genotype 28 | b               | c  | b                | b                |
| Genotype 29 | a               | c  | b                | b                |

Source: Own authorship

**Table suppl. 4.2.** Mean comparison of Grain number, Pod number and Pod length of cowpea genotypes with and without application of Se. Different letters indicate difference between means according to Scott Knott test ( $p \leq 0.05$ ). Uppercase letters correspond to absence or presence of Se application, lowercase letters correspond to genotypes.

| Treatment   | Grains number | Pod number | Pod length |
|-------------|---------------|------------|------------|
| Genotype 1  | b             | b          | b          |
| Genotype 2  | b             | b          | b          |
| Genotype 3  | a             | b          | b          |
| Genotype 4  | a             | a          | a          |
| Genotype 5  | b             | b          | b          |
| Genotype 6  | a             | a          | a          |
| Genotype 7  | b             | b          | b          |
| Genotype 8  | a             | b          | b          |
| Genotype 9  | b             | b          | b          |
| Genotype 10 | b             | a          | a          |
| Genotype 11 | b             | b          | b          |
| Genotype 12 | b             | b          | b          |
| Genotype 13 | b             | b          | b          |
| Genotype 14 | b             | b          | b          |
| Genotype 15 | b             | a          | b          |
| Genotype 16 | a             | b          | b          |
| Genotype 17 | a             | a          | a          |
| Genotype 18 | b             | b          | b          |
| Genotype 19 | a             | b          | a          |
| Genotype 20 | b             | b          | b          |
| Genotype 21 | a             | b          | a          |
| Genotype 22 | b             | b          | b          |
| Genotype 23 | a             | a          | a          |
| Genotype 24 | a             | a          | a          |
| Genotype 25 | a             | a          | a          |
| Genotype 26 | a             | b          | b          |
| Genotype 27 | b             | b          | b          |
| Genotype 28 | b             | b          | b          |
| Genotype 29 | a             | b          | b          |

Source: Own authorship

**Table suppl. 4.3.** Mean comparison of Se concentration in cowpea genotypes seed, shoot and roots with and without application of Se. Different letters indicate difference between means according to Scott-Knott test ( $p \leq 0.05$ ). Uppercase letters correspond to absence or presence of Se application, and lowercase letters correspond to genotypes.

| Treatment   | Root    |    | Shoot   |    | Grain   |    |
|-------------|---------|----|---------|----|---------|----|
|             | Control | Se | Control | Se | Control | Se |
| Genotype 1  | a       | c  | aB      | bA | aB      | cA |
| Genotype 2  | aB      | cA | aB      | cA | aB      | cA |
| Genotype 3  | aB      | cA | aB      | bA | aB      | cA |
| Genotype 4  | aB      | cA | aB      | bA | aB      | dA |
| Genotype 5  | aB      | cA | aB      | aA | aB      | aA |
| Genotype 6  | aB      | bA | aB      | dA | aB      | dA |
| Genotype 7  | aB      | cA | aB      | cA | aB      | eA |
| Genotype 8  | aB      | cA | a       | d  | aB      | dA |
| Genotype 9  | aB      | cA | a       | d  | aB      | eA |
| Genotype 10 | aB      | cA | aB      | cA | aB      | eA |
| Genotype 11 | aB      | bA | aB      | cA | aB      | bA |
| Genotype 12 | aB      | bA | aB      | aA | aB      | bA |
| Genotype 13 | aB      | aA | aB      | bA | aB      | dA |
| Genotype 14 | aB      | bA | aB      | bA | aB      | cA |
| Genotype 15 | aB      | bA | aB      | cA | aB      | cA |
| Genotype 16 | aB      | cA | aB      | dA | aB      | eA |
| Genotype 17 | aB      | bA | aB      | bA | aB      | dA |
| Genotype 18 | a       | c  | aB      | cA | aB      | dA |
| Genotype 19 | aB      | cA | aB      | dA | aB      | eA |
| Genotype 20 | aB      | cA | aB      | dA | aB      | eA |
| Genotype 21 | a       | c  | a       | d  | aB      | eA |
| Genotype 22 | a       | c  | a       | d  | aB      | eA |
| Genotype 23 | aB      | cA | aB      | cA | aB      | cA |
| Genotype 24 | aB      | aA | aB      | bA | aB      | bA |
| Genotype 25 | aB      | bA | aB      | cA | aB      | cA |
| Genotype 26 | aB      | cA | aB      | cA | aB      | cA |
| Genotype 27 | aB      | cA | a       | d  | aB      | eA |
| Genotype 28 | aB      | aA | a       | b  | aB      | cA |
| Genotype 29 | a       | c  | a       | c  | aB      | dA |

