DISSERTATION

SELENIUM ACCUMULATION IN PLANTS AND IMPLICATIONS FOR HUMAN HEALTH: A SURVEY OF MOLECULAR, BIOCHEMICAL, AND ECOLOGICAL CUES

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ABSTRACT

SELENIUM ACCUMULATION IN PLANTS AND IMPLICATIONS FOR HUMAN HEALTH: A SURVEY OF MOLECULAR, BIOCHEMICAL, AND ECOLOGICAL CUES

The element Selenium (Se) is required in trace amounts by many life forms, including prokaryotes, some microalgae, and many animals, including mammals. However, Se intake higher than a certain threshold may be harmful because of oxidative stress and by replacement of sulfur (S) with Se in proteins. Selenium deficiency affects more than one billion people worldwide. Because plants represent the main portal for Se into human diets, biofortification programs are increasingly carried out to enrich staple crops with Se and thus overcome the deficiency. The success of these fortification programs benefits from understanding the mechanisms of plant Se uptake, assimilation, and tolerance. Chapter 1 gives an overview of our current knowledge.

While biofortification can increase Se levels in crop species, it usually requires costly fertilizer supplementation. Therefore, natural high-Se food can be a simple solution to providing sufficient Se to populations in low-Se areas. Brazil nut (*Bertholletia excelsa* H.B.K), contains the highest reported Se concentration among food sources, and the regular intake of these nuts may be a strategy to prevent Se deficiency. Chapter 2 describes a study that characterized the chemical form of Se, its localization, and concentration in different commercially available batches of Brazil nut. Furthermore, levels of macronutrients and micronutrients in these nuts were characterized, and their interactions with Se were investigated. The data presented revealed the main form of Se in Brazil nuts is organic; up to 8-fold variation in Se concentration was found, and the average Se concentration ranged from 25 to 76 mg Se kg^{-1}. The consumption of one nut was enough to meet

or even exceed the recommended daily allowance for Se. While this forms an excellent source of dietary Se, it is recommended to limit the intake of Brazil nuts to several per day, to avoid toxicity.

An exciting group of plant species from different families called Se hyperaccumulators, have even higher Se levels than the Brazil nut, up to 15,000 mg kg^{-1} dry weight in all organs. Earlier studies have shown that hyperaccumulators such as the North American *Stanleya pinnata* (Brassicaceae) benefit from Se hyperaccumulation through ecological benefits (herbivory protection) and enhanced growth. However, no investigation has so far assayed population-level variation in Se accumulation in the field and the effects of Se accumulation on plant fitness. The research presented in chapter 3 analyzed variation in Se accumulation in two populations of *S. pinnata* and how the plant-Se correlates with fitness parameters, judged from physiological and biochemical performance parameters and herbivory while growing naturally on two seleniferous sites. Natural variation in Se concentration in vegetative and reproductive tissues was determined, and correlations were explored between Se levels with fitness parameters, herbivory damage, and plant defense compounds. Overall, a 34-fold variation was observed in leaf tissues, and a 4-fold averaged variation in seed and siliques was observed. Most importantly, most tissues analyzed showed hyperaccumulator levels of Se (> 1000 mg kg^{-1} DW). This variation in Se hyperaccumulation (ranging from high-Se to extremely high-Se) does not appear to enhance or compromise *S. pinnata* fitness when growing in its natural habitat on seleniferous soil. Plant size and reproductive parameters were not correlated with Se concentration, so the physiological capacity for Se tolerance does not appear to be a constraint for Se hyperaccumulation in this species. Significant herbivory pressure was found even on the highest-Se plants, likely from Se-resistant invertebrate herbivores. Earlier studies reported different species of Se-resistant moth larvae, seed wasps, and seed weevils in this area. In non-seleniferous areas, including Se

biofortification settings, specialized herbivores likely do not occur, and different outcomes of Se-dependent fitness studies may be expected; this question awaits further investigation.

The efficient Se biofortification of crop species depends on Se supply, the presence of competing ions such as sulfate, and on plant uptake and assimilation properties. Organic forms of Se offer a better dietary source of Se and are less toxic. In this context, Se hyperaccumulators show some interesting properties that may make them useful in biofortification. They accumulate high levels of Se in nutritious, organic forms. Most interestingly, hyperaccumulators have a higher Se/S ratio in their tissues compared to their growth medium or to surrounding vegetation, pointing to possible Se-specific transporter. In earlier studies, the hyperaccumulator *S. pinnata* showed an elevated expression of different sulfate/selenate transporters (SULTR), including the main root transporter SULTR1;2. This may explain their higher Se levels and possibly their Se/S enrichment if the *S. pinnata* SULTR1;2 transporter is selenate-specific. In chapter 4 we investigate this hypothesis. The cDNA from the corresponding gene, as well as from related non-hyperaccumulator *Stanleya elata* were amplified, sequenced, and expressed in the model species *Arabidopsis thaliana*. Homozygous transgenic *A. thaliana* lines expressing SULTR1;2 from either species were compared with untransformed control plants for Se uptake via accumulation and tolerance experiments. The speciation and location of Se using x-ray microprobe analysis were also analyzed. The predicted *S. pinnata* SULTR1;2 possesses several unique amino acids compared to both nonaccumulators, which may affect protein function or regulation. However, based on the studies so far, Se tissue distribution and chemical speciation were similar in all plants analyzed, and no evidence for Se specificity of the hyperaccumulator transporter was found. Perhaps another SULTR mediates selenate specificity or one of the Se assimilation enzymes.

Previous studies comparing hyperaccumulator *S. pinnata* to *S. elata* showed massive overexpression of a gene predicted to encode ATPS2, the first enzyme in the reduction of sulfate/selenate to organic forms, in the roots and leaves of the hyperaccumulator. Chapter 5 investigates the role of this *S. pinnata* ATPS2 on the formation of organic and less toxic forms of Se, the overall plant's tolerance against toxic levels of Se, and ultimately its contribution to the Se hyperaccumulation syndrome. The cDNA from the *S. pinnata* gene, as well as *S. elata* ATPS2, were amplified, sequenced, and compared. Homozygous transgenic *A. thaliana* expressing the ATPS2 from either species were compared with untransformed control plants for Se accumulation and tolerance experiments. The speciation and location of Se using x-ray microprobe analysis were also analyzed. Overall, our data showed that *A. thaliana* transgenics overexpressing ATPS from either *Stanleya* species demonstrate enhanced Se tolerance, which is probably caused by an observed enhanced ability to reduce selenate and accumulate organic Se. No evidence was found that the *S. pinnata* ATPS2 is selenate-specific. Nevertheless, ATPS2 likely plays an important role in Se hyperaccumulation *in S. pinnata* and its expression in crops can potentially benefit biofortification, through an enhanced accumulation of organic Se, with is an excellent source of dietary Se.

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CHAPTER 1: INTRODUCTION TO SELENIUM: BIOGEOCHEMISTRY, PLANT METABOLISM, AND IMPLICATIONS FOR HUMAN HEALTH

1.1 INTRODUCTION

The element Selenium (Se) is characterized by several intriguing properties. It is required in trace amounts for the healthy metabolism of many life forms like many prokaryotes, some microalgae, and many animals, including mammals (Novoselov *et al.,* 2002; Rayman, 2012; Schiavon and Pilon-Smits 2017). Selenium has a structural role in these organisms in the form of selenocysteine in the active site of a select number of essential proteins. However, Se intake higher than a certain threshold may be harmful to these -and other- organisms. Inorganic Se anions can be pro-oxidants in cells, causing oxidative stress through depletion of intracellular glutathione; protein misfolding may also occur due to replacement of sulfur (S) by Se in amino acids (Van Hoewyk *et al.,* 2008; Zhang and Gladyshev, 2009).

In humans, the window between deficiency and toxicity for Se is extremely narrow as compared to other micronutrients (Stadtman, 1990). Selenium deficiency has been estimated to affect at least one billion people (Lyons *et al.,* 2003), especially in parts of China, North-West Europe, Australia, New Zealand, sub-Saharan Africa, Southern Brazil, and parts of the USA (Oldfield, 2002; Zhu *et al.,* 2009; Gupta and Gupta, 2017). This number may be growing, according to a moderate climate-change model scenario. Jones *et al.* (2017) analyzed several environmental variables that may influence Se distribution worldwide and predicted that climate and soil organic matter changes will be responsible for a significant reduction of soil Se

concentration in 2080–2099 as compared to a more recent situation (1980–1999), especially in agricultural regions.

Selenium concentration in soil, which mostly ranges between 0.01 and 2.0 ppm of Se, primarily correlates with Se availability in the human diet (Lyons *et al.,* 2003). Plants represent the main portal for Se into the food web, as well as into human diets. Therefore, Se biofortification programs are increasingly carried out to enrich staple crops with Se in order to overcome the Se-deficiency issue (Malagoli *et al.,* 2015; Wu *et al.,* 2015). The success of these programs largely depends on understanding the mechanisms of Se uptake, assimilation, and tolerance by plants (Malagoli *et al.,* 2015; Wu *et al.,* 2015; Schiavon and Pilon-Smits 2017). On the other side of the spectrum, in parts of the USA, Canada, China, and India, soils occur that are rich in Se and are named seleniferous soils; these contain 4–1200 ppm Se, which may be harmful to humans and livestock (Oldfield, 2002; Dhillon and Dhillon, 2003, Fordyce, 2013; Pilbeam *et al.,* 2015). Anthropogenic activities may exacerbate the toxic effects of Se, through e.g., irrigated agriculture or mining. Plants may be used for the remediation of naturally occurring or polluted high-Se soils and waters (Schiavon and Pilon-Smits, 2017).

Selenium deficiency and toxicity concerns are not only related to Se concentration in soil, but also to its chemical form (Zhu *et al.,* 2009; Gupta and Gupta, 2017; Schiavon and Pilon-Smits 2017). Selenium in soil and organisms can exist in different oxidation states and in various inorganic and organic forms, which can interconvert via chemical or biochemical processes (Stadtman, 1990; Schiavon and Pilon-Smits 2017). Owing to its chemical similarity to Sulfur (S), the conversion of inorganic Se into organic compounds can be realized via a non-specific route that involves the S assimilation pathway, as described for plants (Sors *et al.,* 2005a; White 2016, White, 2018). In addition, in organisms that have an essential requirement for Se, its conversion

can be mediated by Se-specific enzymes, particularly its Se-specific incorporation into selenoproteins (Wilber, 1980; Brown and Shrift, 1982; Stadtman, 1990; Anderson, 1993; Mihara *et al.,* 2000, Mihara *et al*., 2006).

Evolutionary analyses support the assumption that essential Se metabolism in animals and certain algae (e.g., *Chlamydomonas reinhardtii*) evolved early and the environment played a crucial role in its further evolution, loss, or persistence in different clades (Lobanov *et al.,* 2007; Schiavon and Pilon-Smits 2017). The loss of selenoproteomes in plants, fungi and some animals arguably happened via independent events and because of one or more undetermined environmental factors (Novoselov *et al.,* 2002; Lobanov *et al.,* 2007). It has been hypothesized that aquatic life preserved Se metabolism in photosynthetic organisms, while terrestrial habitats dramatically reduced the metabolic dependence on Se because of its restricted availability (Lobanov *et al.,* 2007; Zhang and Gladyshev, 2009).

As far as it is known, plants do not possess essential selenoproteins, and therefore they lack systems that specifically incorporate Se-amino acids into selenoproteins structures (Van Hoewyk *et al.,* 2008; Schiavon and Pilon-Smits, 2017). Conversely, organisms that need Se have evolved a machinery that cotranslationally inserts SeCys into the active site of selenoproteins via the recoding of the opal stop codon UGA to function as a SeCys codon (Driscoll and Copeland, 2003; Papp *et al.,* 2010). SeCys is also referred to as the 21st protein amino acid. Selenoproteins generally function in redox reactions; use of Se in the catalytic site of proteins offers an advantage over Cys owing to improved redox activity (see part 6.2 of this chapter for more details).

Although lacking essential Se metabolism, plants can experience an array of beneficial properties from Se (Hartikainen, 2005; Pilon-Smits *et al.,* 2009; Ashraf *et al.,* 2017). At low tissue concentrations, the antioxidant properties of Se can promote plant growth, productivity and

enhances resistance against different types of stresses. With increasing tissue Se concentrations, plants additionally benefit from increasing protection against herbivores and pathogens, owing to toxicity of the accumulated Se.

Reportedly, the average Se concentration worldwide is 0.44 mg kg^{-1} (Kabata-Pendias, 2011). Soil Se concentration, composition and availability varies dramatically in relation to the physicochemical characteristics of soils (see part 2 of this chapter). The accumulation of Se by plants is, to a large extent, influenced by Se concentration and phytoavailability in soils. In addition, differences between plant species exist with respect to their capacity to accumulate Se under the same environmental conditions. Plants readily take up Se even though they do not require it, owing to its similarity to S, and plants that are known for their high tissue S levels, such as members of the onion and cabbage families, also tend to accumulate more Se (White *et al.,* 2004; White *et al.,* 2007; White, 2016). Plant species thriving on seleniferous soils hold a special position in this respect. Around 50 taxa from different families are so-called Se hyperaccumulators: they have evolved strategies to prevent Se toxicity while accumulating high tissue Se concentrations (Rosenfeld and Beath, 1964; Brown and Shrift 1982; El Mehdawi and Pilon-Smits, 2011; El Mehdawi *et al.,* 2018).

Plants absorb Se using different types of transporters depending on the form of Se available for uptake (White *et al.,* 2004; Zhang *et al.,* 2006; Li *et al.,* 2017; Cabannes *et al.,* 2011; Zhang *et al.,* 2014; Schiavon *et al.,* 2015). The expression of these transporters and their kinetic properties and substrate specificity vary in the plant kingdom and contribute to plant adaptation to high-Se environments. Plant species not only differ in their capacity to accumulate Se, but also in their Se metabolic properties, including the ability to produce Se volatile compounds, as well as in their preferential strategy to avoid Se toxicity (Zayed and Terry, 1992; White *et al.,* 2007; Schiavon and

Pilon-Smits, 2017). Variation in Se metabolic properties is observed between genera, species, and even ecotypes within species (Feist and Parker, 2001; White *et al.,* 2004; White *et al.,* 2007; Watanabe *et al.,* 2007; Cappa *et al.,* 2014; El Mehdawi *et al.,* 2015; White, 2016).

The observed differences in physiology and biochemistry between plant taxa in response to Se might have ecological significance and raises the question of which benefits, and potential constraints are associated with high concentrations of Se in plants, both physiologically and with respect to interactions with ecologic partners (Schiavon and Pilon-Smits, 2017). Selenium may enhance plant fitness via enhanced growth and abiotic stress resistance, protection from pathogens and herbivores, or via elemental allelopathy, i.e., competition toward other plant species that are sensitive to Se (El Mehdawi and Pilon-Smits, 2011; White, 2016; Schiavon and Pilon-Smits, 2017). Plants that exhibit the fascinating trait of Se hyperaccumulation can accumulate Se to more than 0.1% of their dry weight. These taxa are of great interest in the field of Se research, not only for intrinsic scientific value but because their study may benefit applications in Se phytotechnologies, i.e., biofortification and phytoremediation (Schiavon and Pilon-Smits, 2017). A particularly interesting trait of hyperaccumulators is their capacity to accumulate Se specifically, even in the presence of high S concentration.

In the next sections of this first chapter, I will introduce a diversity of topics related to Se in more depth, so the reader is familiarized with this fascinating and complex topic. First, I provide information about Se in the environment, where sources of Se and chemical forms of Se in the soil are discussed. Next, I will review Se uptake, transport, and assimilation by plants, and the distinguishing properties of Se hyperaccumulating species, as well as evolutionary aspects of Se hyperaccumulation. Finally, the last part of this chapter discusses the beneficial aspects of plant-derived Se to human health.

1.2 SELENIUM IN THE ENVIRONMENT

The natural occurrence and distribution of Se in soil is a result of early geological soil formation and deposition, mainly as a response to volcanic activity in the Cretaceous period in the Mesozoic era (145 million years ago), in which ashes and gases containing Se were deposited in the ocean due to rain, largely ending up in the clay section of sedimentary rocks in the earth's crust from this geological period (Kabata-Pendias, 2011).

Interestingly, the presence of Se in the soil varies greatly, not only due to geologic processes, but also due to different anthropogenic activities, mainly mining and agriculture. Additionally, Se naturally and continuously cycles through the environment. Its concentration in the soil is governed by a multitude of processes, including precipitation (via atmospheric Se deposition) and Se speciation, and the soil properties like pH, redox potential, structure, and organic matter content and composition (Golberg, 2014; Saha *et al.,* 2017; Statwick and Sher, 2017). According to the literature the Se variation in soils follows the relationship between anthropogenic and natural sources and different sinks (Wen and Carignan, 2007; Winkel *et al.,* 2015).

Soil is formed by parenting rock weathering, naturally composed of different trace elements and minerals. Se is typically found at high concentrations in clay-rich sedimentary rocks like shale, as an example, formed by volcanic activity (Saha *et al.,* 2017; Statwick and Sher, 2017). Furthermore, the concentration of Se tends to be naturally high in other soil formations where the S concentration is also elevated. It is important to note the adsorption of Se to soil is correlated with the oxidation state of the atom and the pH of the soil solution, with increased adsorption to soil particles at lower pH values (Golberg, 2014).

While the weathering of parenting rocks can be considered one of the primary natural sources of Se, different atmospheric and geogenic sources of Se to soil have been extensively studied

(Winkel *et al.,* 2015). It is estimated that a minimum of 13,000 tons of Se is cycled in the troposphere yearly (Mosher *et al.,* 1987; Wen and Carignan, 2007). from different natural and anthropogenic sources. Volcanic activity (Floor and Román-Ross, 2012; Golberg, 2014) and industrial processes, such as waste from the crude oil refining process and fossil fuel combustion, are the principal source of atmospheric Se (Wen and Carignan, 2007; Golberg, 2014).

In the soil, Se can be found at different oxidation states and under organic or inorganic forms. The oxyanions selenate (VI), as SeO_4^{2-}, and selenite (IV), mainly as HSe_3O^{3-} and SeO_3^{2-}, are commonly found in drained soil at pH values between 4 and 9. These forms are soluble and thus largely bioavailable to plants; however, their retention to soil particles increases when the pH decreases. The most reduced inorganic forms of Se that occur in natural environments are elemental Se (Se_0) and selenides, including hydrogen selenide and different metallic selenides, produced by microbial activity (Qin *et al.,* 2012). However, these Se species are insoluble and not bioavailable. Generally, the bioavailability of Se increases in more oxidizing environments, where selenate ions tend to be highly soluble and mobile in aerated, alkaline, and oxidized soils. Selenite, instead, predominates in more acidic and reducing environments (Li *et al.,* 2017; Favorito *et al.,* 2021).

Organic matter (OM) also plays a role in the retention, bioavailability, and mobilization of Se in the soil. The OM can form colloids with Se and increase its retention, and some studies suggest the OM-Se colloids might correspond up to 50% of the total soil Se in seleniferous areas (Qin *et al.,* 2012, Li *et al.,* 2017). The immobilization of Se by the soil OM is more prominent when the Se levels are relatively low; however, the type of soil and the composition of the OM is more relevant to Se mobilization than its concentration (Li *et al.,* 2017).

1.3 SELENIUM UPTAKE AND TRANSPORT BY PLANTS.

The most important aspect to be considered when studying Se accumulation by plants is the chemical similarity between Se and S. These two elements can be found in group 16 or the oxygen family (the chalcogens) in the periodic table. Their ionic radius, redox potentials, and electronegativity are similar (Wessjohann *et al.,* 2007). Selenate, the most common form of Se taken up by plants in soils, is taken up by the root system via sulfate transporters, SULTR (Gigolashvili and Kopriva, 2014; White, 2016; White, 2018), while selenite uptake is mediated by phosphate and silicon transporters (Hopper and Parker, 1999; Zhao *et al.,* 2010; Zhang *et al.,* 2014). Sulfate transporters were first characterized in Se resistant mutants of *Arabidopsis thaliana*, Sel1-8, and Sel-11 (mutations in the SUTR1;2 coding sequence), and Sel1-9 (T-DNA insertion in the SULTR1;2 promoter), (Shibagaki *et al.,* 2002; El kassis *et al.,* 2007). Four groups of sulfate transporters have been identified in plants and are responsible for the uptake and translocation of Se. SULTR 1;1 and SULTR1;2 are high-affinity H^+ co-transporters localized at the root hairs, cortex, and epidermis (Buchner, 2004) (Check item 4.2 for more information); SULTR2;1 is expressed in the xylem parenchyma and pericycle, while SULTR2;2 in the phloem and bundle sheath cells (Takahashi *et al.,* 2000); SULTR3;1 is a chloroplast transporter (Cao *et al.,* 2013), and SULTR4;1 and SULTR4;2 are efflux transporters found in the tonoplast (Gigolashvili and Kopriva, 2014).

Plants can also take up organic Se compounds, especially in the form of seleno-aminoacids acids. They do not show substantial uptake capacity for the less bioavailable forms: elemental Se, metal selenide compounds, or colloidal elemental Se (White and Broadley, 2009; White, 2016). Once inside plant cells, selenate can be assimilated into selenocysteine (SeCys) and selenomethionine (SeMet) through the biochemical pathway that is normally involved in sulfate

reduction and assimilation (Figure 1-1) (Anderson, 1993; Sors *et al.,* 2005a; White, 2016; Guignardi *et al.,* 2017; Gupta and Gupta, 2017). The non-specific incorporation of these two Se-amino acids in proteins in the place of the analogs cysteine (Cys) and methionine (Met) causes the disruption of protein folding, which is considered the main cause of Se toxicity to plants (Van Hoewyk, 2013). In this respect, plants have evolved a range of strategies to mitigate Se toxicity, which include conversion of SeCys to elemental Se and alanine, methylation of SeCys and SeMet, and conversion of these compounds to volatile dimethyl(di)selenide (DMDSe) (Shrift, 1969; Sors *et al.,* 2005a). Accumulation of Se in plant tissues and production of methylated volatile Se species are both critical for Se cycling in the environment (Winkel *et al.,* 2015). Selenium volatilization into the atmosphere by plants and microalgae may be responsible for a significant portion of Se fluxes and may contribute to the formation of seleniferous regions (Blazina *et al.,* 2014; Winkel *et al.,* 2015).

SELENIUM ACCUMULATION AND HYPERACCUMULATION IN PLANTS

There is broad variation in the capacity of plants to accumulate Se in their organs, which is largely affected by soil Se content and phytoavailability (White, 2016; White, 2018). Plants thriving in soils either naturally rich in available Se or contaminated with Se due to anthropogenic activities or dust depositions sacked by coal-burning areas are inclined to accumulate more Se than plants colonizing low Se areas (White 2016; Schiavon and Pilon-Smits 2017). The Se content in soils is commonly below 2 μg g-1 but can reach several hundred μg g-1 (up to 1.2 mg g-1) in soils derived from sedimentary rocks, especially Cretaceous sediments rich in selenites and selenides associated with sulfide minerals (Winkel *et al.,* 2015). These soils are termed seleniferous and are located in the Great Plains of the USA, Canada, Brazil, Australia, India, China, and Russia, and usually support a distinctive pattern of vegetation (Oldfield, 2002; Winkel *et al.,* 2015). Plants

growing in seleniferous areas actively remove Se from sensitive tissues, or hyperaccumulate and tolerate high internal Se concentrations (White, 2018). Despite such a different behavior towards Se, all these plants exhibit a minimal ability to tolerate elevated Se concentrations.

According to their capacity to accumulate Se, plants can be divided in three main categories: non-accumulators, which include species that accumulate less than 100 μg Se g^{-1} dry weight; secondary accumulators (or accumulators) like *Brassica juncea* and *Brassica napus*, which can contain up to 1000 μg Se kg^{-1} dry weight, can thrive on both non-seleniferous and seleniferous soils, and their tissue Se concentration is directly indicative of the Se phytoavailability in the soil (Se-indicators); hyperaccumulators, such as certain species of the genera *Stanleya* (Brassicaceae) and *Astragalus* (Fabaceae), able to accumulate over 1000 μg Se g^{-1} dry weight in all organs (0.1–1.5%) when growing on seleniferous soils (Terry *et al.,* 2000; Moreno Rodriguez *et al.,* 2005; Galeas *et al.,* 2007; White *et al.,* 2007; Pilon-Smits *et al.,* 2009; White, 2016; Schiavon and Pilon-Smits, 2017) (Check Figure 1-2 for more examples of hyperaccumulator species).