Source: Own authorship

**Table suppl. 4.4.** Mean comparison of phytic acid, total sugars and sucrose concentration in grains of cowpea genotypes with and without application of Se. Different letters indicate difference between means according to Scott Knott test ( $p \leq 0.05$ ). Uppercase letters correspond to absence or presence of Se application, lowercase letters correspond to genotypes.

| Treatment   | Phytic acid |    | Total sugars |    | Sucrose |    |
|-------------|-------------|----|--------------|----|---------|----|
|             | Control     | Se | Control      | Se | Control | Zn |
| Genotype 1  | cA          | eB | aB           | aA | eB      | bA |
| Genotype 2  | fB          | bA | bB           | cA | c       | c  |
| Genotype 3  | cA          | dB | aA           | eB | cA      | dB |
| Genotype 4  | gA          | gB | b            | e  | d       | d  |
| Genotype 5  | b           | a  | a            | c  | d       | d  |
| Genotype 6  | d           | d  | aA           | dB | e       | c  |
| Genotype 7  | aA          | aB | b            | d  | d       | c  |
| Genotype 8  | cA          | dB | b            | d  | aA      | dB |
| Genotype 9  | dA          | hB | bA           | fB | dB      | bA |
| Genotype 10 | dB          | bA | a            | b  | bB      | aA |
| Genotype 11 | fB          | dA | bA           | eB | e       | d  |
| Genotype 12 | eB          | cA | bA           | eB | e       | e  |
| Genotype 13 | cA          | dB | b            | e  | dA      | eB |
| Genotype 14 | f           | e  | b            | d  | d       | c  |
| Genotype 15 | aA          | aB | b            | d  | dB      | bA |
| Genotype 16 | eB          | aA | a            | c  | d       | d  |
| Genotype 17 | hB          | fA | aA           | eB | dA      | eB |
| Genotype 18 | h           | f  | b            | d  | e       | e  |
| Genotype 19 | hB          | eA | b            | d  | c       | c  |
| Genotype 20 | h           | f  | b            | d  | d       | c  |
| Genotype 21 | cA          | dB | a            | c  | b       | b  |
| Genotype 22 | e           | d  | bA           | eB | e       | e  |
| Genotype 23 | cB          | aA | bA           | fB | dA      | eB |
| Genotype 24 | dA          | dB | a            | c  | d       | d  |
| Genotype 25 | g           | e  | bA           | fB | e       | e  |
| Genotype 26 | eB          | eA | b            | d  | cA      | eB |
| Genotype 27 | dA          | eB | b            | d  | dA      | eB |
| Genotype 28 | g           | f  | b            | e  | b       | c  |
| Genotype 29 | c           | a  | bA           | eB | d       | d  |

Source: Own authorship

**Table suppl. 4.5.** Mean comparison of Albumin, Globulin, Prolamin and Glutelin in grains of cowpea genotypes with and without application of Se. Different letters indicate difference between means according to Scott Knott test ( $p \leq 0.05$ ). Uppercase letters correspond to absence or presence of Se application, lowercase letters correspond to genotypes.