From studies so far (reviewed by El Mehdawi and Pilon-Smits, 2012), the non-accumulators and the (secondary) accumulators appear to be physiologically similar, mainly differing in the degree of uptake of S, and consequently of non-specific Se uptake. However, the hyperaccumulator plants are physiologically different, showing Se-specific uptake and metabolism to avoid the misincorporation of Se into proteins, via methylation and volatilization, and also different patterns of Se sequestration.

SELENATE TRANSPORT AND EVIDENCE FOR SPECIFIC MECHANISMS OF SE UPTAKE IN HYPERACCUMULATORS

Generally, selenate is more common and bioavailable than selenite in well-drained/oxidized and alkaline soils, while selenite is the prevalent water-soluble species in wetlands and anaerobic

soils with a neutral to acidic pH (Mikkelsen *et al.,* 1989; White *et al.,* 2007; Fordyce, 2012). Selenate is a chemical analog of sulfate (S), and thus it can enter the root cells and move throughout the plant via sulfate transporters (White *et al.,* 2004; El Kassis *et al.,* 2007). Solid evidence for the role of the sulfate transport system in selenate movement across cell membranes derives from a study conducted in *Arabidopsis thaliana* selenate-resistant mutants by Shibagaki *et al.* (2002) and El Kassis *et al.* (2007). SULTR1;2 was identified as the major portal for selenate entry into the plants, as *A. thaliana* SULTR1;2 mutants were more tolerant to selenate than wild-type plants and SULTR1;1 mutants (Barberon *et al.,* 2008). In addition to SULTR1;2, under low external S concentration or in the absence of selenate/sulfate competition, another member of the group 1 root high affinity sulfate transporters, SULTR1;1, seems to mediate selenate transport as well (El Kassis *et al.,* 2007; Rouached *et al.,* 2008; Shinmachi *et al.,* 2010; El Mehdawi *et al.,* 2018). The expression of these sulfate/selenate transporters is regulated by several factors, including the S status of the plant, the Se:S ratio in the plant organs and growth medium, and also the plant species (White *et al.,* 2004; Cabannes *et al.,* 2011; Schiavon *et al.,* 2015; White, 2016; El Mehdawi *et al.,* 2018).

Non-hyperaccumulators and hyperaccumulators often exhibit different expression levels of sulfate transporters in response to external Se and S availability, which in turn influences Se accumulation in their organs (White *et al.,* 2004; Cabannes *et al.,* 2011; Schiavon *et al.,* 2015; El Mehdawi *et al.,* 2018; Wang *et al.,* 2018). Hyperaccumulators typically show more abundant expression of sulfate transporters than non-hyperaccumulators (Figure 1-3); while this explains their high Se concentrations, it does not explain their high tissue Se:S ratio (Cabannes *et al.,* 2011).

The Se-hyperaccumulator *Stanleya pinnata* (Brassicaceae) has been recently reported to display greater root and shoot Se accumulation and less competitive inhibition by sulfate in the

short (1 h) and long term (9 days) than non-accumulator *Stanleya elata* and accumulator *Brassica juncea* (El Mehdawi *et al.,* 2018). Specifically, selenate uptake rates for *S. pinnata* were not appreciably decreased by 100-fold excess sulfate over selenate in the short term, whereas they dramatically declined for non-hyperaccumulators. These results are well correlated with the expression of different sulfate transporter genes: *S. pinnata* SULTR1;2 (expressed in root hairs, cortex, and epidermis in related model species *Arabidopsis thaliana*) and SULTR2;1 (reportedly expressed in pericycle and xylem parenchyma), were constitutively expressed at very high levels, and therefore may be responsible for higher selenate uptake and translocation to aerial parts, respectively, as compared to non-hyperaccumulators.

Constitutive expression of SULTR2;1 homolog was previously observed in hyperaccumulator spp. of the genus *Astragalus* as well, while the transcript abundance of group 1 sulfate transporters was slightly affected by S starvation (Cabannes *et al.,* 2011). Also, *S. pinnata* SULTR1;2 expression was not upregulated in the absence of S as normally observed for sulfate transporters under S deficiency in non-hyperaccumulators (Barberon *et al.,* 2008; Rouached *et al.,* 2008; Yoshimoto *et al.,* 2002). Based on these findings, the two SULTR genes are likely crucial for the Se hyperaccumulation trait in *S. pinnata* (Buchner, 2004; Rouached *et al.,* 2009; Takahashi *et al.,* 2011). The question why *S. pinnata* SULTR1;2 does not show up-regulation in response to S limitation still remains to be elucidated, but it can be hypothesized that constitutive and elevated expression of this transporter might be in part the result of gene duplication events that induced elevated selenate uptake capacity and promoted the evolution of at least one of the gene copies towards greater specificity of transport for selenate over sulfate (Hanikenne *et al.,* 2008; Lochlainn *et al.,* 2011; Craciun *et al.,* 2012). Mutations in specific cis-regulatory sequences and changes in one or more trans-regulatory elements of the transporters may be responsible for their high and

steady expression, while mutations in their coding sequence that affect protein-protein interactions and carrier function, especially in regulatory domains (e.g., STAS domain) are possible mechanisms through which selenate specificity may have evolved (Shibagaki *et al.,* 2006; Takahashi *et al.,* 2011). SULTR1 sequences identified in other Se hyperaccumulator species within the genus *Astragalus* (Fabaceae) for instance, possess one alanine residue in place of the glycine found in SULTR1 isoforms of non-accumulators, which may contribute to the preferential uptake of selenate over sulfate observed in these species (Cabannes *et al.,* 2011).

Further indication for higher selenate specificity of sulfate transporters in *S. pinnata* than in non-hyperaccumulators is suggested by the decrease of S accumulation in *S. pinnata* supplied with increasing external selenate concentrations (high Se:S ratio) (El Mehdawi *et al.,* 2018). Interestingly, SULTR1;1 for *S. pinnata* exhibited lower expression than in non-hyperaccumulators and was up-regulated by S starvation (Figure 1-1), thus suggesting its minor role in selenate acquisition in this species and SULTR1;2 as the unique root route for sulfate/selenate uptake (El Mehdawi *et al.,* 2018). The hypothesis that other sulfate/selenate transporters beside SULTR1;2 may possess higher specificity for selenate over sulfate cannot be excluded and needs more investigation. One possible candidate could be *S. pinnata* SULTR2;1, which mediates the movement of sulfate into pericycle and xylem parenchyma cells for their translocation to the aerial parts of the plant and could preferentially transport selenate over sulfate (Kataoka *et al.,* 2004; Takahashi *et al.,* 2011; Gigolashvili and Kopriva, 2014).

MECHANISMS FOR UPTAKE OF SELENITE AND ORGANIC SE-COMPOUNDS

Selenite is generally less bioavailable than selenate in most soils because it is strongly absorbed by iron and aluminum oxides/hydroxides, as well as by clays and organic matter (Fordyce, 2013; Pilbeam *et al.,* 2015). Plants take up selenite and organic forms of Se using transport pathways

that are distinct from those mediating selenate fluxes. For selenite in particular, although a passive diffusion mechanism was initially hypothesized (Shrift, 1969; Arvy, 1989; Arvy, 1993), it is now well-accepted that its transport is largely mediated by an active mechanism that involves phosphate transporters (Li *et al.,* 2017; Zhang *et al.,* 2014).

Discrepancies between old and more recent studies were likely due to the effect of pH on the formation of selenite species in the rhizosphere solution (Zhang *et al.,* 2010). Indeed, at different pH values, selenite exists in varying proportions and chemical forms as H_2SeO_3, SeO_3^{2-}, and $HSeO_3^-$ (Zhang *et al.,* 2006, Zhang *et al.,* 2010; Zhao *et al.,* 2010). Selenite in the form of H_2SeO_3 was found to be absorbed in rice via aquaporins (Zhang *et al.,* 2006) and silicon (Si) influx transporter OsNIP2;1 (Lsi1), a nodulin 26-like intrinsic membrane protein (NIP) subfamily of aquaporins (Zhao *et al.,* 2010). In the form of HSeO3, selenite enters roots sharing common transporters with phosphate (Li *et al.,* 2017; Zhang *et al.,* 2014).

In rice, the most abundant phosphate transporter expressed in roots, OsPT2, has been shown to possess selenite transport capacity because OsPT2-overexpressing and knockdown mutants exhibited a substantial increase or reduction in selenite uptake rates, respectively (Zhang *et al.,* 2014). Furthermore, Se accumulation in rice grains was higher in OsPT2-overexpressing plants compared to wild-type plants. Additionally, evidence for a pivotal role of phosphate transporters in selenite uptake is provided by a number of studies that have pointed out the decrease of selenite uptake by increases in phosphate concentration in the growth medium (Broyer *et al.,* 1972; Hopper and Parker, 1999; Zhang *et al.,* 2006). For instance, in perennial ryegrass (*Lolium perenne* L. cv. Evening Shade) and strawberry clover (*Trifolium fragiferrum* L. cv. O'Conner) selenite uptake dropped by about 50% when external phosphate concentration was increased 10- fold (Hopper and

Parker, 1999), and in wheat (*Triticum aestivum*) the affinity for selenite transport was reduced by the presence of phosphate (Li *et al.,* 2017).

Plants are also able to absorb organic forms of Se directly, primarily Se-amino acids selenocysteine (SeCys), selenomethionine (SeMet), and methylselenocysteine (MeSeCys) (Kikkert and Berkelaar, 2013; White, 2016). Studies performed with durum wheat (*Triticum turgidum*) and spring canola (*Brassica napus*) showed that SeCys and SeMet were preferentially absorbed over either selenate or selenite (Zayed *et al.,* 1998; Kikkert and Berkelaar, 2013).

Broad specificity amino acid permeases likely play a major role in the uptake of Se amino acids, as suggested by competition studies of proline uptake in *A. thaliana* using Cys and Met as substrate competitors (Frommer *et al.,* 1993). Interestingly, in an RNA-Seq study, an amino acid transporter with sequence similarity to *A. thaliana* LHT1 (lysine-histidine transporter 1) was found to display significantly higher expression in Se-hyperaccumulator *S. pinnata* than in non-accumulator *S. elata* (Figure 1-1), and its transcription increased in roots of hyperaccumulator by selenate (Wang *et al.,* 2018). It is feasible that seleno-amino acids are taken up and translocated by this amino acid transporter as well, and that this transporter contributes to Se hyperaccumulation in *S. pinnata*.

1.4 SELENIUM BIOCHEMISTRY

SELENOPROTEIN SYNTHESIS IN ORGANISMS THAT REQUIRE SELENIUM

Plants do not require Se, as far as is currently known, although some algae do, as well as many animals and prokaryotes. Selenoprotein formation is an interesting process, worth mentioning here. A selenocysteine insertion sequence (SECIS) in the 3′ untranslated region (UTR) of selenoproteins drives the UGA recoding as SeCys, and selenocysteine-tRNA([Ser]Sec) holds the anticodon complementary to this UGA codon (Zhang and Gladyshev, 2009; Bulteau and Chavatte,

2015). The SeCys-tRNA initially binds the amino acid serine, which is further enzymatically converted to SeCys by modification of the hydroxyl(-OH) group to selenol (SeH) (Stadtman, 1990). Genes containing SECIS elements are fairly similar but not identical between animals and aquatic photosynthetic organisms that need Se, and those from the microalga *C. reinhardtii* in particular were shown to direct the synthesis of selenoproteins in mammals, thus reinforcing the hypothesis that Sec insertion mechanisms in photosynthetic organisms and animals share a common origin (Novoselov *et al.,* 2002).

SELENIUM ASSIMILATION IN PLANTS: FROM INORGANIC SELENIUM TO SE-AMINO ACIDS

Once absorbed by plants, inorganic Se is assimilated into Se-amino acids via the S assimilation pathway by virtue of its chemical similarity to S (White *et al.,* 2004; Sors *et al.,* 2005a; Bulteau and Chavatte, 2015; Schiavon and Pilon-Smits, 2017). Most enzymes involved in this pathway are upregulated by S limitation in plants, but in hyperaccumulators they often show constitutive expression (Van Hoewyk *et al.,* 2005; Van Hoewyk *et al.,* 2008; Takahashi *et al.,* 2011; Freeman *et al.,* 2010; White, 2016). The assimilation process happens in part in the plastid, and the envelope-localized sulfate transporter SULTR3;1 delivers selenate from the cytosol to the stroma of the organelle (Cao *et al.,* 2013). The first step in selenate reduction is mediated by the enzyme ATP sulfurylase, which couples selenate (or sulfate) to ATP with formation of adenosine 5′-phosphosulfate/selenate (APS/APSe) (Sors *et al.,* 2005a; Pilon-Smits *et al.,* 2009; Schiavon *et al.,* 2015). This step seems to be rate limiting for Se assimilation (Pilon-Smits *et al.,* 1999). It can take place in both the cytosol and plastids (Takahashi *et al.,* 2011; Bohrer *et al.,* 2015), because different isoforms of ATP sulfurylase exist in these compartments. In *A. thaliana*, for instance, four ATP sulfurylase isoforms have been identified, three of them localizing only to the plastid (APS1, 3 and

4) and one, isoform 2 (APS2), having dual localization (cytosol and plastids) (Anjum *et al.,* 2015; Bohrer *et al.,* 2015).

Interestingly, the gene encoding APS2 showed extremely elevated expression in roots of Se-hyperaccumulator *S. pinnata* compared to non-hyperaccumulator *S. elata* (over 120-fold), while in leaves its expression was 2–4 fold higher in *S. pinnata* than *S. elata* (Schiavon *et al.,* 2015; Wang *et al.,* 2018). This observation suggests that overexpression of APS2 may be in part responsible for hypertolerance and hyperaccumulation traits in *S. pinnata*. APS2 may be envisioned as a target for genetic engineering to develop plants with superior Se uptake, accumulation, and tolerance capacity to use in both biofortification and phytoremediation technologies. Previously, only the isoform APS1 from *A. thaliana* has been overexpressed in plants (Pilon-Smits *et al.,* 1999; Sors *et al.,* 2005b;). *Brassica juncea* transgenics overexpressing APS1 exhibited increased selenate reduction and assimilation into organic Se compounds as compared to wild-type plants, which mainly contained selenate in their organs (Pilon-Smits *et al.,* 1999). The enhanced capacity of APS-overexpressing transgenics to accumulate Se was further confirmed in greenhouse and field experiments (Van Huysen *et al.,* 2004; Banuelos *et al.,* 2005).

Once APSe is produced, Se assimilation proceeds towards the conversion of this compound to selenite in a rate-limiting step catalyzed by the enzyme APS reductase (APR) (Sors *et al.,* 2005a; Suter *et al.,* 2000) (Figure 1-1). Evidence in support of a role for APR in this respect derives from studies on *A. thaliana* transgenics. apr2-1 mutants in particular, were shown to contain high concentration of selenate and negligible amounts of selenite (Grant *et al.,* 2011; Chao *et al.,* 2014), as well as low S flux from sulfate to reduced S compounds and proteins (Chao *et al.,* 2014), while plants overexpressing APR had increased Se flux throughout the plant and high rate of selenate assimilation into amino acids (Sors *et al.,* 2005a). The catalytic capacity of APR2 was found to

vary by 4 orders of magnitude across the *A. thaliana* species range and corresponds with significant differences in S and Se metabolism (Chao *et al.,* 2014), However, among eight *Astragalus* species with varying abilities to accumulate Se, no correlation was observed between Se hyperaccumulation and APR expression (Sors *et al.,* 2005b). Thus, APR may be more rate-limiting for Se assimilation in non-hyperaccumulators than in hyperaccumulators.

In the next step of Se assimilation, selenite is converted to selenide (Se^{2-}). This conversion has been proposed to occur enzymatically by sulfite reductase (SiR) (Yarmolinsky *et al.,* 2012; White, 2016), or non-enzymatically via glutathione mediated reduction, with formation of selenodiglutathione (GSSeSG) and selenopersulfide (GSSeH) as intermediates, and superoxide as a byproduct (Terry *et al.,* 2000; Anderson *et al.,* 2001). GSSeH is then converted to selenide by the enzyme glutathione reductase (GR) (Hsieh and Ganther, 1975). Ultimately, selenide is incorporated into SeCys by the enzyme complex cysteine synthase, also named O-acetylserine (thiol) lyase (OASTL), which catalyzes the formation of SeCys from O-acetylserine (OAS) and selenide (Sors *et al.,* 2005a; Terry *et al.,* 2000; White, 2016). The conversion of SeCys to SeMet implies the formation of the intermediates selenocystathionine and selenohomocysteine (SeHCys) and is catalyzed in series by three enzymes: cystathionine γ synthase (CGS), which catalyzes the formation of Se-cystathionine through condensation of O-phosphohomoserine (OPH) and SeCys (Huysen *et al.,* 2003; Sors *et al.,* 2005a) and is rate-limiting for conversion of SeCys to volatile DMSe (Huysen *et al.,* 2003), cystathionine β-lyase (CBL) and methionine synthase (McCluskey *et al.,* 1986; Cossins and Chen, 1997; Sors *et al.,* 2005a). Interestingly, several Se hyperaccumulator Stanleya species accumulate high concentrations of selenocystathionine in their tissues (Birringer *et al.,* 2002; Freeman *et al.,* 2006; Freeman *et al.,* 2010).

BENEFICIAL EFFECTS OF SE-INDUCED ANTIOXIDANTS ON PLANT PRODUCTIVITY AND OXIDATIVE STRESS RESISTANCE

Plants can be faced with different environmental conditions that generate oxidative stress via production of Reactive Oxygen Species (ROS) and must activate different strategies to overcome it. ROS are the unstable and partially reduced forms of atmospheric oxygen (O_2), which show a great capacity to oxidize other cell compounds. These molecules are formed from the transfer of one, two or three electrons to the O_2 molecule, thus forming the superoxide radical ($O2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$), respectively. This is particularly prone to happen in electron transfer processes in mitochondria, chloroplasts, and peroxisomes (Shieber and Chandel, 2014).

Various cellular defense responses are important for maintaining low concentrations of ROS and involve both enzymatic and non-enzymatic antioxidant mechanisms (Figure 1-3). Superoxide dismutases (SOD) constitute the first enzymatic barrier against oxidative stress by the dismutation reaction of $O2^{-\bullet}$ in order to form O_2 and H_2O_2 (Shieber and Chandel, 2014). Subsequently, H_2O_2 can be quickly converted into H_2O and O_2 by specific peroxidases (POX), enzymes such as catalase (CAT) and glutathione peroxidase (GSH-Px) (Roychoudhury *et al.,* 2012). High concentration of H_2O_2 in the cellular environment as a response to a stressful condition or SOD activity can cause oxidative damage. Non-enzymatic molecules implied in ROS detoxification are also important to preserve the cellular redox state, and mainly include the reduced form of glutathione (GSH), ascorbate, phytochelatins (PCs), proline, flavonoids, alkaloids, and carotenoids (Foyer and Noctor, 2012).

Selenium has been reported to help plants cope with stress by stimulating the plant cell antioxidant capacity through the enhancement of the activity of antioxidant enzymes (SOD, CAT

and GSH-Px) and the synthesis of GSH, PCs, ascorbate, proline, flavonoids, alkaloids, and carotenoids. Furthermore, Se may induce the spontaneous dismutation of the superoxide radical ($O2^{-\bullet}$) into H_2O_2 (Feng *et al.,* 2013). As a result of Se-increased antioxidant defense systems, lower levels of lipid peroxidation were observed under metal-induced oxidative stress conditions, because of reduced ROS accumulation (Feng and Wei, 2012) (Figure 1-3). In addition to its function in mitigating heavy metal stress in plants, Se at low dosage has been shown to protect plants from a variety of other abiotic stresses including drought, cold, heat, salinity, and UV-B radiation, which also cause oxidative stress (Feng *et al.,* 2013; Kaur *et al.,* 2016).

Even when growing under optimal conditions, plant cells accumulate ROS to some extent, particularly in mitochondria and chloroplasts at the sites of electron transport. Therefore, the ROS scavenging machinery described in the previous section is constitutively important (Figure 1-3). This may explain the reported beneficial effects of Se on plants via promotion of growth, (Terry *et al.,* 2000; Pilon-Smits *et al.,* 2009; White and Broadley 2009) and productivity (Xue *et al.,* 2001; Djanaguiraman *et al.,* 2010; Jiang *et al.,* 2015; Kaur and Nayyar, 2015) under both stress and no stress environments. There is evidence that Se may improve plant productivity via amelioration of photosynthesis, as this process is stimulated in plants by optimal supplementation with Se during the vegetative period. For instance, the application of Se in rice has been reported to positively influence photosynthesis, which resulted in increased rice grain yield and Se grain concentration . Similar results were reported in other plant species treated with Se, like ryegrass (Hartikainen *et al.,* 2000), potato (Turakainen *et al.,* 2004), *B. rapa* (Lyons *et al.,* 2009), and lentil (Ekanayake *et al.,* 2015).

The positive effects of low Se concentrations on the photosynthetic process may be explained via the enhancement of the antioxidant activity in cells at different levels (Figure 1-4). Selenium

can up-regulate the amount and activity of antioxidant enzymes (GSH-Px, GR, SOD, APX and CAT) and metabolites (GSH, ascorbate) resulting in higher ROS scavenging capacity of plants, as well-documented under stress conditions (Germ *et al.,* 2007; Tadina *et al.,* 2007; Djanaguiraman *et al.,* 2010; Feng *et al.,* 2013). In addition to this effect on the antioxidant machinery, appropriate Se concentrations could significantly improve photosynthesis by increasing the production of chlorophyll (Hawrylak-Nowak, 2009; Yao *et al.,* 2011; Liu *et al.,* 2011), stomatal conductance, intercellular CO_2 concentration, and transpiration efficiency (Germ *et al.,* 2007; Djanaguiraman *et al.,* 2010). In other photosynthetic organisms like algae, no significant effect of Se on photosynthesis or modification of chloroplast ultrastructure were observed, with the exception of the increase in content of carotenoids, which are known to act as important intracellular antioxidants (Schiavon *et al.,* 2012).

PLANT MECHANISMS TO AVOID SE TOXICITY: HYPERACCUMULATORS VERSUS NON-HYPERACCUMULATORS

A prominent cause of Se toxicity to plants likely is the misincorporation of Se-amino acids into proteins (Stadtman, 1990; Van Hoewyk *et al.,* 2008; Pilon-Smits, 2012). In addition, inorganic Se anions may cause oxidative stress by depletion of the GSH cellular pool and production of the superoxide radical (O_2^-) that damages cytosolic iron-sulfur (Fe-S) clusters, mitochondrial proteins and chloroplastic iron-sulfur proteins (Fisher *et al.,* 2016). Selenium may also be misincorporated into Fe-Se clusters, since the enzyme that releases elemental S from Cys for the formation of Fe-S clusters can also utilize SeCys as a substrate (Van Hoewyk, 2005).

One key tolerance mechanism of Se hyperaccumulators is likely to be their capacity to prevent the incorporation of seleno-amino acids into proteins (Brown and Shrift 1982). Among the different mechanisms that Se hyperaccumulators exploit, one is the methylation of SeCys to form

MethylSeCys (MeSeCys) via a reaction catalyzed by the enzyme SeCys methyltransferase (SMT) (Figure 1-4) (Neuhierl and Bock, 1996; Pilon-Smits *et al.,* 2009). In this way, the amount of SeCys that non-specifically replaces Cys in proteins is significantly reduced. SMT is chloroplast localized (Sors *et al.,* 2009) and has been identified in both non-accumulator and Se hyperaccumulator species of the genus Astragalus. However, only the functional isoform of this enzyme, found in hyperaccumulators, is able to produce MeSeCys and shows preference for methylation of SeCys over Cys (Neuhierl and Bock, 1996; Neuhierl *et al.,* 1999; Sors *et al.,* 2009). This explains why Se-hyperaccumulators, such as *A. bisulcatus* and *S. pinnata*, contain significantly high concentration of MeSeCys in their tissues than non-accumulator species, which mainly accumulate inorganic Se (Neuhierl *et al.,* 1999; Pickering, 2003; Sors *et al.,* 2005b; Freeman *et al.,* 2006; Freeman *et al.,* 2010) (Figures 1-2, 1-4, and 1-5). Although, SMT is constitutively and highly expressed in hyperaccumulators, it also can be induced by Se in some Se accumulators (e.g., *B. oleracea*) (Lyi *et al.,* 2005; Pilon-Smits, 2012). SMT from *A. bisulcatus* has been overexpressed in non-hyperaccumulators *A. thaliana* and *B. juncea*, leading to enhanced Se accumulation (primarily as MeSeCys and γ glutamyl-MeSeCys), tolerance and volatilization (Ellis *et al.,* 2004; LeDuc *et al.,* 2004; Bañuelos *et al.,* 2007a).