| Treatment   | Albumin |    | Globulin |    | Prolamin |    | Glutelin |    |
|-------------|---------|----|----------|----|----------|----|----------|----|
|             | Control | Se | Control  | Se | Control  | Se | Control  | Se |
| Genotype 1  | dA      | eB | jB       | eA | aA       | dB | c        | c  |
| Genotype 2  | dB      | aA | gB       | cA | cB       | dA | d        | d  |
| Genotype 3  | bA      | dB | g        | e  | a        | c  | cB       | bA |
| Genotype 4  | fB      | cA | iB       | dA | b        | d  | dB       | cA |
| Genotype 5  | cA      | eB | fB       | aA | b        | d  | dB       | bA |
| Genotype 6  | c       | b  | jB       | bA | c        | e  | c        | d  |
| Genotype 7  | c       | a  | bA       | cB | aA       | dB | aA       | cB |
| Genotype 8  | cA      | dB | fB       | cA | c        | e  | cA       | dB |
| Genotype 9  | c       | c  | fA       | eB | aA       | dB | dB       | cA |
| Genotype 10 | d       | d  | iB       | eA | b        | d  | c        | d  |
| Genotype 11 | cA      | dB | fA       | gB | b        | d  | bA       | cB |
| Genotype 12 | dA      | fB | iB       | eA | bB       | cA | cB       | bA |
| Genotype 13 | dA      | hB | iB       | dA | cB       | cA | dB       | bA |
| Genotype 14 | bA      | dB | h        | e  | bA       | dB | cB       | aA |
| Genotype 15 | e       | e  | i        | f  | aA       | eB | c        | d  |
| Genotype 16 | fB      | dA | fB       | dB | a        | d  | bA       | dB |
| Genotype 17 | dA      | dB | fA       | eB | c        | d  | d        | d  |
| Genotype 18 | d       | d  | gB       | dA | aA       | dB | b        | dB |
| Genotype 19 | fB      | bA | e        | b  | c        | e  | bA       | dB |
| Genotype 20 | eA      | hB | hB       | cA | cB       | bA | cB       | aA |
| Genotype 21 | b       | a  | aA       | cB | d        | e  | dB       | cA |
| Genotype 22 | eA      | gB | fB       | cA | cB       | cA | cB       | aA |
| Genotype 23 | c       | c  | dA       | cB | dB       | cA | d        | d  |
| Genotype 24 | dB      | bA | hB       | dA | bB       | eA | bA       | dB |
| Genotype 25 | aA      | aB | c        | b  | a        | c  | d        | d  |
| Genotype 26 | b       | a  | fB       | cA | b        | d  | c        | d  |
| Genotype 27 | cA      | cB | cA       | dB | b        | d  | bB       | bA |
| Genotype 28 | bA      | dB | gB       | bA | aB       | aA | c        | c  |
| Genotype 29 | eB      | cA | d        | b  | bB       | cA | dB       | bA |

Source: Own authorship

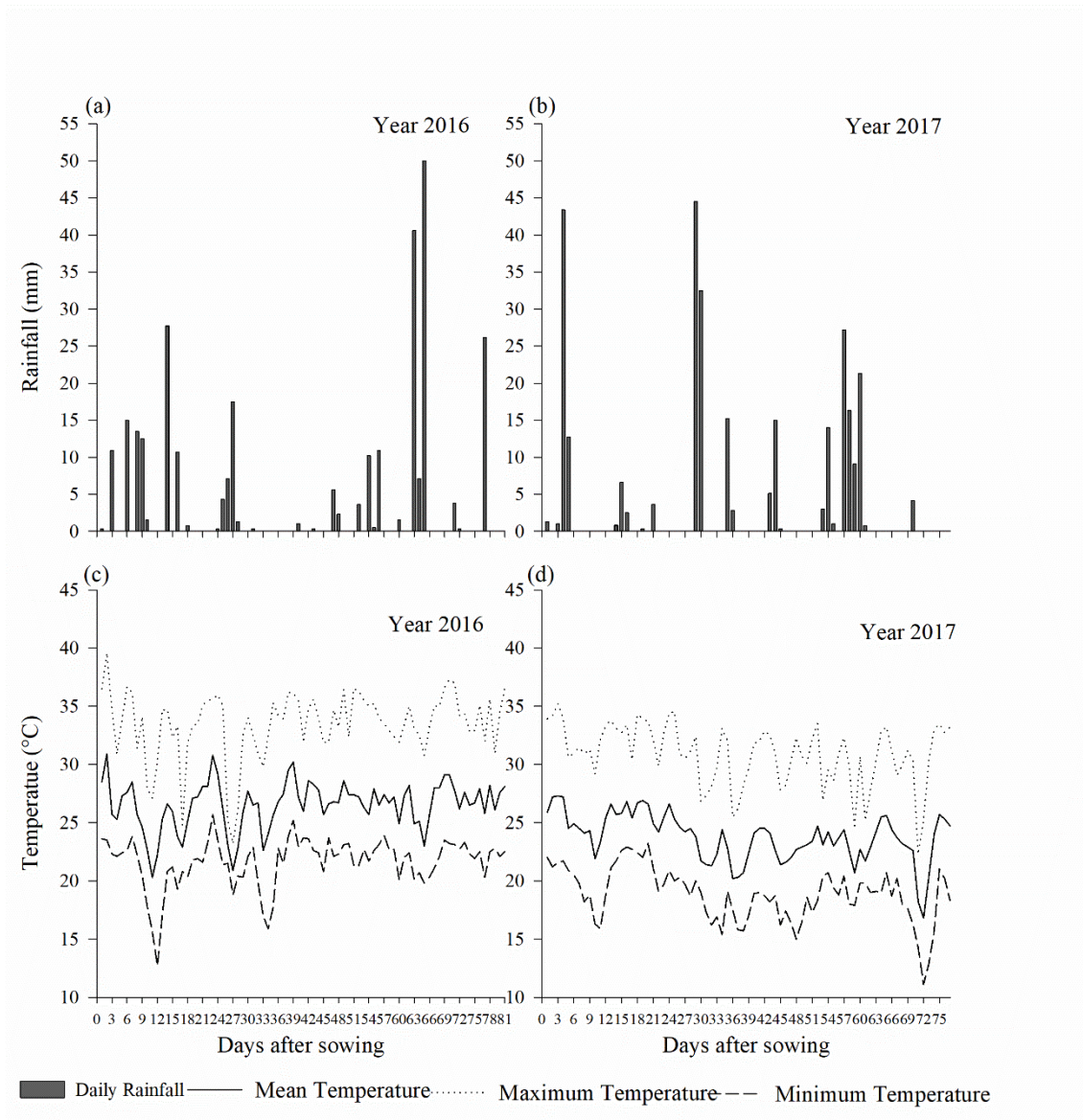
**Table suppl. 4.6.** Mean comparison of Amino acids in grains and Ureides in shoots of cowpea genotypes with and without application of Se. Different letters indicate difference between means according to Scott Knott test ( $p \leq 0,05$ ). Uppercase letters correspond to absence or presence of Se application, lowercase letters correspond to genotypes.

| Treatment   | Amino Acids |    | Ureides |    |
|-------------|-------------|----|---------|----|
|             | Control     | Se | Control | Se |
| Genotype 1  | gB          | dA | aA      | bB |
| Genotype 2  | g           | f  | eB      | dA |
| Genotype 3  | g           | f  | gB      | fA |
| Genotype 4  | f           | e  | g       | f  |
| Genotype 5  | g           | e  | c       | d  |
| Genotype 6  | g           | f  | f       | f  |
| Genotype 7  | g           | f  | c       | d  |
| Genotype 8  | e           | d  | eB      | dA |
| Genotype 9  | dB          | aA | cA      | eB |
| Genotype 10 | bA          | fB | dB      | cA |
| Genotype 11 | dA          | fB | gB      | eA |
| Genotype 12 | fB          | cA | eB      | dA |
| Genotype 13 | f           | e  | bA      | cB |
| Genotype 14 | aA          | cB | d       | d  |
| Genotype 15 | fA          | fB | gB      | fA |
| Genotype 16 | eB          | cA | f       | f  |
| Genotype 17 | g           | e  | e       | f  |
| Genotype 18 | eA          | eB | g       | f  |
| Genotype 19 | fB          | cA | f       | f  |
| Genotype 20 | e           | c  | dA      | eB |
| Genotype 21 | dA          | cB | aB      | aA |
| Genotype 22 | gB          | eA | d       | d  |
| Genotype 23 | gB          | dA | f       | f  |
| Genotype 24 | fB          | cA | g       | f  |
| Genotype 25 | g           | e  | e       | e  |
| Genotype 26 | cA          | aB | c       | e  |
| Genotype 27 | fB          | dA | gB      | eA |
| Genotype 28 | eB          | bA | g       | f  |
| Genotype 29 | f           | d  | g       | f  |