An additional mechanism by which hyperaccumulators tolerate high Se concentration in tissues is the conversion of MeSeCys into volatile dimethyldiselenide (DMDSe) (Figures 1-2, 1-4 and 5) (Pilon-Smits and LeDuc, 2009). This process happens in leaves, where MeSeCys is initially converted to methylselenocysteineselenideoxide (MeSeCysSeO) that is then transformed into methaneselenol (CH_3SeH) by the activity of the enzyme Cys sulfoxide lyase (Ellis and Salt, 2003; Chin and Lindsay, 2004). In non-hyperaccumulators, a possible metabolic shunt to mitigate Se toxicity involves volatilization of SeMet to form dimethylselenide (DMSe) (Schiavon and Pilon-

Smits, 2017; Terry *et al.,* 2000; Tagmount, 2002). The synthesis of DMSe involves first methylation of SeMet to produce Se-methyl Se-Met (SeMM) in a reaction catalyzed by the enzyme S-adenosyl-L-Met: Met-S-methyl transferase (MMT) (Tagmount, 2002), and then proceeds via SeMM conversion to intermediate molecule 3-dimethylselenoniopropionate (DMSeP) or directly from SeMM via the enzyme methylmethionine hydrolase (Mudd and Datko, 1990; Kocsis, 1998; Chin and Lindsay, 2004; Tagmount, 2002). In addition to diverting potentially toxic Se amino acids into less toxic volatile compounds, generation of Se volatile DMDSe and DMSe might also have a role in plant defense against herbivores (Meija *et al.,* 2002; Quinn *et al.,* 2010; Schiavon and Pilon-Smits, 2017).

Aside from the production of MeSeCys and volatile compounds, another metabolic route that prevent the incorporation of SeCys in proteins involves the activity of the enzyme selenocysteine lyase (SL), which breaks down SeCys into elemental Se and alanine (Figure 1-1) (Van Hoewyk *et al.,* 2005). This enzyme is analogous to NifS-like Cys desulfurase proteins characterized in A. thaliana, whose function is to produce free S from Cys for the formation of Fe-S clusters (Ye *et al.,* 2005). Its overexpression in A. thaliana conferred higher Se tolerance and accumulation (2-fold) and decreased Se incorporation into proteins (Van Hoewyk *et al.,* 2005; Bañuelos *et al.,* 2007b). In the same transgenics, S accumulation was increased as well, which may explain why the formation of Fe-S clusters was not affected by higher production of elemental Se (Van Hoewyk *et al.,* 2005).

Another potentially important mechanism for Se tolerance in hyperaccumulators is the ability to sequester organic Se forms (C-Se-C compounds, likely a majority MeSeCys with minor fraction of selenocystathionine) into specific compartments away from sensitive key biochemical processes (Cappa *et al.,* 2015). Selenium in leaves of *A. bisulcatus* was found in leaf hairs (Freeman *et al.,*

2006), and *S. pinnata* stores it in the vacuole of epidermal cells along the leaf periphery (Freeman *et al.,* 2006; Freeman *et al.,* 2010; Cappa *et al.,* 2015). In contrast, in non-hyperaccumulator species, Se was mainly restricted in the vascular tissues (Freeman *et al.,* 2006; Cappa *et al.,* 2014). *S. pinnata* also accumulates high concentrations of Se in the form of MeSeCys in the flowers (Quinn *et al.,* 2011).

In parallel to the assimilation of inorganic Se into non-toxic organic Se compounds and specific sequestration patterns, hyperaccumulator species appear to have constitutive upregulation of antioxidant defense systems to cope with the oxidative damage caused by the excess Se in the cellular environment (Figure 1-1). While low concentrations of Se in tissues can enhance antioxidant defense mechanisms in different plant species, providing protection against abiotic stresses (Pilon-Smits *et al.,* 2009; Feng and Wei, 2012; Hasanuzzaman *et al.,* 2012; Malik *et al.,* 2012; Feng *et al.,* 2013), excess Se can imbalance the cellular redox state due to the generation of reactive oxygen species (ROS) that disrupt proteins, cause peroxidation of membrane lipids and oxidative stress (Gupta and Gupta, 2016; Gupta and Gupta, 2017).

A number of studies suggest that Se is directly involved in antioxidant metabolism in hyperaccumulator plants (Freeman *et al.,* 2010; Hasanuzzaman *et al.,* 2012; Malik *et al.,* 2012). Different defense-related enzymes and hormones are highly expressed in these plants (Figures 1-1, and 1-5). As shown by Freeman *et al.* (2010), hyperaccumulator *S. pinnata* has 1.5-fold higher antioxidant capacity when compared to non-hyperaccumulator *Stanleya albescens*. After 10 weeks of exposure to 20 μM selenate, the leaf concentrations of ROS O_2^- and H_2O_2 in *S. pinnata* were lower when compared to the non-accumulator *S. albescens*. Interestingly, the total glutathione concentration in the hyperaccumulator was 1.3-fold higher than in the non-hyperaccumulator under the same conditions. Specifically, *S. pinnata* had 1.4- fold more reduced glutathione (GSH)

and 1.2-fold more oxidized glutathione (GSSG) when compared to the non-hyperaccumulator *S. albescens*.

GSH, a S-containing metabolite formed from the amino acids glutamate (Glu), cysteine and glycine (Gly), constitutes a major defense mechanism against oxidative stress, participating with the ascorbate peroxidase (APX) enzyme in the ascorbate-GSH cycle (Foyer and Noctor, 2012). The antioxidant activity in the cycle is dependent on glutathione reductase (GR), responsible for the conversion of GSSG into GSH using NADPH as an electron donor. GSH is used by the enzyme dehydroascorbate reductase (DHAR) to produce ascorbate (ASC), which is used as a substrate for the APX antioxidant enzyme activity (Inzé and montage, 1995). Wang *et al.* (2018), found that important genes mediating the synthesis of GSH (glutathione synthetase, gsh1), ROS scavenging (GSH peroxidase, gpx6, thioredoxin peroxidase, tpx1, ascorbate peroxidase, apx1) were highly expressed in *S. pinnata* as compared to non-hyperaccumulator *S. elata*, which could explain the lower ROS concentration and higher GSH content in the hyperaccumulator, suggesting that this species is more likely to deal with oxidative stress.

In addition to antioxidant enzymes, defense phytohormones such as jasmonate (JA), salicylic acid (SA) and ethylene seem to play a central role in Se tolerance and hyperaccumulation (Figure 1-5) (Freeman *et al.,* 2010). Similar processes may be important in non-accumulators. Tamaoki *et al.* (2008) reported that enhanced Se (selenite) resistance in *A. thaliana* could be triggered by higher concentrations of jasmonate (JA) and ethylene, coupled with enhanced S uptake and reduction. Constitutive up-regulation of genes involved in signaling pathways mediated by stress hormones was also described in *S. pinnata* by Freeman *et al.* (2010) and confirmed recently in a RNAseq study by Wang *et al.* (2018). Several genes implied in the biosynthesis of JA were more expressed in *S. pinnata* compared to non-hyperaccumulator *S. elata*.

While these differences between Se hyperaccumulators and non-hyperaccumulators can in part explain their different capacity to tolerate and accumulate Se, still much remains to be discovered about key genes upstream of upregulated pathways, and mechanisms for Se-specific transport. Also, a better understanding of the benefits and potential constraints of Se hyperaccumulation in high-Se or low-Se environments, and the interaction of Se with other defense mechanisms are fascinating questions to address further.

1.5 EVOLUTIONARY ASPECTS OF PLANT SELENIUM HYPERACCUMULATION

SELENIUM HYPERACCUMULATION ACROSS THE PLANT KINGDOM INDICATES CONVERGENT EVOLUTION

Selenium-hyperaccumulating species are rare and denote plants with an ability to accumulate and tolerate extremely high Se (White, 2016; Schiavon and Pilon-Smits, 2017) (Figure 1-2). They have been defined by their capacity to concentrate more than 1 mg Se g^{-1} DW in their shoots while growing in their native environment, which is generally restricted to seleniferous soils. The bioconcentration factor (BF) for these species is generally very high (e.g., over 800:1 in *Astragalus bisulcatus*); BF may perform better than the absolute concentration threshold in discriminating hyperaccumulators from non-accumulators (Statwick and Sher, 2017). The BF, as well as the translocation factor (TF), often negatively correlates with the plant biomass in non-hyperaccumulators because of Se toxicity. Conversely, in hyperaccumulators, despite the apparent costs required to actively concentrate high Se in tissues through energy-dependent mechanisms, Se seems to stimulate plant growth (Statwick and Sher, 2017) (Figures 1-4, and 1-5).

The discovery of Se-hyperaccumulators was made by Orville A. Beath and coworkers in the 1930's in the western United States (Rosenfeld and Beath, 1964), Se-hyperaccumulators that populate natural seleniferous soils in these areas were found to accumulate up to 15 mg Se g^{-1} DW

(Schiavon and Pilon-Smits, 2017). This is probably a derived trait from non-hyperaccumulators that is found in at least 45 taxa in 14 genera from 6 dicot plant families (White, 2016; Schiavon and Pilon-Smits, 2017).

The trait appears to have evolved independently in different lineages, which therefore might possess distinct hyperaccumulation mechanisms. Nevertheless, hyperaccumulators from different families show many similarities in Se hyperaccumulation mechanisms, likely as a result of convergent evolution (El Mehdawi and Pilon-Smits, 2012; Cappa and Pilon-Smits, 2014; White, 2016). Families that include most of hyperaccumulator species are Asteraceae (genera Dieteria, Grindelia, Gutierrezia, Oonopsis, Symphyotrichum, and Xylorhiza), Amaranthaceae (genus Atriplex), Brassicaceae (genera Cardamine and Stanleya), Fabaceae (genera Acacia, Astragalus and Neptunia), Rubiaceae *(Coelospermum decipiens*) and Orobanchaceae (*Castilleja augustifolia* var. dubia) (White, 2016) (Figure 1-2 depicts representative Se hyperaccumulator plants. Table 1-1). An interesting species that deserves mention because of its importance as dietary source of Se is *Bertholletia excelsa* (Brazil nut tree), which belongs to the Lecythidaceae family. *B. excelsa* is reported to accumulate up to about 68 mg Se kg^{-1} nut fresh weight, but this number can vary dramatically according to the soil in which the tree is cultivated (Silva Junior *et al.,* 2017, see chapter 2).

The genus *Astragalus* of the Fabaceae family comprises 25 Se hyperaccumulator species, while the Asteraceae genera *Xylorhiza* and *Symphyotrichum* contain 3 Se hyperaccumulator species each (Table 1). In other cases, there are only one or two species per genus, such as *Stanleya pinnata* and *Stanleya bipinnata* from the genus Stanleya, *Cardamine violifolia* from the genus Cardamine of the Brassicaceae family, and N*eptunia amplexicaulis* from the genus Neptunia of the Fabaceae family (Rosenfeld and Beath 1964; El Mehdawi and Pilon-Smits, 2012; White, 2016). Except for

Cardamine violifolia, which is native to seleniferous soils in the Yutangba region in China, and *Neptunia amplexicaulis*, which grows in seleniferous soils in Queensland (Australia), Se-hyperaccumulators are commonly native to seleniferous soils of the Western USA (Schiavon and Pilon- Smits, 2017).

The species and varieties within the genus Stanleya have been investigated for their capacity to accumulate and metabolize Se (Cappa *et al.,* 2014; Cappa *et al.,* 2015). Tissue Se concentration differed considerably among Stanleya spp., with *Stanleya pinnata* var. pinnata, *S. pinnata* var. integrifolia and *S. bipinnata* being the unique hyperaccumulators. These all are part of the *S. pinnata* species complex, which also contains the non-hyperaccumulator *Stanleya pinnata* var. inyoensis. Among the taxa tested, S. *pinnata* var. inyoensis and *Stanleya elata* showed the lowest Se concentration, and *S. albescens*, *S. viridiflora,* and *S. tomentosa* contained intermediate values. While Se hyperaccumulation appeared restricted to several members of the *S. pinnata* species complex, Se tolerance was widespread within the Stanleya genus (Cappa *et al.,* 2015).

However, it must be noted that within a Se hyperaccumulating species there are populations that largely differ in their capacity to accumulate Se, as well as individuals within the same population (Schiavon and Pilon- Smits, 2017). Such differences are mainly due to genetic variability, local Se availability, and perhaps associated rhizosphere and endophytic microorganisms (Schiavon and Pilon- Smits, 2017).

POSSIBLE SELECTION PRESSURES DRIVING THE EVOLUTION OF SE HYPERACCUMULATION

Different hypotheses have been formulated regarding the selection pressures that may have driven the convergent evolution of Se hyperaccumulation and, more broadly, elemental hyperaccumulation in different taxonomic clades (El Mehdawi and Pilon-Smits, 2011; Cappa and

Pilon-Smits, 2014). Selenium concentration, speciation and phytoavailability in soil may be qualifying conditions for the evolution of Se hyperaccumulation; the geographic distribution of hyperaccumulator species generally is correlated with Se distribution in soil (White, 2016). Only a small proportion of the plant species inhabiting seleniferous soils hyperaccumulate Se, and thus the presence of Se in soil is not sufficient to explain the development of the hyperaccumulation trait, but additional physiological and ecological factors likely play a critical role. The observation that Se hyperaccumulator species mainly occur in seleniferous areas suggests that they rely on Se for their competitive fitness and perhaps their physiology (White, 2016; Schiavon and Pilon-Smits, 2017). Indeed, hyperaccumulators physiologically benefit from Se, as evidenced from a much more pronounced positive growth response to Se than non-hyperaccumulators, yet there is no evidence that they require Se (El Mehdawi and Pilon-Smits, 2012). However, most likely, ecological benefits from elevated Se concentrations, particularly protection from biotic stressors, are the major selective advantage of Se hyperaccumulation (El Mehdawi and Pilon-Smits, 2011).

Mechanistically, the acquisition of Se tolerance likely evolved prior to the capacity to hyperaccumulate Se. In the genus *Stanleya*, early steps in the evolution of the Se hyperaccumulation syndrome may have enhanced Se tolerance due to higher antioxidant levels, tissue-specific Se sequestration, and high conversion of Se to non-toxic organic forms (Feng and Wei, 2012). To mediate these traits, hyperaccumulators have constitutive high expression of genes involved in the synthesis of and responses to stress-related hormones (ethylene, jasmonic acid, salicylic acid), as well as enzymes involved in antioxidant processes, and in metabolic conversion of selenate to MeSeCys (Figures 1-4, and 1-5) (Freeman *et al.,* 2010; Wang *et al.,* 2018). Owing to this upregulated network of abiotic and biotic defense mechanisms, hyperaccumulators have

been selected in evolution to tolerate and accumulate high concentrations of Se in all their organs, with concomitant significant ecological benefits.

The ecological benefit of increased protection from herbivores and pathogens in particular, may have acted as selection pressure for the evolution from non-accumulators via Se accumulators to Se hyperaccumulators. Indeed, Se has been found to protect both Se accumulator plants like *B. junce*a and Se hyperaccumulator plants like *S. pinnata* and *A. bisulcatus* from a wide variety of herbivores and pathogens, via both deterrence and toxicity (El Mehdawi and Pilon-Smits, 2011; Freeman *et al.,* 2009; Quinn *et al.,* 2010). For example, in a greenhouse study, the leaf Se concentrations as low as 10 mg kg^{-1} DW already offered *B. juncea* protection against aphid herbivory (Hanson *et al.,* 2003) and in a 2-year manipulative field study, the Se concentrations up to 750 mg kg^{-1} DW were shown to protect *S. pinnata* against herbivory by black-tailed prairie dogs (*Cynomys ludovicianus*) (Freeman *et al.,* 2009). High leaf Se concentrations were also found to protect *B. juncea* from two pathogenic fungi (Hanson *et al.,* 2003).

Interestingly, the protection by Se against herbivory seems to extend to plants growing close to hyperaccumulators (El Mehdawi *et al.,* 2011). Leaf damage and arthropod load was lower in *Artemisia ludoviciana* and *Symphyotrichum ericoides* individuals growing in close proximity to hyperaccumulators *S. pinnata* or *A. bisulcatus*. This was associated with higher leaf Se concentrations. In a further laboratory experiment, the protective effect of growing next to hyperaccumulators was confirmed. Grasshoppers from the same site were collected and used in choice- and non-choice feeding studies with high or low leaf Se *A. ludoviciana* and *S. ericoides* plants also collected in the field either in proximity to Se hyperaccumulator *A. bisulcatus* or away from it (El Mehdawi *et al.,* 2011). The grasshoppers chose to feed on the low Se plants collected away from hyperaccumulators and suffered toxicity when forced to feed on high-Se plants

collected next to hyperaccumulators (El Mehdawi *et al.,* 2011). The elevated Se content found in neighboring vegetation around hyperaccumulators was associated with 7 to 10-fold elevated soil Se concentration.

Perhaps hyperaccumulators can increase their surrounding soil Se concentration via litter deposition or root exudation. While, as in the case of *A. ludoviciana* and *S. ericoides*, this may benefit neighboring plants if they are tolerant to the Se, it may mediate elemental allelopathy to Se-sensitive neighbors and thus help avoid plant-plant competition (Fig. 3) (El Mehdawi *et al.,* 2011; El Mehdawi and Pilon-Smits, 2012; El Mehdawi *et al.,* 2015). Indeed, soil collected next to hyperaccumulators was toxic to Se-sensitive *A. thaliana* (El Mehdawi *et al.,* 2011). Thus, hyperaccumulators can negatively or positively affect different members of the plant community nearby through the enrichment of soil with Se: The Se-sensitive neighboring species will suffer from Se toxicity, while Se-tolerant species will experience less herbivory (El Mehdawi *et al.,* 2011; El Mehdawi and Pilon-Smits, 2012). Because hyperaccumulators transform inorganic to organic Se, they can change not only the concentration but also the Se speciation in the soil, which can additionally promote Se uptake by other plants (Figure 1-5) (El Mehdawi and Pilon-Smits, 2012; El Mehdawi *et al.,* 2015).

Elevated Se concentrations in vegetation around Se hyperaccumulators may not only offer protection from herbivory but may also promote growth. Indeed, *S. ericoides* showed a positive growth response to Se in controlled greenhouse studies (El Mehdawi *et al.,* 2014). As mentioned in earlier sections, low Se concentrations can promote plant growth via enhance photosynthesis and induce a variety of antioxidant and defense mechanisms (Zembala *et al.,* 2010; Feng and Wei, 2012; Feng and Wei, 2013). Selenium at low concentration in leaves has been reported to lead to decreased lipid peroxidation and to restoration of the membrane and overall structure of

chloroplasts (Feng and Wei, 2013), via stimulation of the cellular antioxidant systems (Djanaguiraman *et al.,* 2010; Walaa *et al.,* 2010; Feng and Wei, 2013). Selenium can also reduce osmotic stress via enhanced proline concentration (Hawrylak-Nowak, 2009; Walaa *et al.,* 2010). Thus, low Se concentrations in plant tissues may prime plants to overcome stress conditions by upregulating plant defense systems.

POSSIBLE EVOLUTIONARY CONSTRAINTS ON PLANT SE HYPERACCUMULATION

Plants maintain intimate and necessary relations with their environment through interactions with abiotic factors and mutualistic relationships with biotic partners such as pollinators and their microbiome. These interactions are important for plant physiology and reproduction. The ecological benefits of having a high Se concentration in organs could be offset by ecological constraints if they impair mutualistic relationships. Therefore, understanding how Se can affect these important ecological interactions deserves special attention. In addition, it is possible that extreme Se accumulation carries a physiological burden due to toxicity.

One of the first studies analyzing the potential constraints of Se hyperaccumulation in relation to reproductive fitness was done by Quinn *et al.* (2011). The authors observed differences in Se speciation and allocation between the Se hyperaccumulator *S. pinnata* and non-hyperaccumulator *B. juncea*. The *S. pinnata* plants allocated Se preferentially to flowers rather than to leaves. A very specific Se distribution pattern was identified in the flowers of this hyperaccumulator, within the ovules in the pistil, and in the pollen grains, primarily as MeSeCys. In contrast, *B. juncea* showed higher Se concentration in leaves than in flowers, and different chemical forms of Se were found in the flowers, including SeCys, SeMet, MeSeCys, and the non-organic forms selenate and selenite, which could be toxic to the plant. The high Se concentration in the pollen grains of *S. pinnata* did not affect the germination rate. Conversely, high Se concentration in *B. juncea* (2200

mg Se kg^{-1}) considerably decreased pollen germination. These findings suggested that there is no physiological cost of Se hyperaccumulation for reproduction and plant fitness related to pollen germination in the hyperaccumulator. Rather, high concentrations of Se in the pollen grain might be a trait evolved for protection against herbivory in the reproductive organs of hyperaccumulators (Quinn *et al.,* 2011).

In the same study by Quinn *et al.* (2011) no evidence was observed of any negative effects of high Se concentration in flowers of *S. pinnata* or *B. juncea* on pollinator visitation. Plants from both species with high or low Se concentrations received similar numbers of visits from the European honeybees (*Apis mellifera*) or other potential pollinators. Intriguingly, while honeybees collected from *S. pinnata* growing in seleniferous habitat contained Se concentration below 20 mg Se kg^{-1} DW, native bumble bees were found to contain more than 270 mg Se kg^{-1} DW, in the form of non-toxic MeSeCys, and were found to carry high-Se pollen in its pollen baskets. This may suggest that this native species has evolved mechanisms to tolerate the high Se concentration and may serve the ecological niche of *S. pinnata* pollinator. Somewhat similarly, Freeman *et al.* (2006) found Se tolerant herbivores (*Plutella xylostella*) that are able to feed on *S. pinnata* and accumulate high concentrations of Se in their body, also as MeSeCys. Additionally, as mentioned above, certain Se-tolerant plant species benefit from growing close to Se hyperaccumulators. Therefore, among ecological partners of various types, there appear to be some that (co-)evolve Se tolerance so that they can have symbiotic relationships with Se hyperaccumulators.

Other mutualist symbionts that could potentially be affected by the extreme Se concentrations of hyperaccumulator plant species are rhizospheric and endophytic microorganisms. This would harm the plant, since the plant microbiome can affect the bioavailability of nutrients in the soil, influence plant growth and development and confer abiotic stress resistance (Jha *et al.,* 2013). A

study by Sura-de Jong *et al.* (2015), however, found no evidence of any negative effects of high Se in *S. pinnata* and *A. bisucatus* on the colonization and diversity of bacterial endophytic species. The main genera found were Bacillus, Pantoea, Pseudomonas, Paenibacillus, Variovorax, Advenella, Arthrobacter, and Staphylococcus. Similarly, in another study Alford *et al.* (2012) found no evidence of any negative effects of Se concentration in plant tissues on the nodulation process in different *Astragalus* species (Fabaceae) in the field. Furthermore, a greenhouse experiment showed no evidence of the effects of high Se in plants on nodulation index in the hyperaccumulator *A. bisulcatus* when compared to non-hyperaccumulators *A. convallarius* and *A. shortianus*. Indeed, the nodulation index increased in the hyperaccumulators (*A. praelongus* and *A. racesmosus*) with higher Se concentration in plants, which was indicative of a positive relationship between Se and the symbiotic rhizobia in these hyperaccumulator species.