Source: Own authorship

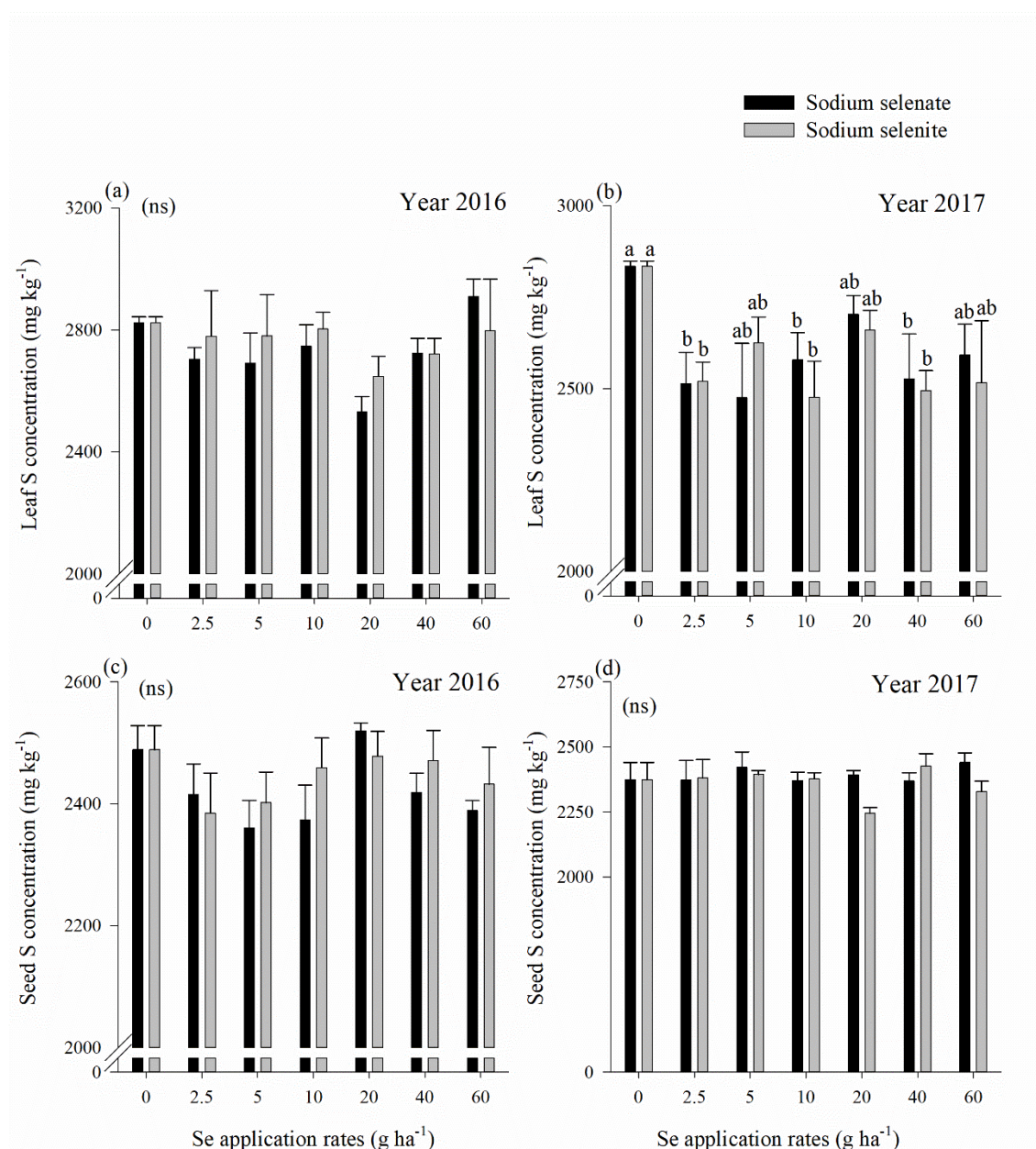
## SUPPLEMENTARY MATERIAL – FIGURES

**Figure suppl. 3.1.** Rainfall (a-b) and temperature (c-d) recorded throughout cowpea field experiments.



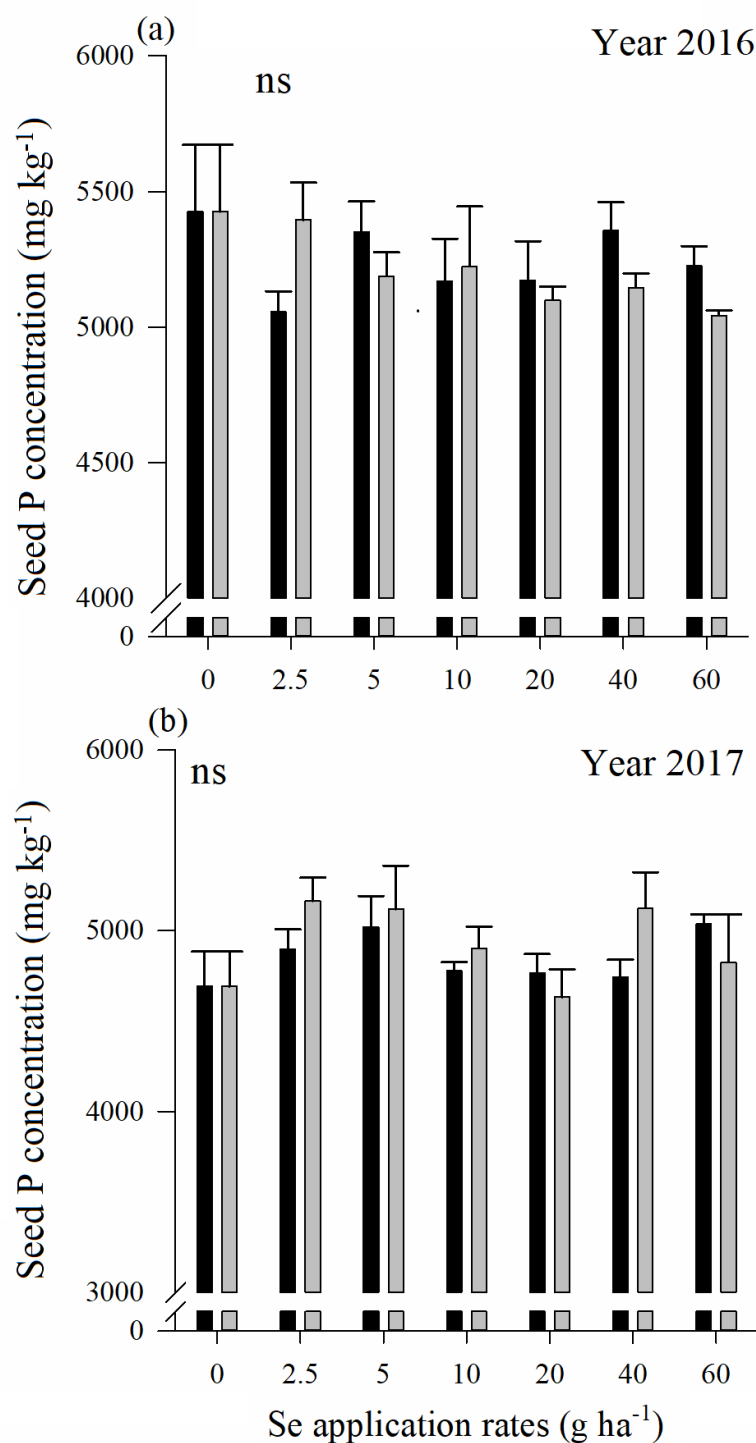
Source: Own authorship

**Figure suppl. 3.2.** Leaf S concentration in the first (a) and second (b) year and seed S concentration in the first (c) and second (d) year of cowpea plants in response to levels of sodium selenate sodium selenite application. Error bars indicates the standard error of mean (n=4). Different letters indicate difference between means according to tukey test ( $p \leq 0,05$ ). Uppercase letters correspond to Se sources, and lowercase letters correspond to Se application rates. ns = not significant according to tukey test ( $p \leq 0.05$ ). CV (%) = 6.46 (a); 7.11 (b); 3.84 (c); 3.60 (d). Se application rates effect regardless of sources according to tukey test ( $p \leq 0,05$ ) observed in: b.