Thus, while more studies are needed to investigate all the possible constraints of Se hyperaccumulation better, studies to date do not show any evidence of selection pressures constraining plant Se hyperaccumulation. Perhaps one-time constraints due to the toxicity of hyperaccumulated Se have since been overcome by the evolution of Se tolerance mechanisms, in the hyperaccumulators themselves and in ecological partners (Quinn *et al.,* 2011).

1.6 SELENIUM ACCUMULATION IN FOOD CROPS

Selenium in crop food is directly related to the level of Se found in the edible parts of crops, which is determined by plant properties and affected by Se concentration and bioavailability in the soil and water. Several non-agricultural areas worldwide are known to contain very high Se concentration in their soil (seleniferous), including San Joaquin Valley in California/USA (Oldfield, 2002; Bañuelos *et al.,* 2007b), Pine Ridge natural area In Fort Collins/USA (El Mehdawi *et al.,* 2012), Wyoming/USA (El Mehdawi *et al.,* 2012), Hubei/China (Wang and Gao, 2001), and

Punjab/India (Sharma *et al.,* 2015). However, low-Se areas are more commonly documented, and Se deficiency is estimated to affect 1 billion people worldwide (Combs and Combs, 1984; Combs, 2001).

Practices of Biofortification can be adopted to increase the levels of Se in crops, to overcome the low dietary Se intake by the population living in low-Se areas (White and Broadley, 2009). The most direct way to increase the Se levels in the soil is the application of Se through inorganic and organic fertilizers. However, physicochemical properties of the soil can pose a challenge to the proper fortification of crops via soil fertilizers, so other strategies, such as foliar application of Se, can be alternatively utilized.

Some plants of the Brassicales order (broccoli, cabbage, mustard, and cauliflower, among others) can produce an extensive group of more than 130 aliphatic, indolic or aromatic secondary metabolites, namely glucosinolates (GLS), synthesized in different vascular tissues (Wiesner-Reinhold *et al.,* 2017). GLS are sulfur and nitrogen compounds that use different amino acids as precursors, including Met in the case of the aliphatic GLS. These secondary metabolites are used by plants as a defense mechanism against herbivores and different pathogens and afford benefits to consumers. Each subtype of GLS has its precursors and is synthesized independently; however, all biosynthetic pathways follow the same general steps in the following order: side-chain elongation, formation of core molecule structure, and secondary modification (Ishida *et al.,* 2014; Harun *et al.,* 2021). SeMet can be used as a precursor of aliphatic GLS in place of Met, and the resulting (methylseleno)glucosinolates, as well as their Se-containing aglycons, are supposed to possess superior bioactivity as anticancer and antimicrobial agents

Different reports support the evidence of positive health effects of GLS on human health and advise the regular consumption of cruciferous vegetables like broccoli (*Brassica oleracea* L. var.

Italica), reduce the risk of different forms of cancer and myocardial infarction (Melrose, 2019). It is suggested that GLS might have promising applications for other areas of medicine, including the potential against viral infections, considering the protective nature of these compounds to plants (Melrose, 2019).

Interestingly, broccoli and forage rape, *Brassica napus* L., supplemented with sodium selenate, can synthesize selenoglucosinolates (SeGLS) by utilizing the analog amino acid SeMet as an aliphatic GLS precursor (Matich *et al.,* 2012). Three different forms of GLS were identified using liquid chromatography-mass spectrometry (LC-MS), described as glucoselenoiberverin, glucoselenoerucin, and glucoselenoberteroin.

Other studies identified the incorporation of Se to other GLS compounds, including 2-phenylethylglucosinolate in roots of *Nasturtium officinale* (Wielanek *et al.,* 2009) 3-butenylselenoglucosinolate in *Stanleya pinnata*, Prince's Plume plants grown on hydroponics supplied with high Se concentration (Bertelsen *et al.,* 1988). Other forms of SeGLS include glucoselenoraphanin and glucoselenoerucin in broccoli, glucoselenoiberverin in cauliflower, in a more recent study *Brassica oleracea* L. var. botrytis, and finally glucoselenonasturtiin, glucoselenoerucin, and glucoselenoberteroin in forage rape roots (Matich *et al.,* 2012). It was demonstrated in the past that the consumption of broccoli enriched with Se induced beneficial immune responses (Bentley-Hewitt *et al.,* 2014).

In the past fifteen years, several crop species were biofortified with Se on the field or via greenhouse experiments, where different sources of Se, as well as application methods, were studied. Organic and healthy forms of Se, the amino acids SeMet and SeCys, were identified in corn (*Zea Mays* L.) grains after supplementation with sodium selenite via fertigation, utilizing 200 g of Se Ha^{-1} (D'Amato *et al.,* 2020). Other cereal species were also studied due to their nutritional

and economic importance. As an example, another study reported the presence of SeMet in mature bread wheat (*Triticum aestivum*) L. grains, and durum wheat grains, *Triticum durum* Desf., after soil and foliar application using either sodium selenate or sodium selenite, 4, 20, and 100 g of Se Ha^{-1} (Galinha *et al.,* 2015). Interestingly, the amino acid SeMet was found in all samples analyzed, regardless of the form of Se or mode of application. Another study reported similar results, where SeMet was determined in durum wheat grains after foliar spray in the field, using 0, 10, 20, and 40 g Ha^{-1} of sodium selenate or sodium selenite (Poblaciones *et al.,* 2014).

The Se biofortification of legumes, bulb and root plants and other relevant crops were also extensively analyzed in recent years. Chickpea (*Cicer arietinum* L.) grains, supplemented with sodium selenate or sodium selenite via foliar spray in the field, using a range of 0, 10, 20, and 40 g of Se Ha^{-1}, incorporated >70%, of organic SeMet (Poblaciones *et al.,* 2014). Soybean (Glycine max L.) accumulated SeMet and SeCys, after with sodium selenite supplementation, 0.9 mg of Se Kg^{-1} of soil, in a greenhouse experiment (Chan *et al.,* 2010).

Although the amino acids SeMet and SeCys are more commonly found in Se biofortified crops, other distinct organic forms of Se were reported in the literature. Carrot (*Daucus carota* L.), accumulated SeMet, and gamma-glutamyl-selenomethyl-selenocysteine (γ-glutamyl-SeMet-SeCys), after foliar application with sodium selenate or sodium selenite, 10 and 100 µg of Se ml-1, in a greenhouse experiment (Kápolna *et al.,* 2009). Broccoli and carrot grown on field-installed lysimeters, containing soil treated with Stanleya pinnata (selenium hyperaccumulator) powdered plant material, with a concentration of 700 µg of Se g-1 of DW, showed around 7% of MeSeCys among the total soluble seleno compounds in the broccoli florets and carrot roots (Bañuelos *et al.,* 2015).

Realistically, the implementation of Se-enriched fertilizers can be an expensive process for producers in low-Se areas. Alternatively, consumers can obtain their necessary Se from naturally Se-enriched food, from crops that have a tendency to accumulate Se. As mentioned, crops from the Brassica and Allium genera naturally accumulate high S and Se levels. However, the highest Se levels compared to any other plant-based food are found in the Brazil nut (*Bertholletia excelsa* H.B.K.). Importantly, its Se was found to consist mainly of nutritious C-Se-C forms, possibly SeMet, MetSeCys, or Se-lanthionine (Silva Junior *et al.,* 2017; Lima *et al.,* 2019) (for more detailed information see chapter 2).

1.7 CONTEXT AND SCOPE OF THIS DISSERTATION

Extensive work has been carried out by the Pilon-Smits lab in the last decade that helped elucidate parts of the complex mechanisms of Se hyperaccumulation in *Stanleya pinnata*. To contextualize the research questions presented in this dissertation, I will briefly revisit pertinent findings from former researchers. Freeman *et al.* (2010) studied the Se hyperaccumulation trait in *S. pinnata* at the physiological, biochemical and transcript level. The novel data brought insight of different molecular mechanisms of the Se hyperaccumulation syndrome, showing different expression of genes in the hyperaccumulator compared to a secondary accumulator Stanleya albescens, either constitutively, in the absence of Se, or induced with the application of 40 μm of Se (selenate). Some of the genes described to be upregulated in the hyperaccumulator were associated with antioxidant activity and redox homeostasis, defense mechanisms, defense hormone synthesis, and sulfate transport/assimilation (including the SULTR1;2 transporter and the APS2 enzyme).

Further research aimed to investigate the selenate uptake mechanisms by the hyperaccumulator S. pinnata, comparing this plant to the related non-hyperaccumulators *B. juncea* and *Stanleya elata*.

Harris et al. (2014) and El Mehdawi et al. (2018) compared the effect of high sulfate supply (up to 5mM sulfate) on the selenate uptake, translocation, and the expression of different sulfate transporters. The results showed that Se uptake in the hyperaccumulator is sulfate-independent, judged from less competitive inhibition of selenate uptake by the higher sulfate treatments, which points to a possible Se-specific transporter in the roots of the hyperaccumulator S. pinnata. RT-PCR data from the same research showed a constitutive higher expression of the SULTR1;2 and SULTR 2;1 genes in the hyperaccumulator.

Subsequently, Wang et al. (2018) found transcriptome-wide differences in shoot and root gene expression levels between the hyperaccumulator S. pinnata and the non-hyperaccumulator S. elata. The authors proposed that the tolerance to high levels of Se by S. pinnata might be mediated by the up-regulation of defense-related hormone synthesis and signaling genes, leading to upregulation of pathways involved in antioxidant activity, defense, and sulfate/selenate uptake and assimilation. The transcript levels of the SULTR1;2 transporter and the APS2 enzyme were remarkably higher, by >100 fold, in the roots of the hyperaccumulator when compared to the non-hyperaccumulator.

The combined findings above prompted further research to investigate the possible Se specificity of the transporter, and the role of the APS2 enzyme in Se tolerance, as presented in this dissertation. An elaborate genetic engineering approach was carried out with the aim to understand how hyperaccumulator plants can bioconcentrate selenate specifically over sulfate, and how they can tolerate such high tissue levels of Se. Two *S. pinnata* genes were expressed in *A. thaliana*: the putative sulfate transporter SpSULTR1;2 and the putative ATP sulfurylase SpAPS2, mediating the first step in selenate assimilation toward organic and less toxic chemical forms.

Other work described in this dissertation broadly focus on ecological significance of plant Se hyperaccumulation and on nutritional aspects of plant-based Se for consumers. The second experimental chapter builds on earlier ecological-evolutionary studies from the group and describes a field and lab study that aimed to answer the question why plants evolved the Se hyperaccumulation trait, analyzing how the high tissue Se levels affect different fitness parameters of *S. pinnata* plants in the field. Furthermore, I describe an investigation of Se accumulation, speciation, and localization in the Brazil nut; it appears as a healthy and natural source of dietary Se but should be consumed in moderation to avoid Se toxicity.

1.8 TABLES AND FIGURES

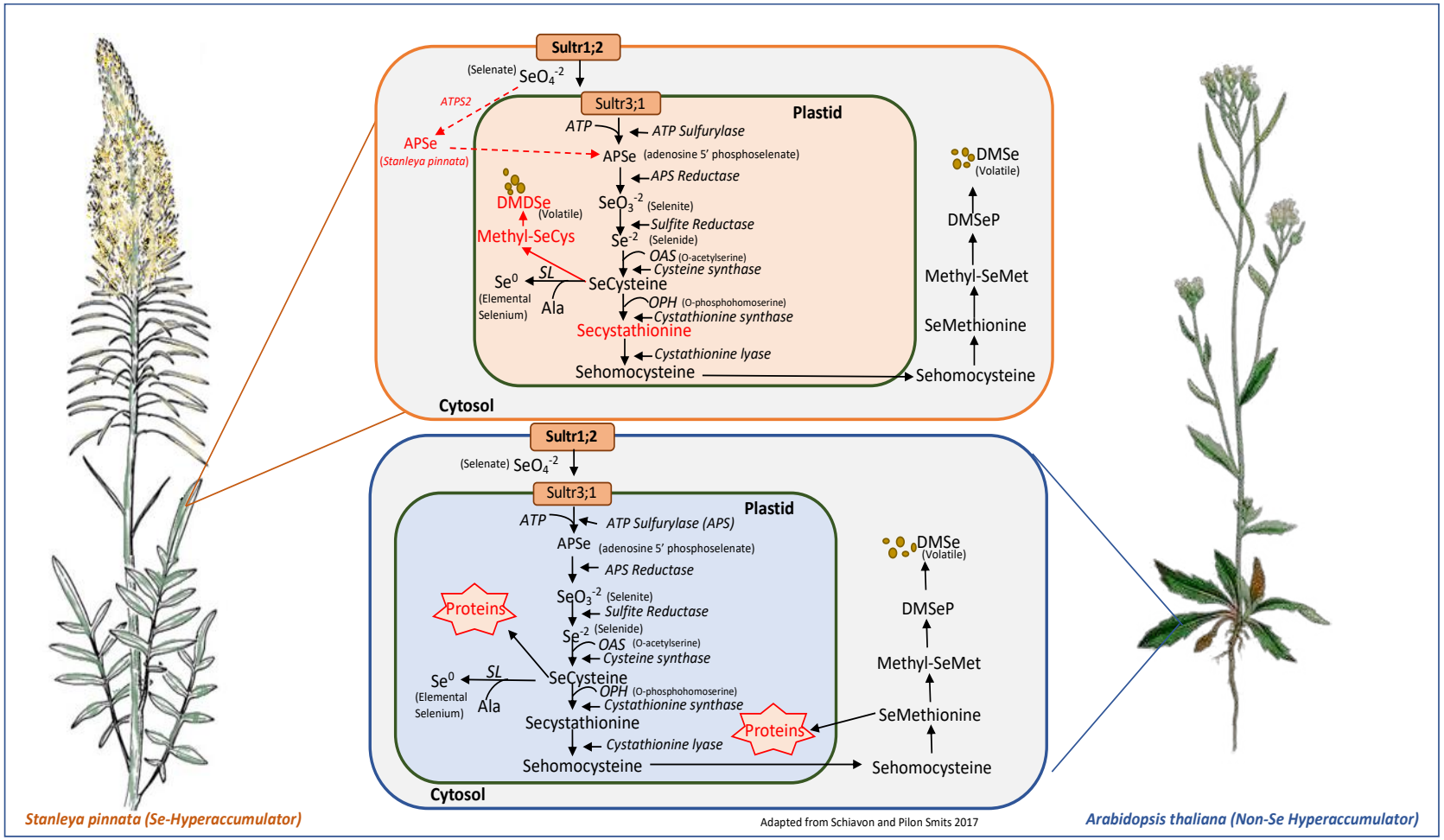

Figure 1-1. Selenium assimilation in hyperaccumulators and non Se-hyperaccumulators. Biochemical routes specific to hyperaccumulators are highlighted in red. ATPS2= ATP sulfurylase isoform 2, SL= selenocysteine lyase, Ala= Alanine, DMSe = dimethylselenide, DMDSe= Dimethyldiselenide (Lima and Schiavon, 2021).

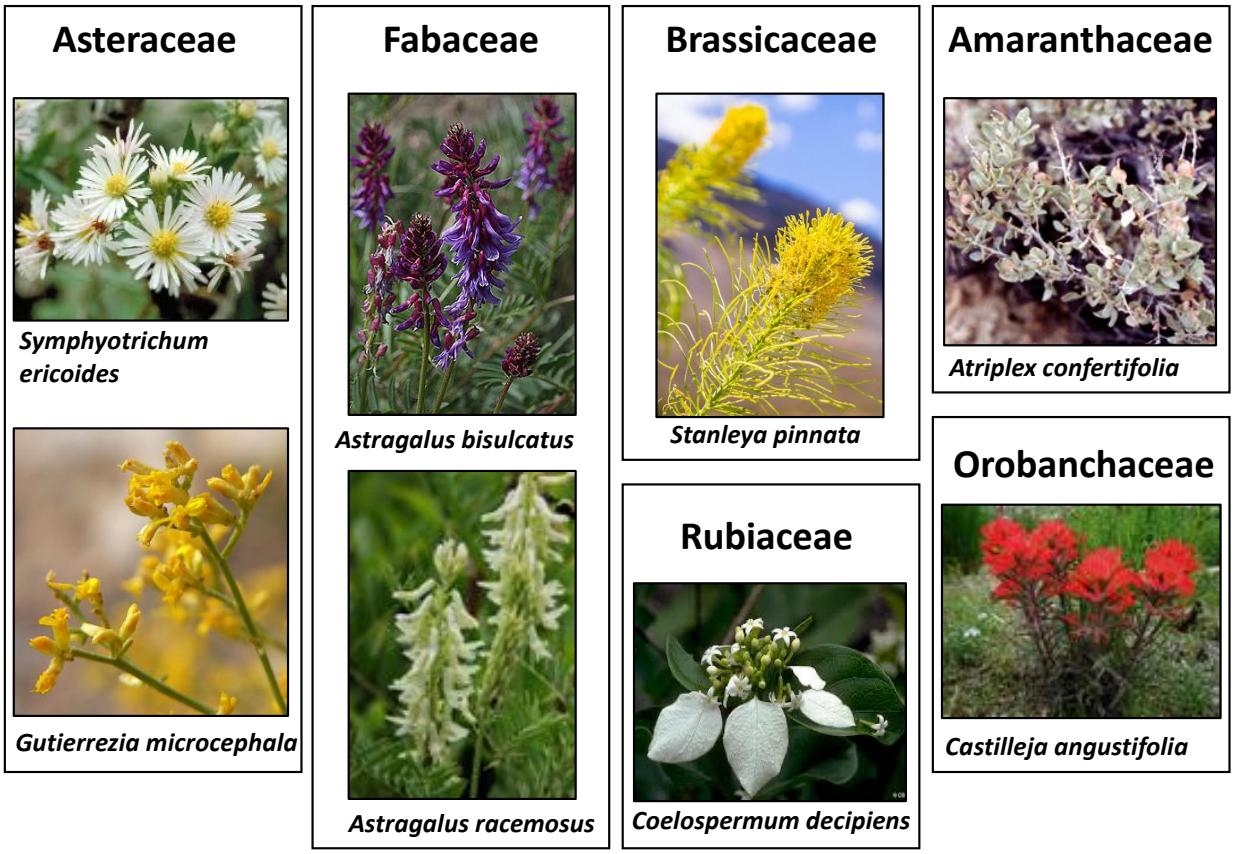

Figure 1-2. Representative Se-hyperaccumulators within different families (Lima and Schiavon, 2021).

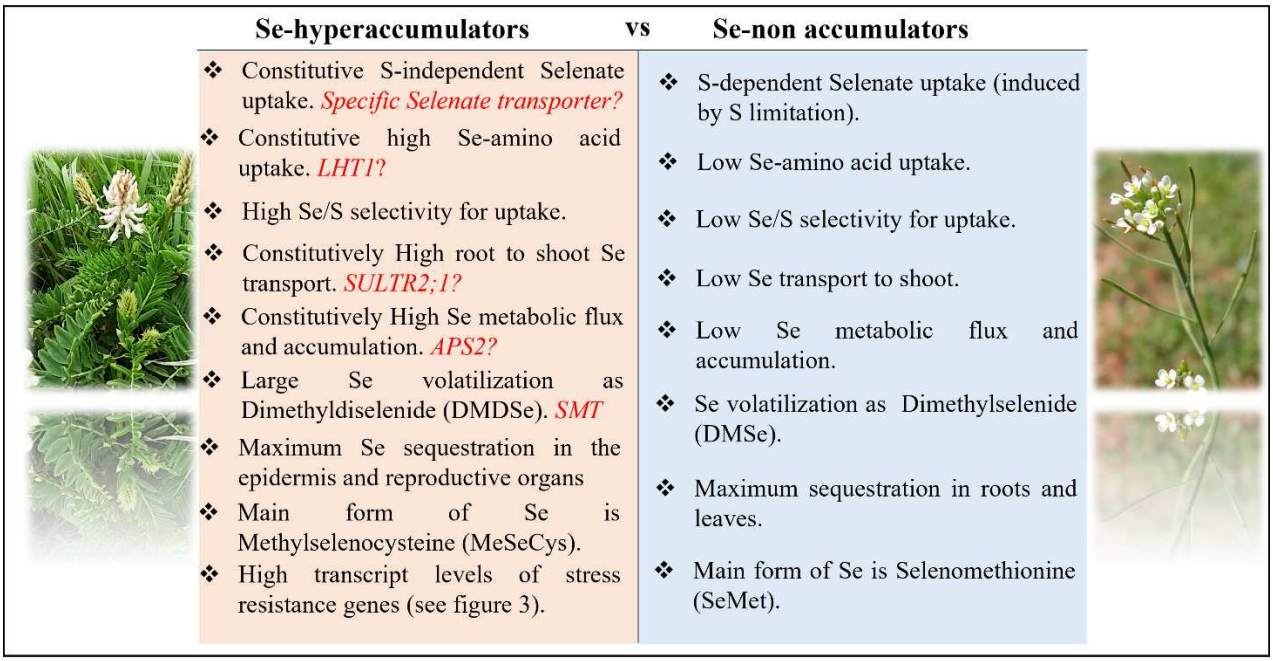

Figure 1-3. Main physiological differences between Se-hyperaccumulators and non-hyperaccumulator plant species (modified from Lima *et al.*, 2018).

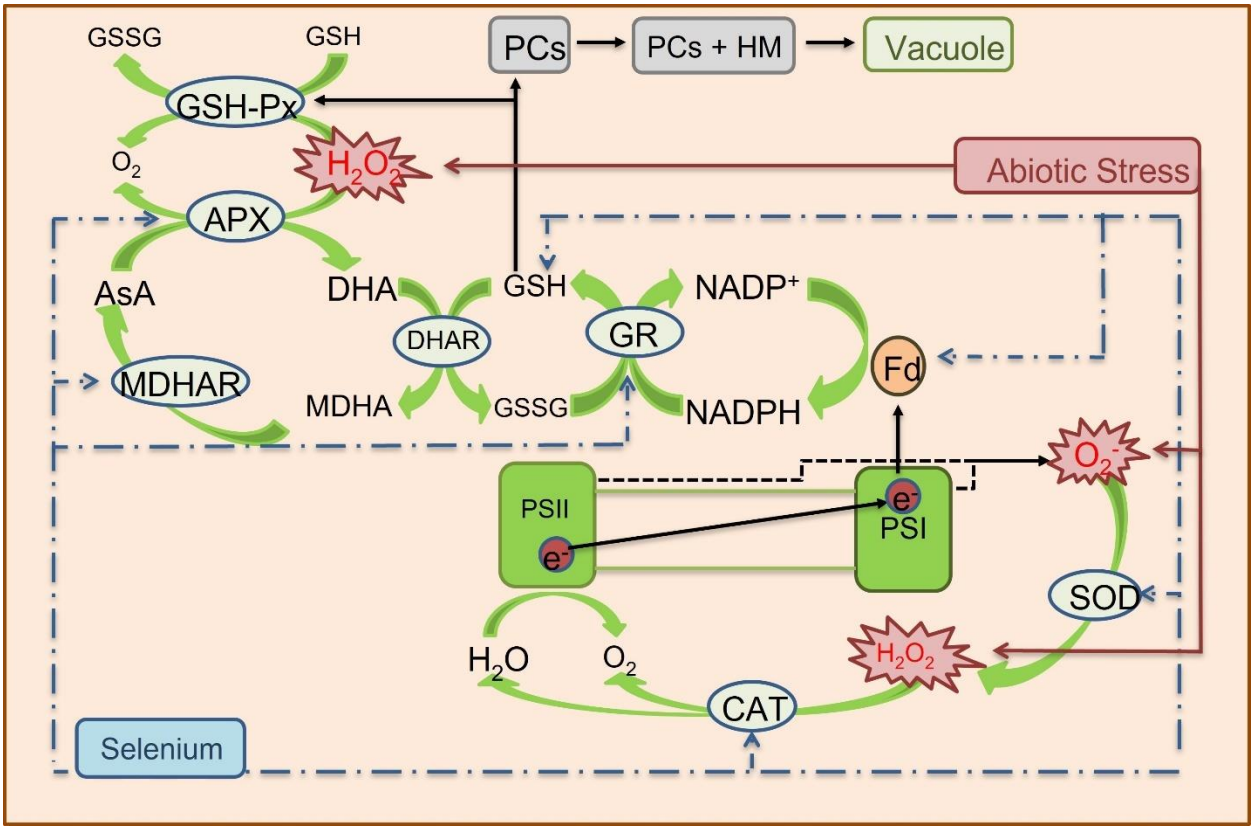

Figure 1-4. Selenium induces enhanced antioxidant activity which stimulates plant productivity and resistance to oxidative stress. The image illustrates the pathway in photosynthetic tissues. Enzymes: Ascorbate peroxidase (APX), Catalase (CAT), Dehydroascorbate reductase (DHAR), Glutathione peroxidase (GSH-Px) Glutathione reductase (GR), Monodehydroascorbate reductase (MDHAR), SOD (Superoxide dismutase). Metabolites: AsA (Ascorbate), GSH (reduced glutathione), PCs (Phytochelatins). Reactive Oxygen Species: Superoxide radical ($O2^{-\bullet}$); Hydrogen peroxide (H_2O_2) (Schiavon *et al.,* 2017).

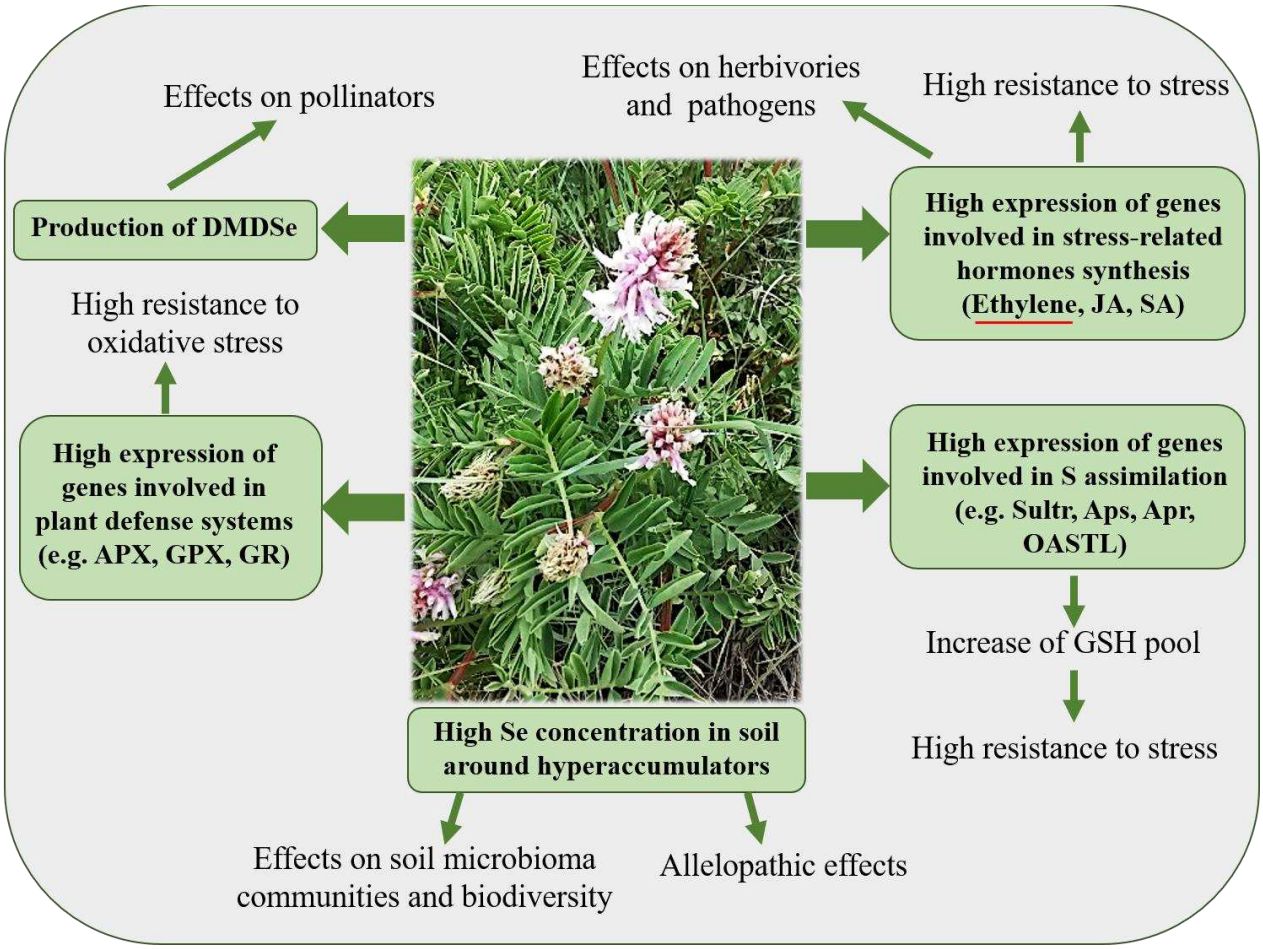

Figure 1-5. Hyperaccumulator plants have constitutive high expression of defense genes and genes associates to stress-related hormones synthesis and signaling, which confers protection against herbivore and pathogen attack and protects plants from oxidative stress potentially caused by high Se concentrations in the cell. In addition to upregulation of defense networks DMDSe may have a role in protection from herbivory and influence pollination (Lima *et al.,* 2018).

Table 1-1. Species, distribution, and maximum Se shoot concentration of several Se hyperaccumulators as retrieved by White (2016). Se hyperaccumulators whose Se shoot concentration and distribution are unknown are not reported (Lima and Schiavon, 2021).

Species	Plant distribution	Se concentration (mg Se kg^{-1} DW)
Asteraceae (Asterales)		
Dieteria canescens (Pursh) Nutt.	Midwest USA	1600
Grindelia squarrosa (Pursh) Dunal	Lower Brule Reservation, SD, USA	930
Gutierrezia microcephala (DC.) A.Gray	Thompson, UT, USA	1287
Oonopsis foliosa Greene	Lascar, CO, USA	3630
Oonopsis wardii (A.Gray) Greene	Albany County, WY, USA	9120
Symphyotrichum ascendens (Lindl.) G.L.Nesom	Soda Springs, ID, USA	4455
Symphyotrichum ericoides (L.) G.L.Nesom	Pine Ridge, Fort Collins, CO, USA	1378
Symphyotrichum lateriflorum (L.) Á.Löve & D.Löve	SD, USA	1800
Xylorhiza glabriuscula Nutt.	Huerfano County, CO, USA	1750
Xylorhiza parryi Greene	Albany County, WY, USA	5390
Xylorhiza venusta (M.E.Jones) A.Heller	Midwest USA	3486
Fabaceae		
Acacia cana Maiden	NW Queensland, Australia	1121
Astragalus albulus Wooton & Standl.	La Ventana, NM, USA	530
Astragalus beckwithii var. *purpureus* M.E.Jones	Cameron, AZ, USA	3135
Astragalus bisulcatus (Hook.) A.Gray	Pine Ridge, Fort Collins, CO, USA	13 685
Astragalus bisulcatus var. *haydenianus* (A. Grey) Barneby	Cuba, NM, USA	2377
Astragalus canadensis L.	Las Vegas, NE, USA	1110
Astragalus crotalariae A.Gray	Truckhaven, CA, USA	2175
Astragalus eastwoodiae M.E.Jones	Utah, USA	1664
Astragalus flavus Torr. & A.Gray	Aztec, NM, USA	1361
Astragalus flavus var. *argillosus* (M.E.Jones) Barneby	Greenriver, UT, USA	631
Astragalus flavus var. *candicans* A.Gray	Thompson, UT, USA	1322
Astragalus grayi S.Watson	Carbon County, WY, USA	4450
Astragalus osterhoutii M.E.Jones	Kremmling, CO, USA	2678
Astragalus pattersonii A.Gray	Thompson, UT, USA	8512
Astragalus pectinatus (Hook.) G.Don	Teton County, MT, USA	5170
Astragalus praelongus E.Sheld.	Leupp, AZ, USA	4835
Astragalus praelongus var. *ellisiae* (Rydb.) B.L.Turner	Valmont, NM, USA.	656
Astragalus preussii A.Gray	Thompson, UT, USA	4188
Astragalus racemosus Pursh.	WY, USA	14 920
Astragalus rafaelensis M.E.Jones	Jensen, TX, USA	716
Astragalus sabulosus M.E.Jones	Thompson, UT, USA	2210
Astragalus toanus M.E.Jones	ID, USA	990
Neptunia amplexicaulis Domin	Richmond, Queensland, Australia	4334
Brassicaceae (Brassicales)		
Cardamine hupingshanensis	Yutangba, Enshi, China	1965
Cardamine violofolia	Yutangba, China	2700
Stanleya bipinnata Greene	Laramie, WY, USA	2490
Stanleya pinnata (Pursh) Britton	Pine Ridge, Fort Collins, CO, USA	>4000
Stanleya pinnata var. *integrifolia* (E. James) Rollins	Vernal, UT, USA	977
Amaranthaceae (Caryophyllales)		
Atriplex confertifolia (Torr. & Frém.) S.Watson	Thompson, UT, USA	1734
Atriplex nutallii S.Watson	WY, USA	930
Rubiaceae (Gentianales)		
Coelospermum decipiens Baill.	Cape York Peninsula, Queensland, Australia	1141
Orobanchaceae (Lamiales)		
Castilleja angustifolia var. dubia	Lysite, WY, USA	3460

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CHAPTER 2: SELENIUM ACCUMULATION, SPECIATION AND LOCALIZATION IN BRAZIL NUTS (*BERTHOLLETIA EXCELSA* H.B.K.)

2.1 INTRODUCTION

This study characterizes the chemical form of Selenium (Se) and its localization in the Brazil nut (*Bertholletia excelsa* H.B.K., Lecythidaceae), as well as the variation in Se concentration within and among different commercially available batches. Bertholletia is a monotypic tree genus in the Lecythidaceae family, and its only species, *B. excelsa*, produces large, oil-rich seeds. These, known as Brazil nuts, are of biological and nutritional interest, because they accumulate extraordinarily high Se levels. Selenium (Se) is an essential micronutrient for humans and other mammals. This element plays an important role in the organism, and its inadequate nutritional supplementation can cause a number of health disorders (Mehdi *et al.,* 2013). The main forms of Se found in humans are organic, in the form of the amino acids selenocysteine (SeCys), analog to cysteine (Cys), and selenomethionine (SeMet), analog to methionine (Met). SeCys is a structural part of the active site of twenty-five different selenoproteins (Qazi *et al.,* 2018), which play roles in the maintenance of physiological homeostasis, including the cellular redox state regulation and hormonal biosynthesis.

Adequate Se intake varies and depends on personal physiological and biological parameters such as body weight, age, and sex (Dos Reis *et al.,* 2017; IOM, 2020). Therefore, the Recommended Dietary Allowance (RDA) of Se in the United States and Canada ranges from 15 μg Se/day (infants from 0 to 6 months old) to 70 μg Se/day (women from 14 to 50 years old during lactation), while the recommendation for male and female adults, between 18 and 71 years old, corresponds to 55 μg Se/day (IOM, 2020). The RDA varies in different countries (Hurst, *et al.,*

2013). The Austrian, German and Swiss nutrition societies recommend higher Se intake for adult women, 60 μg Se/day, and for adult men, 70 μg Se/day (Kipp *et al.,* 2015), while in Japan the recommended Se intake is 25 μg Se/day for adult women and 35 μg Se/day for adult men (Hurst, *et al.,* 2013).

Despite its importance to human metabolism, Se can become toxic above a certain threshold, due to its interference with sulfur (S) metabolism (Rayman, *et al.,* 2012). There is a narrow window between Se deficiency, adequacy, and toxicity. The tolerable Se intake limit is considered 400 μg Se/day (IOM, 2000; ATSDR, 2003), while the intake of Se associated with toxicity (selenosis) was estimated to be around 1200 μg Se/day (in people exposed to large amounts of organic Se in China) (ATSDR, 2003). Long-term exposure to moderate Se levels can result in chronic Se toxicity, and exposure to high Se levels can in some cases cause death due to acute toxicity. Chronic selenosis symptoms range from fragile or depigmented hair and nails to loss of these parts (Renwick, 2006), and characteristic acute selenosis symptoms include diarrhea, nausea, skin rash, disorders to the nervous system, fatigue, and irritability (IOM, 2020). There are also possible risks of supra-nutritional Se levels, as suggested by several recent papers on the complex U-shaped relationship between Se dose and diseases such as type 2 diabetes or cancers (Kohler , 1924; Rayman and Stranges, 2013; Rocourt and Cheng, 2013).

While Se toxicity is a great concern, deficiency is an even bigger problem worldwide. Low dietary Se intake, less than 40 μg/day (Winkel *et al.,* 2012), is estimated to negatively affect more than one billion people worldwide (Combs, 2001) including areas in China, Eastern Europe, Brazil and Sub-Saharan Africa, Australia, and New Zealand (Dos Reis *et al.,* 2017). In low Se areas in China, two specific diseases related to Se deficiency occur, i.e., Keshan disease (Winkel *et al.,* 2012; Collipp and Chen, 1981) and Kaschin-Beck disease (Ge and Yang, 1993; Li *et al.,* 1984).

The general symptoms of Se deficiency are related to impaired cellular redox capacity, thyroid function, and immune defense (Rayman, 2012; Li *et al.,* 1984; Olivieri *et al.,* 1996). Not surprisingly, several studies have found a positive correlation between Se deficiency and incidence of different types of cancer (Rayman, 2000; Amaral *et al.,* 2010; Etminan et al.,2005) as well as decreased survival in HIV-positive patients (Rayman, *et al.,* 2012). Deficiency symptoms may also include muscle weakness, muscle pain (myalgia) and heart dysfunction (Kabata-Pendias and Mukherjee, 2007), irreversible brain injury and impaired fertility (Rayman, *et al.,* 2012).

Selenium enters the food chain via plants, so an important source of Se to the human diet is plant-based food; this is particularly important for populations in low Se areas that rely on a vegetarian diet (Hurst, *et al.,* 2013). The Se concentration in crops varies greatly, not only due to species differences, but also due to variation in soil Se concentration worldwide, which is determined by geological processes (Dos Reis *et al.,*2017). In addition, local Se speciation and bioavailability are influenced by physico-chemical aspects of the soil (Rocourt and Cheng, 2013). The most common bioavailable Se forms are inorganic selenate (SeO_4^{2-}), found in well aerated, alkaline, and oxidized soils, or selenite (SeO_3^{2-}), present in more acidic and reducing environments like wetlands (Kabata-Pendias and Mukherjee, 2007). Organic forms of Se, such as SeCys and selenomethionine (SeMet), analog to methionine (Met), can also be present in soil, through plant decomposition and microbiome activity; selenides and elemental Se can also be present, but are not very bioavailable (Lima *et al*., 2017; Chasteen and Bentley, 2003).

To better provide Se to populations in low Se areas, different strategies are used to augment Se levels in crops, practices called biofortification (Hawrylak-Nowak. 2013; Boldrin *et al.,* 2013; Yasin *et al.,* 2015; Chávez-Santoscoy *et al*., 2015). An important factor in biofortification is the plant physiological capacity to take up, metabolize, translocate, and accumulate Se. Plant species

differ in their capacity to take up and assimilate Se. Selenium is not considered a nutrient for plants, but it is a beneficial element in low concentrations (~10 mg kg^{-1} dry weight, DW), owing to increased antioxidant capacity, which may lead to increased photosynthesis, stress resistance and ultimately growth (Longchamp *et al.,* 2015). Selenium can become toxic to plants at tissue levels above 100 mg kg^{-1} DW, because they non-specifically take up selenate (SeO_4^{2-}) via sulfate (SO_4^{2-}) transporters and assimilate it into the Se analogs of the amino acids Cys (SeCys) and Met (SeMet) (Feng *et al.,* 2013). The misincorporation of SeCys and SeMet results in protein malfunction and systemic oxidative stress (Bodnar *et al.,* 2012).

While biofortification can overcome the physiological limitations of crop species, it usually requires costly fertilizer supplementation in agricultural areas with low soil Se concentration (Dos reis *et al.,* 2017). Naturally high-Se food sources can be a simple solution to the challenge of providing sufficient Se to populations in low-Se areas. Selenium accumulation capacity varies dramatically among plant species; in natural Se-containing areas the Se levels in vegetation can differ 100-fold (Gupta and Gupta, 2017). Plants can be generally divided into three large groups based on Se content found in all their organs in natural environments: hyperaccumulators are plants that can exceed the threshold of 1000 mg Se kg^{-1} (DW), while secondary accumulators can accumulate from 100–1000 mg kg^{-1} DW and non-accumulators do not exceed 100 mg kg^{-1} DW (Lima *et al*., 2017). Non-accumulators, i.e., most species, including crops, and secondary accumulators, e.g., Brassica crop species and several wild Brassicaceae and Asteraceae, tend to contain more inorganic Se, while hyperaccumulators such as *Stanleya pinnata* (Brassicacea) and *Astragalus bisulcatus* (Fabaceae) and *Lecythis ollaria* (Lecythidaceae, the monkey pot tree), typically sequester organic forms of Se that do not interfere with S metabolism and therefore are less toxic (Galeas *et al*., 2007). Selenium hyperaccumulation likely evolved independently in

different plant families (Schiavon and Pilon-Smits, 2017), and it is hypothesized to function in herbivory and pathogen protection as well as allelopathic interaction (Cappa and Pilon-Smits, 2014).

Among different plant-derived food Se sources, the Brazil nut (*B. excelsa* H.B.K), endemic to different countries in South America and a relative of the monkey pot tree, contains the highest reported concentration of Se among other nuts/seeds; a few Brazil nuts are sufficient to provide the Se RDA listed for North America, Asia, and Europe (El Mehdawi and Pilon-Smits, 2012). Nuts are included in the healthy diet recommendations of several countries, due to their high nutritional value, fiber content, unsaturated fatty acids, and minerals (Junior *et al.,* 2017), however moderation consumption of nuts is advised due to high caloric values. Therefore, the regular intake of Brazil nuts could be recommended not only as a suitable strategy to prevent Se deficiency, but for several other health benefits such as anti-inflammatory properties, improvement of the cellular redox homeostasis and the reduced risk of different chronic diseases (Junior *et al.,* 2017). However, the Se content in these seeds can vary greatly according to soil properties with respect to Se concentration and bioavailability (El Mehdawi and Pilon-Smits, 2012; Cardoso *et al.,* 2017). Since there is a very narrow window between adequate and toxic Se intake for humans, it is crucial to determine the variation in Se concentration within commercially available Brazil nut batches and also among batches from different companies, which could directly affect consumers. In addition, it is important to analyze the chemical forms of Se present in the seed, which could affect its nutritional value and potential toxicity to consumers. Last, it is also interesting to investigate any possible correlations between Se and other nutrients in the seed.

In light of these considerations, this study characterized the chemical form of Se and its localization in the nut, as well as the variation in Se concentration within and among two different

commercially available batches (hence referred to as A and B). Furthermore, levels of macronutrients and micronutrients in these nuts were characterized, and their interactions with Se investigated. These studies have significance for Brazil nut consumers, sellers, and producers. This study also has intrinsic value; because this species has such unique properties with respect to Se, it is interesting to study its Se metabolic properties in detail.

2.2 RESULTS AND DISCUSSION

BRAZIL NUT SELENIUM CONCENTRATION AND VARIATION IN RELATION TO HEALTH

There was significant variation in seed Se concentration within each one of the two Brazil nut batches from two different companies. A 2.5-fold difference between the lowest and the highest Se concentration for batch A and around an 8-fold difference for batch B, n = 13 per batch, (Tables 2-1 and 2-2). The variation in Se concentration between these batches A and B was also more than 2-fold (Tables 2-1 and 2-2). The Se levels ranged from 25 to 76 mg Se kg^{-1} in batch A and 10 to 79 mg Se kg^{-1} in batch B, while the averages were 49 and 28 mg Se kg^{-1}, respectively (Tables 2-1 and 2-2). These average levels are higher than the 19 ± 2.3 mg Se kg^{-1} reported to be present in Brazil nuts (Secor and Lisk, 1989), and widely used as a public resource. They are also higher than the average level of 14.66 mg Se kg^{-1} found in another study using 72 nuts, however, the levels found here fell within the 0.2–253 mg Se kg^{-1} range that was reported (Cardoso *et al.,* 2017). To put these Se values into perspective, the RDA for the National Institutes of Health (NIH), U.S. Department of Health and Human Services, and also the U.S. Department of Agriculture (USDA) is 55 μg Se/day for adults. Consequently, the consumption of one seed (average of 5 g) from either batch A or batch B would most likely already meet or exceed this RDA.

The commonly recommended serving size for Brazil nuts according to the NIH, the USDA and to the labeling on batch A and B bags, is ~30 g (corresponding to 6 seeds). The Se present in

such a serving size would correspond to 1470 μg Se in batch A and 840 μg Se in batch B (considering the average Se per batch). The maximum allowable Se intake is considered 400 μg Se/day (IOM, 2000; ATSDR, 2003), while the intake of Se associated with toxicity is estimated to be around 1200 μg Se/day (ATSDR, 2003). Thus, the amount of Se provided by the recommended 30 g serving size of these two analyzed batches of Brazil nuts, is 2- to 3.5-fold higher than the maximum allowable daily Se intake, and the Se in the serving size of batch A even exceeds the intake of Se associated with toxicity. In the more extreme scenario where a person would consume the entire 454 g bag of shelled Brazil nuts, the Se intake, calculated from the averages shown in the first paragraph, would amount to 22.2 mg Se and 12.7 mg Se for batches A and B, respectively, which is 10 to 20 times the toxic Se intake level.

Selenium toxicity is not only related to Sulfur metabolism dysfunction but can also trigger more intricate and wide responses in the organism. The consumption of high-Se Brazil nuts, containing 23 times higher than the RDA of 55 μg Se/day, was reported to be positively correlated with high expression of proinflammatory genes in obese woman, and the high concentration of Se in blood may increase the risk for different chronic diseases (USDA, 2001).

The two batches used in our study were from an unspecified region of Brazil. The biggest commercial Brazil nut producer in the world, made up of more than 1.2 million trees, is located in the region of Manaus, in the state of Amazonas. A large variation in Se concentration in Brazil nuts was reported (Galeas *et al.,* 207), and this variation was correlated with the geographic origin in Brazil. The lowest mean concentrations (~2 mg Se kg^{-1}) were found in the states of Acre and Mato Grosso, intermediate concentrations (~11 mg Se kg^{-1}) in Roraima and the highest in the states of Amapá (51 mg Se kg^{-1}) and Amazonas (68 mg Se kg^{-1}). The Se variation in the seeds was correlated with variation in total soil Se concentration, which was also higher (~0.45 mg Se

kg^{-1}) in Amazonas than in the other states (~0.22 mg Se kg^{-1}). In view of the finding that the world's main Brazil nut producer is in a Se-rich area, and that the Se levels were high in our two tested Brazil nut batches, it is reasonable to assume that most commercially available Brazil nuts originating from Brazil could potentially be high in Se. These findings are important to consumers and sellers because the commercialized products usually do not specify the Brazilian region of origin. In addition, there is substantial variation from seed to seed, possibly caused by genetic variation between trees or by local variation in soil Se concentration or in Se bioavailability due to soil acidity (Galeas *et al.,* 207).

SELENIUM LOCALIZATION AND SPECIATION IN BRAZIL NUTS USING X-RAY MICROPROBE ANALYSIS

In addition to the concentration of total Se, it is important to consider the chemical forms of Se in the Brazil nuts, because these differ in nutritional value. Supplementation with organic forms of Se has been reported to be more effective compared to inorganic forms (Kipp *et al*., 2015; Duarte *et al.,* 2019). Micro X-ray fluorescence (XRF) was used to investigate Se localization in the Brazil nuts. First, a longitudinally cut seed was analyzed (Figures 2-1A, B). The Se was present throughout the seed, with strongest concentration in a tissue layer along the periphery, 1 to 2 mm below the surface. Outside of this high-Se zone, a high Zn concentration was present along the outer 1 mm of the seed, while Ca was most concentrated at the extreme exterior (Figure 2-1A).