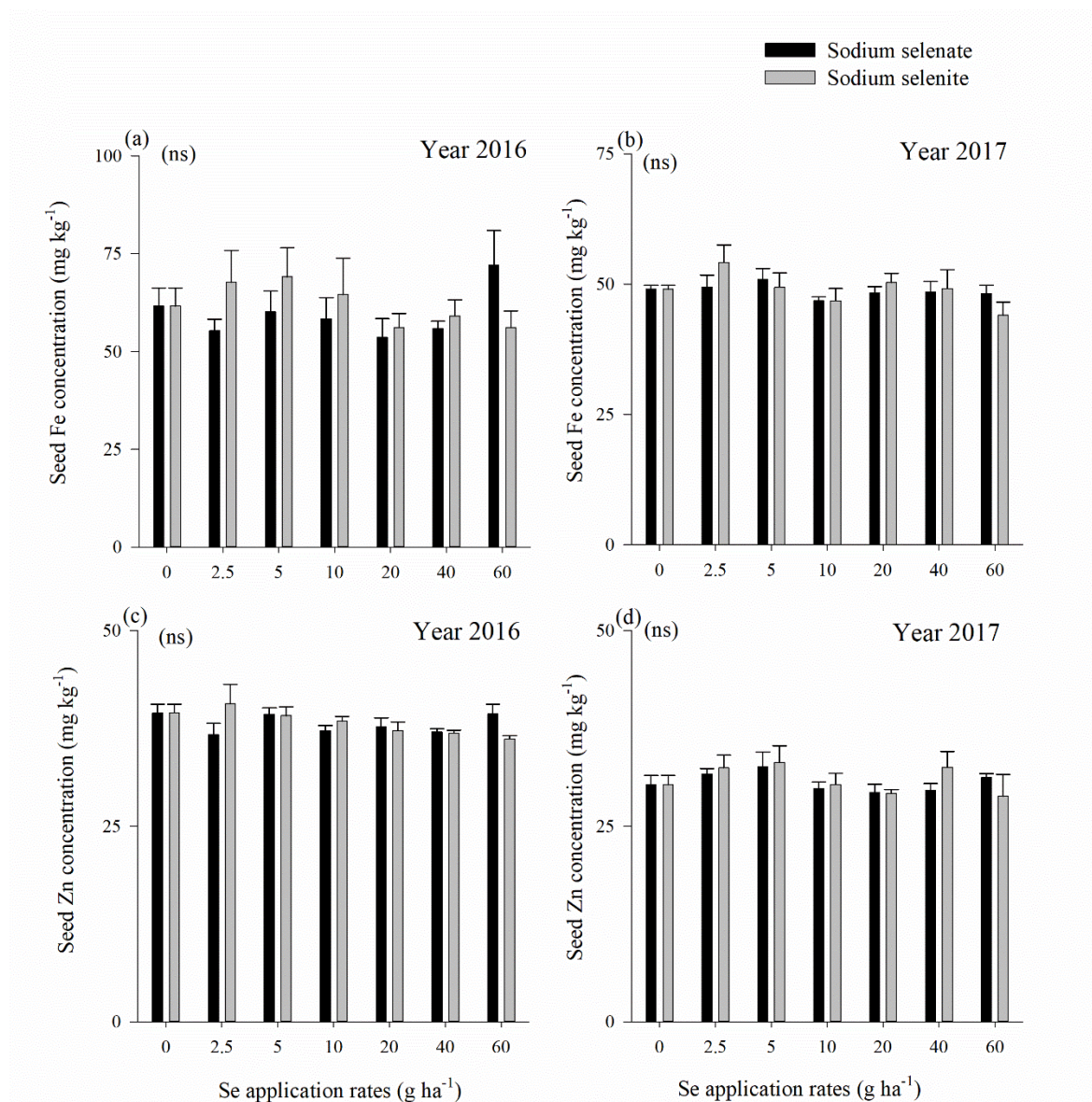
Source: Own authorship

**Figure suppl. 3.3.** Seed P concentration in the first (a) and second (b) year of cowpea plants in response to levels of sodium selenate sodium selenite application. Error bars indicates the standard error of mean (n=4). ns = not significant according to tukey test ( $p \leq 0.05$ ). CV (%) = 4.53 (a); 6.45 (b)