The vital staining with triphenyl tetrazolium chloride (Figure 2-1C) shows that all of the analyzed tissues in the embryo were alive, and also revealed the unusual seed anatomy of this species, which has been described earlier (Schrauzer, 2000). Most of the tissues were reported to consist of undifferentiated embryo cells, surrounded by a thin layer of tubular cells, possibly endosperm (Corner, 1976), and covered by a hard, lignified testa (mostly non-living cells). The

embryo is classified as macropodial, in which the cotyledons are very rudimentary, even if present, and most of the tissue is considered to be the hypocotyl (Corner, 1976; Prance and Mori, 1978). The outermost cells of the hypocotyl have large oil bodies and surround a procambium ring which is four to six cell layers thick. The ring of cells forming the procambium is the only evidence of a meristematic region within the embryo. Cells of the inner core of the embryo, comprising most of the volume, are undifferentiated parenchyma (Camargo *et al.,* 2000).

In Figure 2-1C, the endosperm may correspond with tissue #1; the thin layer of cells below it (#2, indicated by an arrow) may correspond to the procambium ring (meristematic tissue giving rise to vascular tissues in the seedling), and tissues #3a and b may be the undifferentiated parenchyma of the hypocotyl, making up most of the embryo. The area of concentrated Se appears to be along the outside of the embryo tissues, just below the endosperm, in tissue #3A (undifferentiated parenchyma) and possibly tissue #2 (procambium) (Figures 2-1B, C). Therefore, we speculated that the Se accumulates in such a way that it can readily be distributed to the growing meristems during seed germination. This may serve to protect these tissues from biotic stresses. Selenium has been found to protect the plant from herbivores and pathogens, also at the levels found in these nuts (Cappa and Pilon-Smits, 2014).

Further μXRF analysis of a different, cross-cut Brazil nut showed a ring-shaped Se concentration 1 to 2 mm from the seed's exterior, in agreement with the distribution found in the longitudinally cut seed (Figure 2-2).

At the locations indicated (Figure 2-2), 11 micro-X-ray absorption near-edge structure (μXANES) spectra were collected across this seed, for Se speciation analysis (Figure 2-3A). Se valence scatter plots of the μXANES data along with 52 standard compounds showed that Se throughout the Brazil nut was mainly in organic forms (Figure 2-3B). Least squares combination

fitting further revealed that the Se in the seed consisted predominantly (91% on average) of organic C-Se-C species, in all tested areas (Table 2-3); other, minor forms of Se were fitted as elemental Se (Se^0) and Se (IV) species. The detected C-Se-C compounds may include selenomethionine (SeMet), methyl-selenocysteine (MetSeCys) and/or Se-lanthionine, which are indistinguishable by μXANES. The SeMet form, could either be present as a free amino acid or incorporated into proteins, which is also indistinguishable by μXANES. Studies (Vaughan, 1970; Vonderheide *et al.,* 2002; Németh *et al*., 2013), that used combinations of liquid chromatography and mass spectrometry, found the main form of Se in Brazil nuts to be SeMet. These studies detected this form after proteinase K treatment, suggesting that SeMet was incorporated into proteins. These findings were in agreement with our μXANES data. Incorporation of SeMet in proteins is non-specific, replacing Met, and this is less toxic to organisms than non-specific SeCys incorporation in proteins, or the accumulation of inorganic forms of Se (Kipp *et al*., 2015; Kabata-Pendias and Mukherjee, 2007). For mammals, SeMet is a good source of dietary Se, whether incorporated into protein, or as a free amino acid (Kipp *et al*., 2015; Olivieri *et al*., 1996; Duarte *et al.,* 2019).

INTERACTIONS OF SE WITH OTHER ELEMENTS IN BRAZIL NUT

A few significant correlations were found between Se and other elements (Tables 2-4 to 2-6, $P < 0.05$ levels in bold). In batch A (Table 2-4), Se was negatively correlated with magnesium (Mg, $R = 0.47$); some patterns (non-significant, NS) were also found supporting a negative relationship of Se with phosphorus (P), calcium (Ca), zinc (Zn) and iron (Fe). In contrast, batch B (Table 2-5) showed no significant correlations with Se and other elements, only a NS tendency for Se to positively interact with Zn, Fe, and nickel (Ni). Across both batches (Table 2-6), Se was positively correlated with sulfur (S, $R = 0.53$) and copper (Cu, $R = 0.42$), and there was a NS pattern of positive interaction with Ni. These elements were all present at higher levels in batch A

than B, by 34% (S), 85% (Cu) and 42% (Ni), respectively (Tables 2-1 and 2-2). Among the other nutrients in the seeds, there were consistent positive correlations ($P < 0.05$) between the levels of Mg, Zn, Fe, and P (Tables 2-4 to 2-6).

Thus, apart from the negative correlation between Se and Mg in batch A, there was no evidence that Se accumulation in the Brazil nuts may negatively affect levels of other healthy nutrients for consumers. Although across both batches, there was a positive correlation between Se, S and Cu, there were no such correlations within batches A or B. Rather, this correlation could be explained by differences between the batches: batch A had higher levels than batch B for Se, S, and Cu. Molybdenum (Mo) levels were also higher in batch A, but no statistics could be done because the levels were too low to be detectable in batch B. In this context it was interesting to note that Se, S and Mo were similar oxyanions that could be taken up by the same transporters (Galeas *et al.,* 207).

2.3 MATERIALS AND METHODS

BIOLOGICAL MATERIAL

Two different samples of commercially available in-shell Brazil nuts (*B. excelsa* H.B.K.) were used in this experiment. Both of the 454 g bags listed Brazil as the country of origin. The first batch was purchased from a U.S.A. website (company A) specializing in nuts, hence referred to as "batch A". The second batch was purchased in a local store from a big U.S.A. supermarket franchise (company B), hence referred to as "batch B". Thirteen seeds from each batch were randomly picked for elemental composition analysis. All were in good condition for consumption (no indication of browning or other degradation).

ELEMENTAL COMPOSITION

Fresh samples of 13 different Brazil nuts samples from each seed batch were weighed to 100 mg and dried at 50 °C until constant weight. These samples were then digested with 1 mL of nitric acid (Németh *et al*., 2015); samples were heated for 2 h at 60 °C and 6 h at 125 °C, then diluted to 10 mL with distilled water. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to analyze the digested seed samples' elemental composition (K, P, S, Mg, Ca, Cu, Zn, Fe, Mn, Ni and Mo).

SELENIUM LOCALIZATION AND SPECIATION

Selenium (and calcium, zinc) localization and speciation were analyzed in two different biological replicates of Brazil nut samples, from batch A (seed #12 and #13), using X-ray microprobe imaging (Zarcinas *et al*., 1987); batch B was not yet available at the time. Analyses were performed at beamline 10.3.2 (X-ray Fluorescence Microprobe) of the Advanced Light Source (ALS), at Lawrence Berkeley National Lab (Berkeley, CA, USA) using a Peltier cooling stage (−25 °C). Localization of Se, Ca and Zn was determined on a longitudinal section of sample #13 and a cross section of sample #12, cut fresh with a single-edged carbon steel blade, and then kept frozen during analysis. Micro-focused X-ray fluorescence (μXRF) maps were recorded at 13 keV incident energy, using 20 μm × 20 μm pixel size, a beam spot size of 7 μm × 7 μm, using 70 ms dwell time (Figure 2-1) and 50 ms dwell time (Figure 2-2). Maps were then deadtime-corrected and decontaminated. Selenium K-edge micro-X-ray absorption near-edge structure (μXANES) spectroscopy (in the range 12,500–13,070 eV) was used to analyze Se speciation on eleven different spots on sample #12, close to areas showing high Se concentration in the μXRF maps. Because of time constraints, only one of the nuts could be analyzed for speciation, and the cross-section was chosen because it would give information about speciation in different tissues. Spectra were energy calibrated using a red amorphous Se standard, with the main peak set at 12,660 eV.

Least-square linear combination fitting of the μXANES data was performed in the range of 12,630 to 12,850 eV using a library of 52 standard selenocompounds and procedures described by Fakra (2018). Additionally, a selenium valence scatter plot where kappa and mu represent the normalized absorption values at 12,664.25 and 12,667.8 eV respectively, was extracted from the Brazil nut μXANES data, using procedures described by Németh (2015). All data were recorded in fluorescence mode using a 7-elements Ge solid state detector (Canberra, ON) and processed using custom LabVIEW programs available at the beamline.

TRIPHENYL TETRAZOLIUM STAINING

Triphenyl tetrazolium staining on randomly selected Brazil nuts was performed according to Miller (2018). Representative results from one longitudinally cut seed from batch A is shown.

STATISTICAL ANALYSIS

The software JMP-IN 13.0.0 (SAS Institute, Cary, NC, USA) was used for statistical data analysis. Student t-test was used to compare batch A with B. Bivariate analyses (Fit x by y) were performed to determine correlations between elements, and the correlation coefficients (R) are shown in Tables 2-4 to 2-6. Linear fit was then used to analyze variance and to determine the p-values, which were then plotted in Tables 2-4 to 2-6.

2.4 CONCLUSIONS

This study analyzed the variation in Se concentration, as well as Se tissue localization and chemical speciation, and the relation of Se with other nutrients in 26 seeds in two different commercially available Brazil nut batches, 13 seeds per batch. Several important findings that are of basic biological interest are presented. The Se was found to be present in a tissue layer 1 to 2

mm below the seed surface, along its periphery. Based on μXANES fitting, the forms accumulated were organic C-Se-C compound(s) that may include SeMet, MetSeCys and/or Se-lanthionine. Together, this information provides novel insight into Se physiology and metabolism in this extraordinary Se-accumulating plant species. The findings also have significance for Brazil nut consumers, producers, sellers, and regulatory agencies. Brazil nuts contain the highest Se levels of any plant-based food source (Galeas *et al.,* 207; Schiavon and Pilon-Smits, 2017; USDA, 2001), and are therefore a valuable source of this essential micronutrient. However, Se can easily become toxic at elevated levels, and thus it is vital to inform and protect consumers from possible toxic effects of overconsumption of these high-Se seeds.

The anatomy of *B. excelsa* seeds is unusual: it consists almost entirely of embryo hypocotyl parenchyma, with a thin layer of endosperm around it and a meristematic layer in between (procambium). The area of concentrated Se appears to be below the endosperm along the outside of the embryo hypocotyl, corresponding with the outer parenchyma layer, and possibly the procambium. The Se in the Brazil nuts was mainly organic, reported to be the most effective dietary source of Se (Kipp *et al.,* 2015). There was large seed-to-seed variation (up to 8-fold) in Se concentration and the averages between the batches was ~2-fold. The levels of Se found were such that the consumption of one seed (5 g) was enough to meet or even exceed the recommended daily allowance (RDA) for Se.

While healthy, Brazil nuts should be consumed in moderation, it is important to emphasize that the levels of Se found in these two batches were high enough to exceed the maximum allowable daily intake of Se (400 μg) if consumed at the recommended serving size of 30 g (6 seeds). Depending on the batch, the recommended serving size may even exceed the Se intake level reported to cause toxicity symptoms (1200 μg). Therefore, unless low Se levels in batches of

seeds can be demonstrated, it would be safer for the recommended serving size for Brazil nuts to be reduced to 15 g (3 seeds) to ensure safe Se intake, and to warn consumers to not exceed this limit. In addition, it would be helpful to include on the package the geographic origin of the Brazil nut and ideally the Se concentration of the specific batch, with an indication of the % of RDA for Se. Furthermore, to avoid Se toxicity due to overconsumption, the package size of Brazil nuts from high-Se geographic areas may be reduced, or these seeds could preferentially be sold as part of mixed nuts packages.

2.5 TABLES AND FIGURES

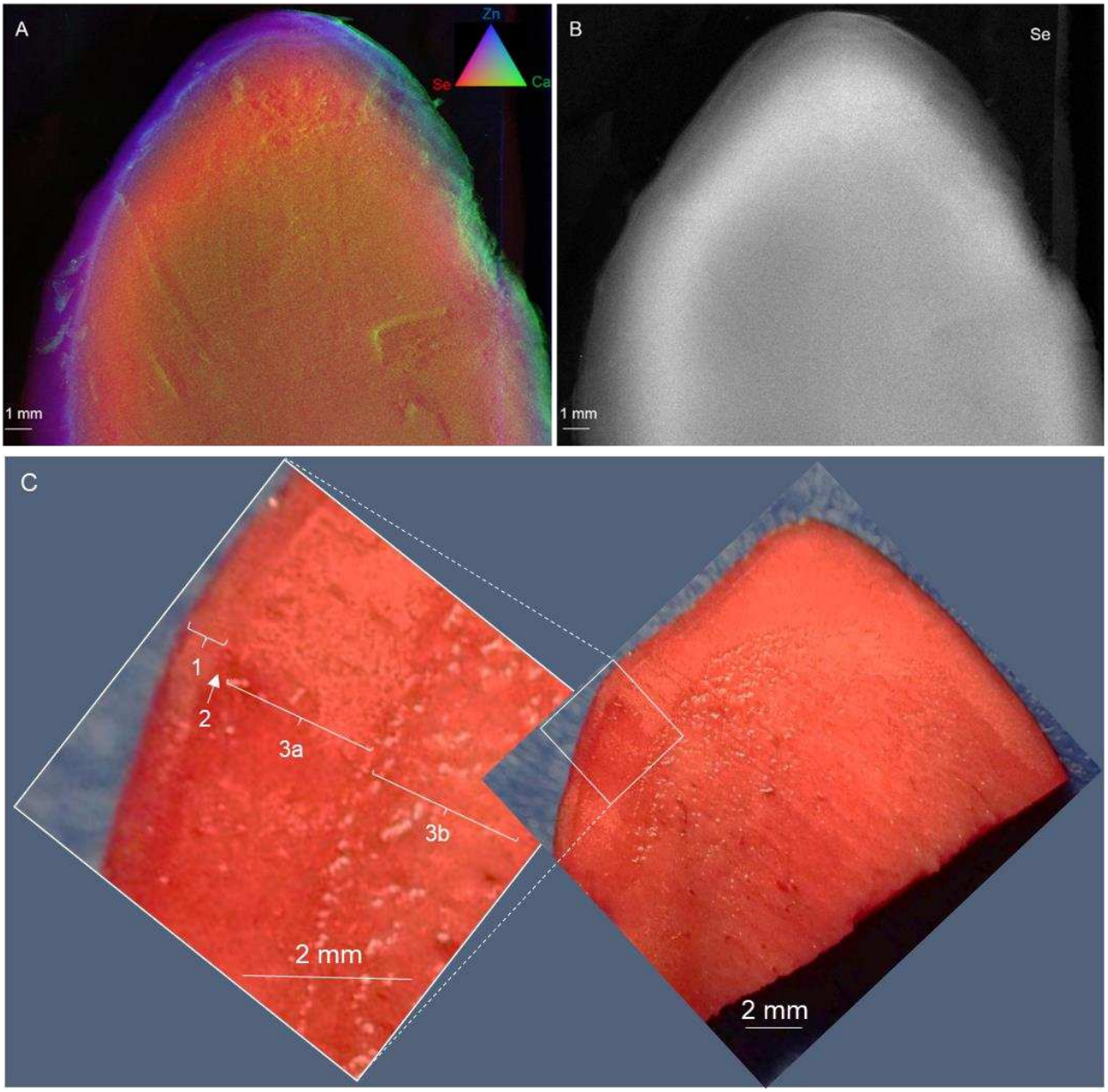

Figure 2-1. Micro X-ray fluorescence elemental distribution maps of a longitudinal section of Brazil nut #13 (25 mg Se kg−1, Table 2-1). Se is shown in red (A) or white (B). Panel A also shows Zn in blue and Ca in green. Panel (C) shows a longitudinal section of another Brazil nut stained with triphenyl tetrazolium (red); Numbered tissue layers are discussed in the text (Lima *et al.,* 2019).

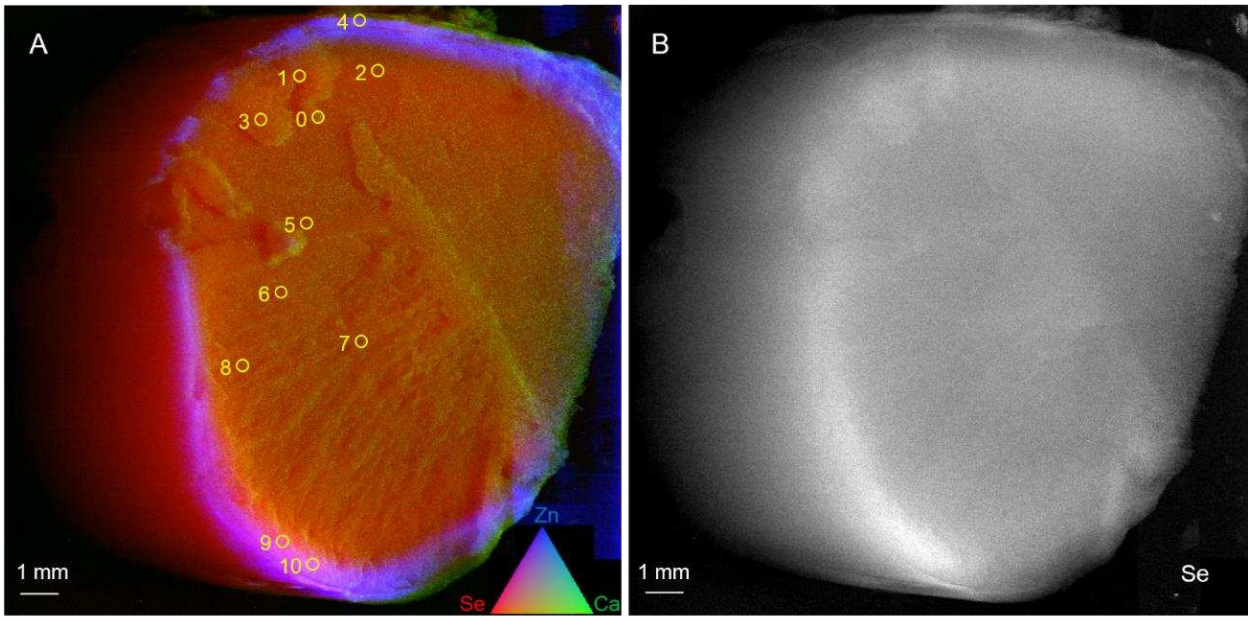

Figure 2-2. Micro X-ray fluorescence elemental distribution maps of a cross section of Brazil nut #12 (48 mg Se kg−1, Table 2-1). Se is shown in red (A) or white (B). Panel A also shows Zn in blue and Ca in green. Micro X-ray absorption near-edge structure spot locations are shown as numbered yellow circles; speciation results are shown in Table 2-3 (Lima *et al.,* 2019).

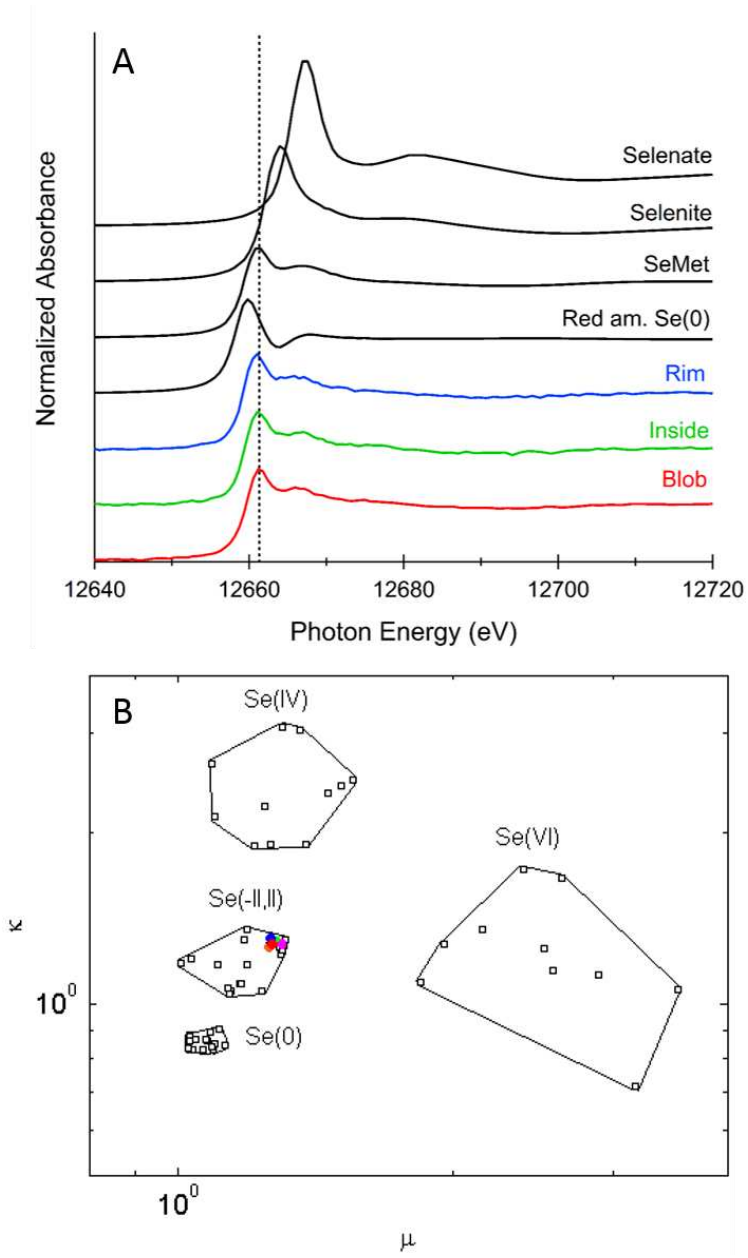

Figure 2-3. (**A**) Se K-edge micro-X-ray absorption near-edge structure spectra of Brazil nut at locations shown in Figure 2-2A. The "Blob" (red graph) is the average spectrum of spots 0, 1 and 3, the "Inside" (green graph) is the average spectrum of spots 6 and 7 and the "Rim" (blue graph) is the average spectrum of spots 9 and 10. Spectra of selected standard compounds are shown in black for comparison. (**B**) Se valence scatter plot of the Brazil nut X-ray absorption near-edge structure data (same color as in panel A), plus spot 4 is in magenta and spot 2 is in orange. Se standard compounds are shown as open black squares (Lima *et al.,* 2019).

Table 2-1. Elemental composition of 13 commercially available Brazil nuts (Batch A), imported from Brazil (Lima *et al.,* 2019).

	Selenium	Macronutrients					Micronutrients					
	(mg/kg)	(mg/g)					(mg/kg)					
Seed#		K	P	S	Mg	Ca	Cu	Zn	Fe	Mn	Ni	Mo
1	48.6	5.6	4.6	3.9	2.2	1.9	34.8	23.8	3.7	6.9	7.0	0.8
2	56.7	6.4	6.4	3.8	2.3	0.8	56.0	18.7	2.5	5.1	3.5	1.1
3	30.0	9.3	5.5	3.0	2.3	1.1	28.2	11.7	1.2	4.4	4.4	2.4
4	47.0	6.7	6.6	4.7	3.0	0.3	18.1	29.5	6.9	3.7	6.2	1.0
5	40.3	7.5	7.9	4.5	2.7	1.0	46.2	16.1	3.5	5.5	2.8	ND
6	64.9	8.3	6.4	4.8	2.6	0.7	28.7	32.4	2.0	7.0	1.9	1.8
7	74.0	3.6	5.5	3.3	2.0	1.0	22.1	15.2	1.9	7.0	2.4	1.0
8	58.4	7.2	5.8	4.0	2.2	0.5	33.8	13.6	2.8	3.3	4.6	2.8
9	75.5	7.8	7.7	3.7	2.0	1.4	19.5	12.9	4.1	12.2	2.9	1.5
10	47.3	5.7	6.0	4.2	2.3	1.3	22.8	12.8	4.3	4.6	1.3	3.7
11	24.4	5.7	8.2	2.6	2.9	1.6	19.4	33.9	12.5	8.5	2.2	ND
12	48.0	7.8	6.6	3.4	2.8	1.0	27.6	36.9	19.6	5.8	2.7	0.3
13	24.9	7.9	10.3	4.1	3.2	2.5	16.9	47	25.5	7.2	2.3	ND
Mean	49*	6.9	6.7	3.9*	2.5	1.2	28.8*	23.4	7.0	6.2	3.4	1.2
SD	16	1.5	1.5	0.6	0.4	0.6	11.4	11.2	7.4	2.3	1.7	1.2
Range	25-76	3.6-9.3	4.6-10.3	2.6-4.8	2.0-3.2	0.5-2.6	17-56	12-47	1.2-26	3.3-12.2	1.3-6.2	0.3-3.7

Seeds 12 and 13 were used for XRF and XANES analysis. ND = not detectable. Asterisks denote significant differences between batch A and B, (t-test, $P<0.05$).

Table 2-2. Elemental composition of 13 commercially available Brazil nuts (Batch B), imported from Brazil (Lima *et al.,* 2019).

Seed#	Selenium (mg/kg)	Macronutrients (mg/g)					Micronutrients (mg/kg)		
		K	P	S	Mg	Ca	Cu	Zn	Fe
14	**12.1**	5.8	6.2	2.9	2.6	0.6	13.2	26.3	7.0
15	**17.4**	7.7	6.1	2.7	2.3	0.4	12.6	22.4	10.7
16	**18.1**	5.7	7.9	2.8	2.9	0.9	21.7	28.1	9.7
17	**11.9**	4.8	6.1	3.2	2.6	1.3	14.2	37.1	6.9
18	**78.7**	4.9	7.8	3.2	3.1	1.2	16.5	46.6	12.0
19	**16.4**	4.3	5.7	2.1	2.2	1.9	19.2	22.4	5.0
20	**15.1**	6.0	7.8	4.3	3.7	1.0	15.8	47.3	12.5
21	**12.6**	6.3	3.6	1.6	1.3	0.6	11.2	9.6	2.3
22	**17.0**	10.8	7.4	2.7	2.8	1.9	20.8	28.5	5.6
23	**23.1**	7.0	5.1	2.4	2.4	0.7	16.2	21.3	2.3
24	**10.0**	6.2	7.3	2.6	2.9	1.3	12.2	38.8	8.8
25	**43.7**	5.8	7.9	3.4	3.4	1.0	12.3	38.2	13.7
26	**27.7**	9.7	6.0	2.2	2.8	0.8	15.0	15.1	1.9
Mean	**27.7***	**6.6**	**6.5**	**2.8***	**2.7**	**1.1**	**15.6***	**29.6**	**7.6**
SD	**19.0**	**1.9**	**1.3**	**0.7**	**0.6**	**0.5**	**3.4**	**11.8**	**4.1**
Range	**10.0–78.7**	**4.3–10.8**	**3.6–7.9**	**1.6–4.3**	**1.3–3.7**	**0.4–1.9**	**11.2–21.7**	**9.6–47.3**	**1.9–13.7**

ND = not detectable. Asterisks denote significant differences between batch A and B (t-test, $P<0.05$).

Table 2-3. Selenium speciation in seed #12 as determined by least-square linear combination fitting of the Micro X-ray absorption near-edge structure (μXANES) spectra collected at locations shown in Figure 2-2A. NSS = normalized sum of squares. C-Se-C may correspond to the organic forms SeMet, MeSeCys and/or Se-lanthionine, which are indistinguishable by μXANES. Errors on fits are +/−10%. N.D: Not detected. Note: The spot 4 spectrum was too noisy to fit, so is not shown in the table (Lima *et al.,* 2019).

XANES Spots	**NSS ($\times 10^{-4}$)**	**C-Se-C**	**Se (IV)**	**Se (0)**
Avg 0,1,3 ("Blob")	3.4	100%	N.D.	N.D.
2	5.2	100%	N.D.	N.D.
5	5.8	64%	10%	26%
Avg 6,7 (Inside)	5.8	100%	N.D.	N.D.
8	6.7	100%	N.D.	N.D.
Avg 9,10 (Rim)	4.1	81%	5%	14%

Table 2-4. P-values for positive (+) and negative (−) correlations between nutrient concentrations in Brazil nut batch A (n=13) (Lima *et al.,* 2019).

	Se	K	P	S	Mg	Ca	Cu	Zn	Fe	Mn	Ni
K	0.368										
P	(-) 0.146	0.351									
S	0.439	0.550	0.879								
Mg	**(-) 0.010[1]**	0.341	**(+) 0.012[2]**	0.527							
Ca	(-) 0.133	0.891	(+) 0.095	0.349	0.447						
Cu	0.735	0.871	0.367	0.599	0.406	0.334					
Zn	(-) 0.134	0.617	**(+) 0.039[3]**	0.804	**(+) 0.004[5]**	(+) 0.178	0.257				
Fe	(-) 0.065	0.580	**(+) 0.007[4]**	0.645	**(+) 0.004[6]**	**(+) 0.047[7]**	(-) 0.170	**(+) 0.0003[8]**			
Mn	0.364	0.891	(+) 0.197	0.370	0.626	(+) 0.083	0.266	0.673	0.616		
Ni	0.924	0.992	(-) 0.113	0.713	0.739	0.591	0.594	0.708	0.427	0.309	

The +/− values are shown for P<0.20; correlations significant at the 0.05 level are in bold. Exponential numbers refer to correlation coefficient (R): **1**=0.68; **2**=0.67; **3**=0.57; **4**=0.70; **5**=0.83; **6**=0.74; **7**=0.55; **8**=0.84.

Table 2-5. P-values for positive (+) and negative (−) correlations between nutrient concentrations in Brazil nut batch B (n=13) (Lima *et al.,* 2019).

	Se	K	P	S	Mg	Ca	Cu	Zn	Fe	Mn
K	0.612									
P	0.204	0.933								
S	0.445	0.467	**(+) 0.004**[1]							
Mg	0.236	0.980	**(+) 0.0001**[2]	**(+) 0.0002**[5]						
Ca	0.909	0.970	0.285	0.882	0.527					
Cu	0.859	0.635	0.223	0.889	0.496	**(+) 0.048**[10]				
Zn	(+) 0.171	0.209	**(+) 0.001**[3]	**(+) 0.0001**[6]	**(+) 0.008**[8]	0.301	0.8431			
Fe	(+) 0.183	0.224	**(+) 0.002**[4]	**(+) 0.001**[7]	**(+) 0.008**[9]	0.992	0.8494	**(+) 0.001**[11]		
Mn	0.714	0.337	0.228	0.745	0.632	0.304	0.3481	0.550	0.506	
Ni	(+) 0.105	0.325	0.364	0.806	0.517	(+) 0.172	0.2344	0.425	0.840	0.942

The +/− values are shown for P<0.20; correlations significant at the 0.05 level are in bold. Exponential numbers refer to correlation coefficient (R): **1**=0.74; **2**=0.90; **3**=0.80 **4**=0.78; **5**=0.85; **6**=0.86; **7**=0.79; **8**=0.81; **9**=0.69; **11**=0.79.

Table 2-6. P-values for positive (+) and negative (−) correlations between nutrient concentrations in Brazil nut batches A+B (n=26) (Lima *et al.,* 2019).

	Se	K	P	S	Mg	Ca	Cu	Zn	Fe	Mn
K	0.636									
P	0.909	0.544								
S	**(+) 0.005**[1]	0.875	(+) 0.094							
Mg	0.490	0.734	**(+) 0.0001**[3]	(+) 0.116						
Ca	0.624	0.945	**(+)0.0327**[4]	0.886	0.438					
Cu	**(+) 0.029**[2]	0.579	0.794	**(+) 0.0143**[7]	0.359	0.906				
Zn	0.471	0.424	**(+) 0.0005**[5]	0.356	**(+) 0.0001**[8]	(+) 0.119	(-) 0.104			
Fe	0.418	0.822	**(+) 0.0001**[6]	0.612	**(+) 0.0006**[9]	(+) 0.057	(-) 0.130	**(+) 0.0001**[11]		
Mn	0.615	0.378	(+) 0.063	0.759	0.915	**(+) 0.0419**[10]	0.850	0.545	0.457	
Ni	(+) 0.085	0.448	0.453	(+) 0.140	0.879	0.756	(+) 0.072	0.735	0.403	0.559

The +/− values are shown for P<0.20; correlations significant at the 0.05 level are in bold. Exponential numbers refer to correlation coefficient (R): **1**= 0.53; **2**= 0.42; **3**= 0.74; 4=0.42; **5**= 0.63; **6**=0.70; **7**=0.47; **8**=0.81; **9**=0.63; **10**=0.40; **11**=0.77.

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CHAPTER 3: HYPERACCUMULATOR *STANLEYA PINNATA:* FITNESS IN RELATION TO TISSUE SELENIUM CONCENTRATION

3.1 INTRODUCTION

Selenium (Se) is recognized as an indispensable nutrient for many animals, prokaryotes, and microalgae (Schiavon and Pilon-Smits, 2017), functioning as a structural component of selenoproteins. The essentiality of Se to higher plants is not yet verified (Schiavon and Pilon-Smits, 2017); instead, this element is considered beneficial (Pilon-Smits *et al.,* 2009). Selenium can induce the cellular antioxidant system at low levels (Feng *et al.,* 2013), with a variety of advantageous responses, such as enhanced growth, more efficient photosynthesis, higher accumulation of starch and sugars, delayed senescence, and protection against oxidative stress (Zembala *et al.,* 2010; Feng and Wei, 2012; Feng *et al.,* 2013). Nonetheless, the threshold between Se adequacy and toxicity is very narrow for many species. Most plants, for instance, experience toxicity above 100 μg g^{-1} DW (dry weight) and are defined as Se-non accumulators.

Plants likely lost their Se-specific metabolism during evolution since no molecular mechanisms have been discovered that specifically insert seleno-amino acids into proteins (Novoselov *et al.,* 2002; Lobanov *et al.,* 2007). In plant cells, Se can be assimilated nonspecifically into the Se-amino acids selenocysteine (SeCys) and selenomethionine (SeMet) by accessing the assimilation pathway of its analog sulfur (S) (Anderson, 1993). The insertion of Se amino acids into proteins in place of the S amino acids cysteine (Cys) and methionine (Met) can produce malformed proteins that lose their function, which has been established as a major cause of Se toxicity to plants (Van Hoewyk *et al.,* 2008; Van Hoewyk, 2013). In addition, inorganic Se can cause oxidative stress at higher tissue concentrations in most species (Van Hoewyk, 2013).

The uptake of Se by roots tightly depends on soil Se concentration, phytoavailability, and Se speciation (White, 2016). The average Se level in the soil is usually below 2 μg g^{-1} but can reach up to 1.2 mg g^{-1} in soils derived from seleniferous sedimentary rocks (Winkel *et al.,* 2015). These areas can be found in the great plains of the United States of America, including the seleniferous shale formations at Coyote Ridge and Pine Ridge Natural Areas in Colorado (Reynolds *et al.,* 2020a; Reynolds *et al.,* 2020b). Some plant taxa growing in these naturally seleniferous areas can efficiently accumulate Se in their leaf tissues but display different physiological and ecological strategies to cope with the high Se concentration. These plants are classified into two major categories, namely accumulators (or secondary accumulators), which can concentrate from 100 - 1,000 μg Se g^{-1} DW, and hyperaccumulators, which can exhibit 1,000-10,000 μg Se g^{-1} DW (up to 0.1% of Se per DW) (Galeas *et al.,* 2017).

To date, more than 500 plant species have been described to hyperaccumulate a specific non-essential element (Cappa and Pilon-Smits, 2014). In the previous 15 years, much knowledge has been gained on Se hyperaccumulator plant species, and, to date, this trait is reported in different families in the orders Malpighiales, Brassicales, and Asterales (Cappa *et al.,* 2014; Cappa *et al.,* 2015), forming a group with no common ancestor (Cappa *et al.,* 2015), where the Brassicaceae constitutes the most represented family with more than 100 taxa (Cappa *et al.,* 2014; Cappa *et al.,* 2015). The Se-hyperaccumulator *Stanleya pinnata* (Brassicaceae), also known as Prince's plume, is a desert perennial plant native to most of the arid western part of the United States, including the great plains, and can be found throughout the state of Colorado (Cappa *et al.,* 2014; Freeman *et al.,* 2010). This particular plant can hyperaccumulate Se up to 0.1% of its dry weight, mainly as organic and less toxic forms of Se (Cappa *et al.,* 2014). Transcriptomic studies have revealed that *S. pinnata* possesses an elevated expression of several genes that have a role in plant defense

against stress, either abiotic or biotic (Freeman *et al.,* 2010; Wang *et al.,* 2018). The overexpression of such genes corresponds with alternative biochemical mechanisms evolved by the Se-hyperaccumulator that efficiently detoxify or remove excessive Se via methylation of SeCys and subsequent volatilization, thus preventing Se misincorporation into proteins (Schiavon and Pilon-Smits, 2017; Gupta and Gupta, 2017).

Boyd and Martens (1992) described the elemental defense hypothesis, stating that the hyperaccumulation trait likely evolved because it confers certain ecological advantages. High levels of Se in plants, in particular, can be toxic to herbivores. Thus, by accumulating extremely high Se levels in their tissues, S. pinnata plants can have greater protection from pathogens or herbivore attacks (Quinn *et al.,* 2007; Freeman *et al.,* 2009; Quinn *et al.,* 2010). Plant Se accumulation can offer protection against herbivory through either deterrence or toxicity (Freeman *et al.,* 2007; El Mehdawi *et al.,* 2011; El Mehdawi *et al.,* 2011); Various invertebrate and vertebrate herbivores were shown to actively avoid plants treated with Se and suffered toxicity when forced to feed on high-Se plants(El Mehdawi *et al.*, 2012). Field surveys also showed a possible protective effect of high Se plants against herbivory: Se hyperaccumulator species growing in the seleniferous Pine Ridge Natural Area sheltered fewer arthropods when compared to non-accumulators (Galeas *et al.,* 2008). Se hyperaccumulator plants may also function in elemental allelopathy against non-accumulator neighboring plants; indeed, by accumulating hyperaccumulator Se levels in their tissues and depositing this element in the litter, *S. pinnata* plants can better compete with the surrounding Se-sensitive vegetation (Schiavon and Pilon-Smits, 2017; El Mehdawi *et al.,* 2011a; El Mehdawi *et al.,* 2011b; El Mehdawi *et al*., 2012; El Mehdawi *et al*., 2015).

While Se protects plants against many generalist herbivores, Se-resistant leaf and seed herbivores were found to live in symbiosis with hyperaccumulators *S. pinnata* and Astragalus

bisulcatus. These herbivores can utilize high-Se plants as a food source without experiencing toxicity, either via Se exclusion or Se tolerance (Freeman *et al.,* 2006a; Freeman *et al.,* 2006b; Freeman *et al.,* 2012; Valdez Barillas *et al.,* 2012). For example, Freeman *et al.* (2006) found that a Colorado population of the diamondback moth (Plutella xylostella) was able to tolerate high tissue levels of Se by avoiding demethylation of the plant's primary form of Se, methyl-SeCys, thus avoiding its incorporation into proteins, in contrast to another population from a non-seleniferous area that was not. In another study, Freeman *et al.* (2012) found a parasitoid chalcid wasp in the seeds of *S. pinnata* that resisted Se via exclusion. Similarly, Valdez Barillas *et al.* (2012) found two different herbivore moth species on *A. bisulcatus*. Various other types of symbionts of hyperaccumulators also appear to have co-evolved with them by developing Se resistance: litter decomposers (Quinn *et al.,* 2010; Quinn *et al.,* 2011a), pollinators (Quinn et al., 2011b), endophytic, and rhizosphere microbes (Lindblom *et al.,* 2014; Cochran *et al.,* 2018). There is also evidence that hyperaccumulators may facilitate Se-tolerant plant species, which were often found growing near hyperaccumulators, where they also had elevated Se levels and suffered less herbivory damage (El Mehdawi *et al.,* 2011a; El Mehdawi *et al.,* 2011b).

Thus, the emerging picture from earlier studies is that Se hyperaccumulator plant species can positively and negatively affect different ecological partners, depending on these partners' Se resistance. Through these combined effects, hyperaccumulators may affect the local species composition of animals ((Freeman *et al.,* 2006; Freeman *et al.*, 2012), plants (Reynolds *et al.,* 2020a; Reynolds *et al.,* 2020b; El Mehdawi *et al.,* 2011a; El Mehdawi et al., 2011b), mycorrhizal, rhizosphere, and endophytic microorganisms (Lindblom *et al.*, 2014; Wangeline *et al.,* 2011).

From collective earlier physiological, biochemical, and ecological studies, it is clear that *S. pinnata* benefits in several ways from Se accumulation through the described ecological benefits

and enhanced growth (El Mehdawi *et al*., 2012; Quinn *et al*., 2011b). So far, there is no evidence for any physiological or ecological constraints. However, no investigation has yet been reported on the effects of Se hyperaccumulation on the overall plant fitness in the field. To investigate to what extent Se hyperaccumulation contributes to *S. pinnata* fitness, a field survey was conducted within two wild populations growing in naturally seleniferous areas. Variation in Se concentration in vegetative and reproductive tissues was determined, and correlations were explored between the observed Se levels with fitness parameters, herbivory damage, and classes of biochemical defense compounds. It is hypothesized that plant Se concentration positively correlates with the various fitness and physiological parameters studied. However, this correlation curve would likely be most pronounced at relatively lower Se tissue levels, perhaps saturating at a certain tissue Se threshold. An inverse correlation between Se concentration and herbivory is predicted.

3.2 RESULTS

SELENIUM ACCUMULATION

As a first step to investigate how Se accumulation affects *S. pinnata* fitness, the degree of variation in Se accumulation was surveyed. Two wild populations were sampled, growing in their native habitat on a naturally seleniferous shale formation at Coyote Ridge Natural Area and Pine Ridge Natural Area near Fort Collins, Colorado, U.S.A. (Figure 3-1). Both populations have been studied extensively (Reynolds *et al*., 2020a; Reynolds *et al*., 2020b; El Mehdawi *et al*., 2011a; El Mehdawi *et al*., 2011b; Freeman *et al*., 2012), but not concerning fitness parameters in the field. The Coyote Ridge population was sampled in spring and the Pine Ridge population in the fall. Substantial variation in leaf Se concentration was found within each population. Leaf Se concentration varied 55-fold among the 23 sampled Coyote Ridge plants (Figure 3-2A) and 13-fold among the 24 sampled Pine Ridge plants (Figure 3-3A). Overall, the leaf Se concentration

was higher in the Pine Ridge plants, which showed an average leaf Se concentration of 2,482 mg kg^{-1} dry weight (DW), while the Coyote Ridge plants showed an average of 868 mg kg^{-1} DW. Furthermore, 83% of the sampled Pine Ridge plants showed Se hyperaccumulator levels in their leaves (> 1000 mg kg^{-1} DW), versus 31% of the Coyote Ridge plants.

The Se variation was more pronounced in the siliques when compared to the seeds in all plants analyzed from both sites. Only 4-fold (Figure 3-2B) and 3-fold (Figure 3-3B) seed Se variations were found, respectively, among Coyote Ridge and Pine Ridge plants. A greater Se variation, 130-fold, was found in the siliques from plants sampled in Coyote Ridge (Figure 3-2B), and a 4-fold variation was determined in the Pine Ridge group (Figure 3-3B). The large silique Se variation in Coyote Ridge reflected the relatively low Se levels found in a few sampled plants; indeed, 17% of all plants from that site showed Se levels below 750 mg kg^{-1} DW.

All the seeds and most of the siliques analyzed contained Se concentrations typical of Se hyperaccumulators for both studied sites. On average, the plants collected from Coyote Ridge accumulated 4,043 mg kg^{-1} DW and 2,267 mg kg^{-1} DW in seeds and siliques, respectively. The Pine Ridge plants contained on average 3,372 mg kg^{-1} DW in seeds and 3,323 mg kg^{-1} DW in the siliques. It should be noted that the seed Se concentration was consistently high for all plants analyzed, even when the silique Se level was low in the same plant.

On average, the Se concentration was higher in the reproductive organs than the leaf tissues for the Coyote Ridge plants. A 3-fold higher Se concentration in the siliques and 5-fold higher Se in seeds were found compared to the leaves. The Pine Ridge plants contained relatively high Se levels in all organs analyzed. However, the slightest Se variation among reproductive organs and leaves was observed in the Pine Ridge population, where the reproductive organs showed on average only 1.5-fold higher Se concentration when compared to the leaves.

Overall, the silique Se concentration showed a positive correlation with the Se levels found in the seeds for Coyote Ridge (Figure 3-4A) (R= 0.5522, P= 0.0266) and Pine Ridge plants (Figure 3-4B) (R= 0.7257. P= 0.0416). Another positive correlation (R= 0.5602, P= 0.0240) was found between leaf Se and Seed Se for the Coyote Ridge plants (Figure 3-4E).

HERBIVORY AND FITNESS PARAMETERS

Further analyses aimed to understand if the leaf Se concentration of *S. pinnata* plants correlated with reduced herbivory percentage, owing to the ecological benefit of reduced herbivory attack. To better understand our results, it is essential to remember that Coyote Ridge samples were collected in the Fall (September), while the Pine Ridge samples were collected during the Spring (May) when the herbivory damage is expected to be lower, and leaf Se concentration is higher (Galeas *et al*., 2007). An indication of a possible positive correlation was found between the percentage of herbivory and the leaf Se accumulation of Coyote Ridge plants (R= 0.4775, P= 0.0526) (Figure 3-5C), which could be attributed to the relatively low Se levels and the activity of Se tolerant herbivores. However, no evident correlation was found in the Pine Ridge plants. A significant (2-fold) difference in the number of leaves per plant was found between the two areas analyzed. On average, the *S. pinnata* plants from Coyote Ridge had 146 leaves (Figure 3-5A), while Pine Ridge plants had 81 leaves (Figure 3-5D). The percentage of leaves with signs of herbivory damage per plant was about fivefold higher in Coyote Ridge, where the plants showed on average more than 85 % of herbivory (Figure 3-5B). In contrast, on average, *S. pinnata* plants grown in Pine Ridge showed only 18% of herbivory (Figure 3-5E).

Overall, no correlation was found between the number of siliques per plant and silique Se. The Coyote Ridge plants had fewer siliques and seeds than those collected at the Pine Ridge site. The average number of siliques per plant was 83 for Coyote Ridge (Figure 3-6A) and 469 for Pine

ridge plants on average (Figure 3-7A), which corresponds to a 5-fold difference. When the seeds were analyzed, no correlation was found between the source Se and the number of seeds per plant (Figures 3-6D and 3-7D) for both sites. A moderate positive correlation (R= 0.4895, P= 0.0543) between the average seed weight per plant and seed Se was found for the Coyote Ridge plants (Figure 3-6F). However, no significant correlation was found for the Pine Ridge plants. The average number of seeds per plant between the two studied sites was more than 30-fold different; the Coyote Ridge plants showed an average of 143 seeds per plant (Figure 3-6C), in contrast to the average of 2,000 seeds found the Pine Ridge plants (Figure 3-7C). The average seed weight per plant did not significantly differ between Coyote Ridge and Pine Ridge plants. The average seed weight per plant in Coyote Ridge was 1.4 mg (Figure 3-6E) and 1.5 mg in Pine Ridge (Figure 3-7E).

TOTAL LEAF PHENOLICS AND ANTIOXIDANT CAPACITY

The antioxidant capacity, expressed in Trolox (vitamin E) equivalents (TEAC), and the amount of leaf total phenolics in terms of gallic acid equivalents (GAE) were analyzed to investigate further the effect of Se hyperaccumulation in *S. pinnata* plants. Overall, the *S. pinnata* plants showed higher total antioxidant capacity when growing at Coyote Ridge than those plants growing at Pine Ridge. The average concentration of vitamin E equivalents found in Coyote Ridge plants was 299 µmol g-1 DW of TEAC, and in Pine Ridge, the number was 1.5-fold lower at 175.63 µmol g-1 DW of TEAC. A significant negative correlation (R= -0.0743, P= 0.0106) between the average seed weight and the total antioxidant capacity was found for the Coyote Ridge plants. However, no significant difference in total leaf phenolics was found between plants from both sites. The average leaf phenolics in Coyote Ridge were 28.54 mg g^{-1} DW of GAE (Figure 3-10A), while in Pine Ridge, this number was 27.5 mg g^{-1} DW of GAE (Figure 3-11A). No

significant correlation was found among the average leaf phenolics and leaf Se (Figures 3-10B and 3-11B), percentage of herbivory (Figures 3-10C and 3-10C), or average seed weight for both sites (Figures 3-10D and 3-11D).