Source: Own authorship

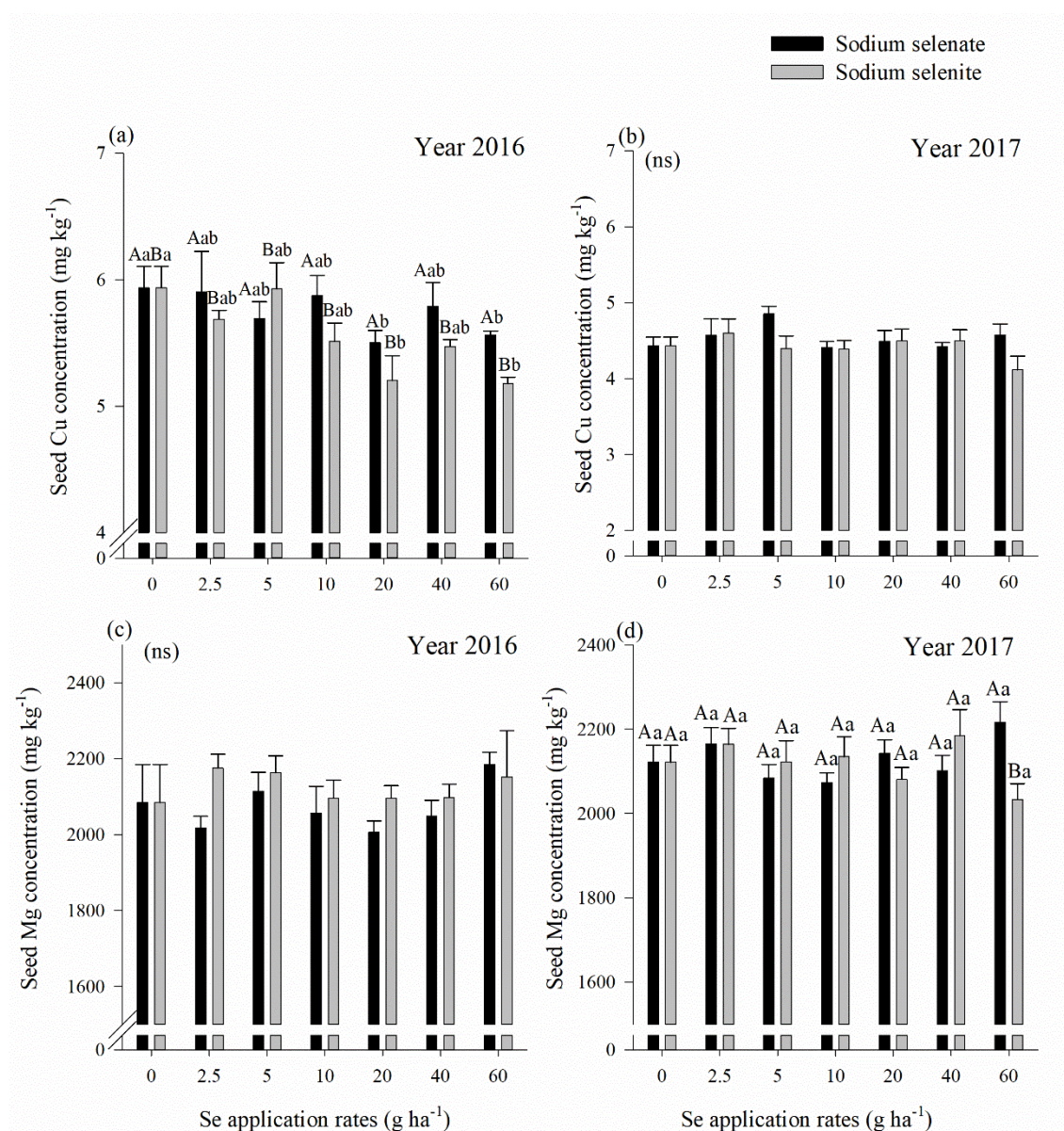
**Figure suppl. 3.4.** Seed Fe concentration in the first (a) and second (b) year and seed Zn concentration in the first (c) and second (d) year of cowpea plants in response to levels of sodium selenate sodium selenite application. Error bars indicates the standard error of mean (n=4). ns = not significant according to tukey test ( $p \leq 0.05$ ). CV (%) = 17.14 (a); 8.64 (b); 5.64 (c); 9.27 (d).



Source: Own authorship

**Figure suppl. 3.5.** Seed Cu concentration in the first (a) and second (b) year and seed Mg concentration in the first (c) and second (d) year of cowpea plants in response to levels of sodium selenate sodium selenite application. Error bars indicates the standard error of mean (n=4). Different letters indicate difference between means according to tukey test ( $p \leq 0,05$ ). Uppercase letters correspond to Se sources, and lowercase letters correspond to

Se application rates. ns = not significant according to tukey test ( $p \leq 0.05$ ). CV (%) = 5.65 (a); 9.27 (b); 5.79 (c); 3.67 (d).



Source: Own authorship