GLUCOSINOLATES QUANTIFICATION

The last set of analyses aimed to understand if the high levels of Se would affect the concentration of glucosinolates (GLS), which are S containing compounds, due to Se and S antagonism for the uptake and assimilation. Also, GLS play critical ecological roles in plants. Progoitrin was the most abundant GLS identified in both leaves and seeds of *S. pinnata*. Our data show no evidence that high Se levels affect the GLS concentration. Furthermore, no negative or positive statistically significant correlation was found between Se and GLS in leaves or seeds of *S. pinnata* (Figures 3-12 and 3-13).

3.3 DISCUSSION

This research aimed to analyze to what extent variation in Se accumulation can affect hyperaccumulator *S. pinnata* fitness, as judged from different parameters for physiological and biochemical performance and herbivory while growing in two seasons on two seleniferous sites, Coyote Ridge and Pine Ridge Natural Areas. While there was substantial variation in Se concentration within each population, most plants within each population had high Se concentration, especially in the reproductive parts. No evidence for positive or negative correlation was found between leaf, silique, or seed Se concentration with any of the fitness or biochemical parameters number of leaves per plant, degree of leaf herbivory, number of siliques and seeds per plant, average seed weight, total leaf phenolics, and total leaf glucosinolates. The lack of correlation between the level of Se concentration and apparent fitness indicates that the observed

variation in Se has no effects. However, it may be that protective effects of Se against biotic stresses or via enhanced antioxidant capacity are already pronounced at low Se tissue levels and that these effects saturate at a particular tissue Se threshold. Most of the plants examined here may have Se levels above this threshold. While there was no indication of Se-conferred enhanced *S. pinnata* fitness within the tissue Se range observed, there was also no indication for fitness being compromised by Se hyperaccumulation due to, e.g., toxicity to the plant itself.

In both populations, there was a clear difference between vegetative and reproductive organs with respect to the degree of variation in tissue Se levels: leaf Se levels varied 55-fold in the Coyote Ridge population and 13-fold in the Pine Ridge population. In contrast, the seeds showed consistently high Se levels, which varied only 4-fold and 3-fold in Coyote Ridge and Pine Ridge populations, respectively. The Se levels found in leaves were not all at hyperaccumulator level (some were below the threshold of 1,000 mg kg^{-1} DW). In contrast, all seeds and siliques samples analyzed from both sites showed hyperaccumulator Se levels.

The observed variation in overall plant Se accumulation may be caused in part by local variation in soil Se concentration and bioavailability (pH, organic matter, microbial composition) (Winkel *et al.*, 2015; White and Broadley, 2009) and in part by variation within populations in expression levels of genes related to hyperaccumulation, such as those involved in sulfate/selenate transport and assimilation (Wang *et al.*, 2018). The physicochemical properties of Pine Ridge soils were investigated (El Mehdawi and Pilon-Smits, 2012), where a relatively high organic matter, and slightly basic soil was reported. Furthermore, some variation in soil Se concentration and distribution in the soil was previously observed. The described soil Se levels in Pine Ridge natural area range from 2 μg g^{-1} DW to 23 μg g^{-1} DW, where the highest Se levels are reported to be present in areas where Se hyperaccumulator plants are present. The reported soil Se levels in

Coyote Ridge is relatively lower, ranging from 0.9 μg g^{-1} DW to 2.2 μg g^{-1} DW, and interestingly, the highest Se levels were found in plots where no hyperaccumulator plants were observed (Reynolds *et al*., 2020a). Regardless of the underlying cause(s), the resulting variation in tissue Se concentration apparently did not affect any of the performance parameters analyzed. Still, evolutionary fitness could only be affected if there is an underlying genetic cause.

The finding that leaf Se was more variable than seed Se may be explained by earlier observations. *Stanleya pinnata* was shown to redistribute Se specifically (independently from S) via remobilization from aging leaves, resulting in Se levels that are highest in young leaves and the pollen and ovules of flowers, as well as in seeds (Galeas *et al*., 2007; Freeman *et al*., 2010; Quinn *et al*., 2011). *S. pinnata* may preferentially store Se in its reproductive organs because it offers seed and seedling protection from biotic and abiotic stresses (Quinn *et al*., 2011). Within young leaves, Se is sequestered in the vacuoles of epidermal cells at the edge of the leaf, achieving maximal herbivory and pathogen protection (Freeman *et al*., 2006; Freeman *et al*., 2010). These Se partitioning preferences may confer plant fitness, associated with a plant's productivity and reproductive success. From a practical perspective, the more consistently high levels of Se in the reproductive organs indicate that these organs are a very reliable indicator of hyperaccumulator status and can be sampled in addition to leaves when available.

Our data show no negative correlation between plant Se concentration and herbivory damage. There was a weak positive correlation between leaf Se accumulation and leaf herbivory for Coyote Ridge plants (R= 0.4775, P= 0.0526, Figure 3-5C). The Coyote Ridge samples were collected in September, when seasonal variation in leaf Se concentration is lowest (Galeas *et al*., 2007), explaining the almost 3-fold lower average Se concentration than the Pine-Ridge samples collected in May, explaining their overall higher degree of herbivory damage. The relatively low

Se levels in those leaves may have made them more edible for herbivores, especially those with elevated Se-resistance. Previous research has shown strong evidence that plant Se accumulation offers protection against a wide variety of different generalist herbivore species through deterrence and toxicity; these include aphids (Hanson *et al*., 2004), moth and butterfly larvae (Lyons *et al*., 2009), grasshoppers (Freeman *et al*., 2007; El Mehdawi *et al*., 2011b), thrips, and spider mites (Quinn *et al*., 2010) and prairie dogs (Freeman *et al*., 2009). However, there is also clear evidence of Se-resistant herbivores occurring in seleniferous areas that feed on hyper-accumulator leaves and seeds (Freeman *et al*., 2006; Freeman *et al*., 2012; Valdez Barillas *et al*., 2012). The new results presented here indicate that these Se-resistant herbivores pose a significant herbivory pressure for *S. pinnata*, considering that most leaves showed herbivory damage, even when the Se tissue levels were high. For instance, 31% of the Coyote Ridge plants showed leaf Se levels above the hyperaccumulator threshold, ranging from 1,018 - 2,565 mg kg^{-1} DW (Figure 3-5A). Many of these plants showed significant herbivory damage (Figure 3-5B). The presence and significant pressure of Se-resistant herbivore populations may obscure any protective effects of the accumulated plant Se against generalist herbivores, offering another reason for the lack of correlation between plant Se concentration and herbivory damage, besides the explanation that most plants had Se levels above a typically protective threshold.

The effect of accumulation of high Se levels in plants goes beyond ecology. Ultimately, toxic levels of Se in tissues could directly impact plant physiology and reproduction. We found no evidence of such a constraint. Plant size in this study did not correlate with leaf Se concentration. The reproductive fitness of the studied *S. pinnata* plants, as estimated from the number of siliques and seeds and the average seed weight per plant, was not compromised by high Se levels.

On the contrary, a moderate positive correlation was observed between the average seed weight per plant and seed Se for the Coyote Ridge plants (Figure 3-6F). A beneficial effect of Se fertilization on plant productivity is well documented at low Se levels, e.g., mustard seeds (Lyons *et al*., 2009) and lentil seeds (Ekanayake *et al*., 2015; Thavarajah *et al*., 2015). Selenium can exert positive physiological effects at low concentrations that could result in higher seed quality, such as improving overall growth and development enhancing photosynthesis resulting in a higher accumulation of starch and sugars (Feng and Wei, 2012; Xue *et al*., 2001).

It has been reported that Se increases the transcript levels and activity of different antioxidant enzymes, thereby regulating the concentration of ROS and overall tissue antioxidant response (Feng *et al*., 2013). Hyperaccumulator species even seem to benefit from Se at tissue levels that are toxic to non-hyperaccumulator species. For example, when Se was supplied at hyperaccumulator levels to *S. pinnata*, the overall growth and reproductive parameters such as pollen tube growth were improved (Quinn *et al*., 2011; El Mehdawi and Pilon-Smits, 2012; Freeman *et al*., 2012). The new field data from this study agree with these lab studies and indicate that the capacity for Se tolerance does not appear to be a physiological constraint for Se hyperaccumulation in *S. pinnata* in the field.

To analyze how tissue Se concentration influences the antioxidant activity in leaves of *S. pinnata* plants grown in the field, the total antioxidant capacity, and the levels of leaf total phenolics were investigated. Under a certain threshold, a relatively higher yield of polyphenols in the extract can indicate a higher cellular antioxidant activity. However, no correlation between the total antioxidant capacity and the total leaf Se or the percentage of herbivory was found. Furthermore, the Coyote Ridge samples showed, on average, a higher antioxidant capacity than those from Pine Ridge, which could be explained by an elevated concentration of antioxidant

compounds due to the higher state of herbivory of those plants, as described before. Interestingly, a significant negative correlation between the average seed weight and the total antioxidant capacity was found for the Coyote Ridge plants. Oxidative stress is characterized by an imbalance in the cellular redox state due to the overproduction of reactive oxygen species (ROS) above the cellular antioxidant capacity (Cuypers *et al*., 2010; Foyer and Noctor, 2012; Shieber and Chandel, 2014). Even though ROS are known to have significant signaling roles in seed germination (El-Maarouf-Bouteau and Bailly, 2008), their overproduction can lead to the destruction of lipids, proteins, and nucleic acids, resulting in impaired development and overall lower seed weight. The cellular enzymatic and non-enzymatic antioxidant defense mechanisms can directly neutralize the excessive levels of ROS. Therefore, if these seeds are experiencing some level of oxidative stress, it is expected that there would be a higher production of different antioxidant enzymes and secondary metabolites, which was not found here.

Furthermore, no correlation was found between the average of the total leaf phenolics and all the other analyzed parameters (total leaf Se, percentage of herbivory, and average seed weight). It seems counterintuitive that a higher Se concentration does not correspond with a higher antioxidant capacity. However, *S. pinnata* plants also have an elevated tissue concentration of sulfur (S) and S-containing metabolites (GSH and GSSG) because of the constitutively high expression of different sulfate transporters and S assimilation pathway enzymes (Wang *et al*., 2018); this might result in a higher reactive oxygen species ROS scavenging capacity and a better response to oxidative stress, independent of the Se-status of the plant. Furthermore, Se at low levels improves the cellular antioxidant response in plants (Feng and Wei, 2012; Feng *et al*., 2013). Perhaps the correlation between Se and antioxidant capacity might be more apparent at a lower Se concentration range or possibly lowered antioxidant capacity when Se is lacking.

Plants utilize different mechanisms to defend their tissues from herbivory and pathogens. Accumulating particular toxic elements from the environment as an elemental defense strategy is generally considered to be relatively cost-efficient (Boyd and Martens, 1992; Boyd, 2007), as compared to other strategies by which some species utilize more energy-costly physiological strategies to cope with biotic stress. The central defense secondary metabolites in the Brassicaceae family are the glucosinolates (GLS), a large group of sulfur and nitrogen-containing compounds responsible for herbivory protection and other ecological roles. The enzyme myrosinase initiates the hydrolysis of the GLS into its active forms, and it is stored in different cells from the GLS. When herbivores damage the tissues, the myrosinase comes into contact with its substrate, forming glucose and the unstable aglycone, which is later converted to the active compounds (Matich *et al*., 2012; Matich *et al*., 2015).

Stanleya pinnata was found here to contain high concentrations of GLS compounds, in addition to its high Se levels. Thus, it seems these plants are using two different mechanisms for herbivory protection. Our study did not find any correlation between Se and GLS in leaves or seeds of *S. pinnata*, indicating the GLS metabolism is not affected by Se in the HA. Since GLS are S-containing metabolites, higher levels of Se could negatively impact the GLS pool in the tissues. Such inhibition was already reported by Tian *et al.* (2018), where the supplementation with 25 μM of sodium selenate lowered the expression of several genes in the GLS biosynthesis pathway and significantly reduced the concentration of the GLS-precursor amino acids methionine and phenylalanine, and the GLS concentration in leaves and florets of broccoli, without affecting the S status of the plant. Similar results were reported by Toler *et al.* (2007), where the concentration of different GLS was reduced in the presence of selenate in Brassica oleracea, even under regular

S supplementation. It appears that the hyperaccumulator *S. pinnata* differs from its Brassicaceae relatives in this respect, as it does in other aspects of Se-S interactions.

Other studies reported the presence of SeGLS in Brassica spp. (secondary Se accumulators), specifically the family of glucosinolates containing the methylthio (CH_3–S–) group (MeS-GLS) (Matich *et al*., 2012). Matich *et al*. (2012) found SeGLS in broccoli florets and leaves as glucoselenoerucin, cauliflower florets, and stems SeGLS was found as glucoselenoiberverin (Matich *et al*., 2015), and in forage rape taproots as glucoselenoerucin (Matich *et al*., 2015), after 5.0 mM sodium selenate supplementation for four weeks. It has been suggested that, at low concentrations, Se can be incorporated into the methylthioalkyl moiety of GLS from the amino acid SeMet in the Brassica spp., replacing its S analog without disrupting the formation of S-GLS (Matich *et al*., 2015). To date, the presence of SeGLS in *S. pinnata* has not yet been reported. Our finding that GLS metabolism is not affected by high levels of Se in this Se-hyperaccumulator may suggest a mechanism for excluding Se from these compounds.

3.4 MATERIALS AND METHODS

PLANT MATERIAL, STUDY SITES, AND GPS COORDINATES

Biological materials of different Stanleya pinnata L. (Brassicaceae) plants were collected at two different sites: Coyote Ridge natural area (Geographic coordinates: Latitude 40°28’51” N, Longitude 105°07’31” W), and Pine Ridge natural area (Geographic coordinates: Latitude 40°32’32” N, Longitude 105°08’04” W). Both natural areas have been described before (Reynolds *et al*., 2020a; Reynolds *et al*., 2020b) and are located on a seleniferous formation West of Fort Collins in the state of Colorado, in the United States of America. Samples from 23 different plants were collected in Pine Ridge natural area, and a total of 24 individual plants from the Pine Ridge natural area were used.

A total of seven field trips were conducted to the study sites during 2017, 2018, and 2019. In 2017 two trips to each location were made to collect leaves (branches), seeds, and siliques. Pine Ridge: May 31st (Spring) and September 18th (Fall). Coyote Ridge: July 05th (Summer) and September 20th (Fall). In 2018 one trip was made to each site to collect Leaves (branches). Pine Ridge: August 3rd (Summer). Coyote Ridge: August 17th (Summer). On May 16th, 2019 (Spring), one last sampling trip was made to Pine Ridge to collect Leaf samples.

The GPS coordinates for each plant were recorded using a Garmin Oregon 650t GPS. The GPS points were managed using EasyGPS (version 6.11 TopoGrafix Edition), and the satellite images shown were generated using Google Earth (Version 9.129.0.1).

DETERMINATION OF SELENIUM CONCENTRATION

After drying at 50 °C until constant weight, 100 mg of *S. pinnata* seed and leaf samples from each study site were weighed for elemental analysis. These samples were then digested with 1 mL of nitric acid as follows (Zarcinas *et al*., 1987): the samples were heated for two hours at 60 °C and six hours at 125 °C, then diluted to 10 ml with distilled water. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to analyze the digested seed samples' elemental composition.

HERBIVORY AND FITNESS PARAMETERS

The following fitness parameters were analyzed: total number of leaves per plant, number of siliques per plant, number of seeds per plant, and average individual seed weight. The total number of leaves with herbivory, number of intact leaves, and percentage of leaves with herbivory were also analyzed per plant. The total number of branches per plant in the field was recorded to help estimate the mentioned parameters. One to three branches per plant were collected and

brought to the lab, and the number of leaves and siliques per branch was counted. The numbers per branch were then multiplied by the total number of branches per plant in the field to estimate the total number of leaves and siliques per plant.

All the siliques from the same plant were opened, and the seeds were collected in one microcentrifuge tube. Ten random seeds from the same plant were then weighed using a precision scale (Mettler Toledo, AB204-S/FACT). This number was then divided by 10 to estimate the average seed weight for that plant.

TOTAL LEAF PHENOLICS AND ANTIOXIDANT CAPACITY

Leaf samples were lyophilized, powdered, and weighed. The freeze-dried material was extracted with 80% acetone at a ratio of 25µL/1mg tissue while rotated in the dark at four °C for 30min. The supernatant was collected, diluted with additional acetone at 1:10 or 1:20 depending on the sample, and stored on ice until used. All samples for this assay were read at 734 nm using a PowerWaveXS2 UV–vis spectrophotometer (BioTek Instruments, Winooski, VT) using the method of Miller and Rice-Evans (1996). Trolox (Vitamin E equivalents) was the standard used for this assay, and results are expressed as micromoles of Trolox-equivalent antioxidant capacity (TEAC) per gram dry weight (µmol g^{-1} DW).

Diluted supernatant collected from the extraction described above was used for total phenolics. Folin-Ciocalteu (Sigma Chemicals, St. Louis, MO) was used as described by Singleton and Rossi (1965). All samples for this assay were read at 765 nm using a PowerWaveXS2 UV–vis spectrophotometer using gallic acid as a standard with results expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight (mg g^{-1} DW).

GLUCOSINOLATES EXTRACTION AND QUANTIFICATION

Total glucosinolates were extracted from leaf and seed samples according to Argentieri *et al.* (2011) with some modifications (Dall'Acqua *et al*., 2019). Seeds (30 mg) were first frozen in liquid nitrogen and then grounded. GLS were extracted by boiling the crushed seeds and leaf samples in 18 mL methanol/water mixture (70:30, v/v) for 10 min to deactivate myrosinase. The supernatants were then dried (two extracts per sample) and resuspended in 500 mL methanol. Sinigrin (1.26 mg/ml concentration) was added to the solution as an internal standard. After 4 minutes, the solution was filtered at 0.45 μM (Millipore). The solutions were then boiled one more time in 70% methanol (v/v) for four minutes to ensure the complete extraction of total glucosinolates from the samples. The two extracts were further combined and purified once more using a Solid-Phase Extraction (SPE) column (0.8 x4 cm, Agilent Technologies), equipped with 0.256 g of an ion exchange resin (DEAE-SE HADES-A25), imbedded in 4 ml of a 0.5 M Na-acetate buffer (pH=5). The system was first washed with 1ml of deionized water and then loaded with 2.5 ml of the extracted samples containing the standard. The column was further treated overnight with the enzyme sulfatase (41.6 mg/ml dilution) extracted from Helix pomatia-Type 1 (Roman snail) to convert the glucosinolates into the corresponding desulfated derivates. These derivates were further eluted from the column using 2 ml of deionized water. Glucoerucin was used as a reference standard at different concentration levels to quantify glucosinolates.

STATISTICAL ANALYSIS

The software JMP Pro 15.0.0 (SAS Institute, Cary, NC, USA) was used for statistical data analysis. Multivariate analysis was used to individually compare the Selenium concentration in different tissues and all the fitness parameters. Pairwise correlations were performed for each

combination of variables. The correlation coefficient R and the P-values are shown in the scatterplots.

3.5 CONCLUSIONS

It was hypothesized that plant Se concentration positively correlates with the various fitness and physiological parameters, particularly at lower Se tissue levels, perhaps saturating at a certain tissue Se threshold. Also, an inverse correlation be-tween Se concentration and herbivory is predicted. The field studies presented here suggest that the observed variation in Se hyperaccumulation does not enhance or compromise *S. pinnata* fitness when growing in its natural habitat on seleniferous soil. Despite the variation, most plants had high to very high Se levels, especially in reproductive organs. Plant size and reproductive parameters were not correlated with Se concentration, so the physiological capacity for Se tolerance does not appear to be a constraint for Se hyperaccumulation in this species. There was significant herbivory pressure even on the highest-Se plants, likely from Se-resistant invertebrate herbivores. Thus, while there may be Se-mediated herbivory protection to the hyperaccumulator from generalist herbivores, Se-resistant herbivores appeared to overcome this protective effect and are a significant presence in this native seleniferous habitat, perhaps limiting the ecological advantage of Se hyperaccumulation.

3.6 FIGURES

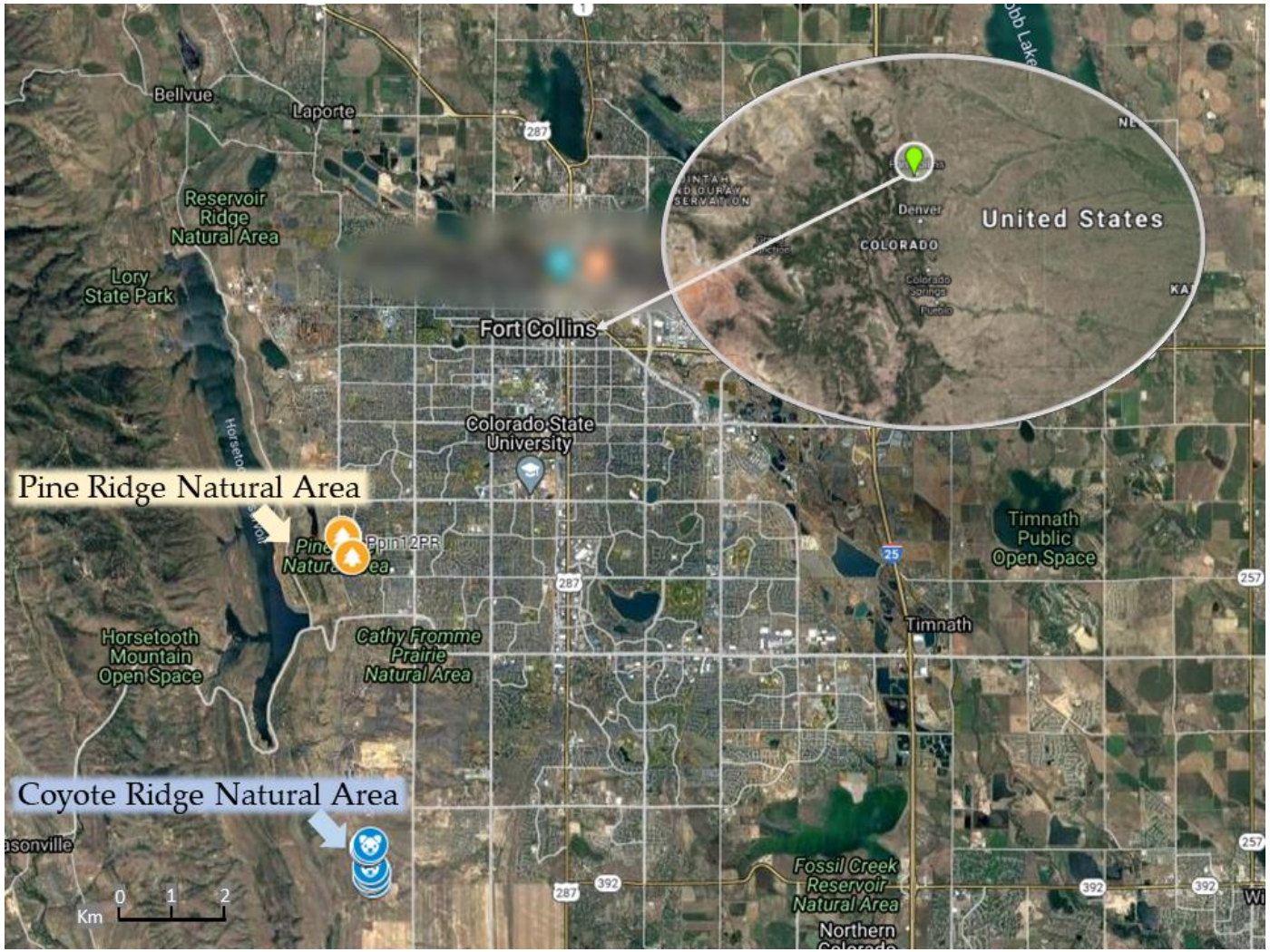

Figure 3-1. Map depicting the two natural areas investigated, namely the Coyote Ridge natural area (Geographic coordinates: Latitude 40°28'51" N, Longitude 105°07'31" W), and the Pine Ridge Natural area (Geographic coordinates: Latitude 40°32'32" N, longitude 105°08'05" W), near the city of Fort Collins, Colorado/U.S.A (Lima *et al*., 2022).

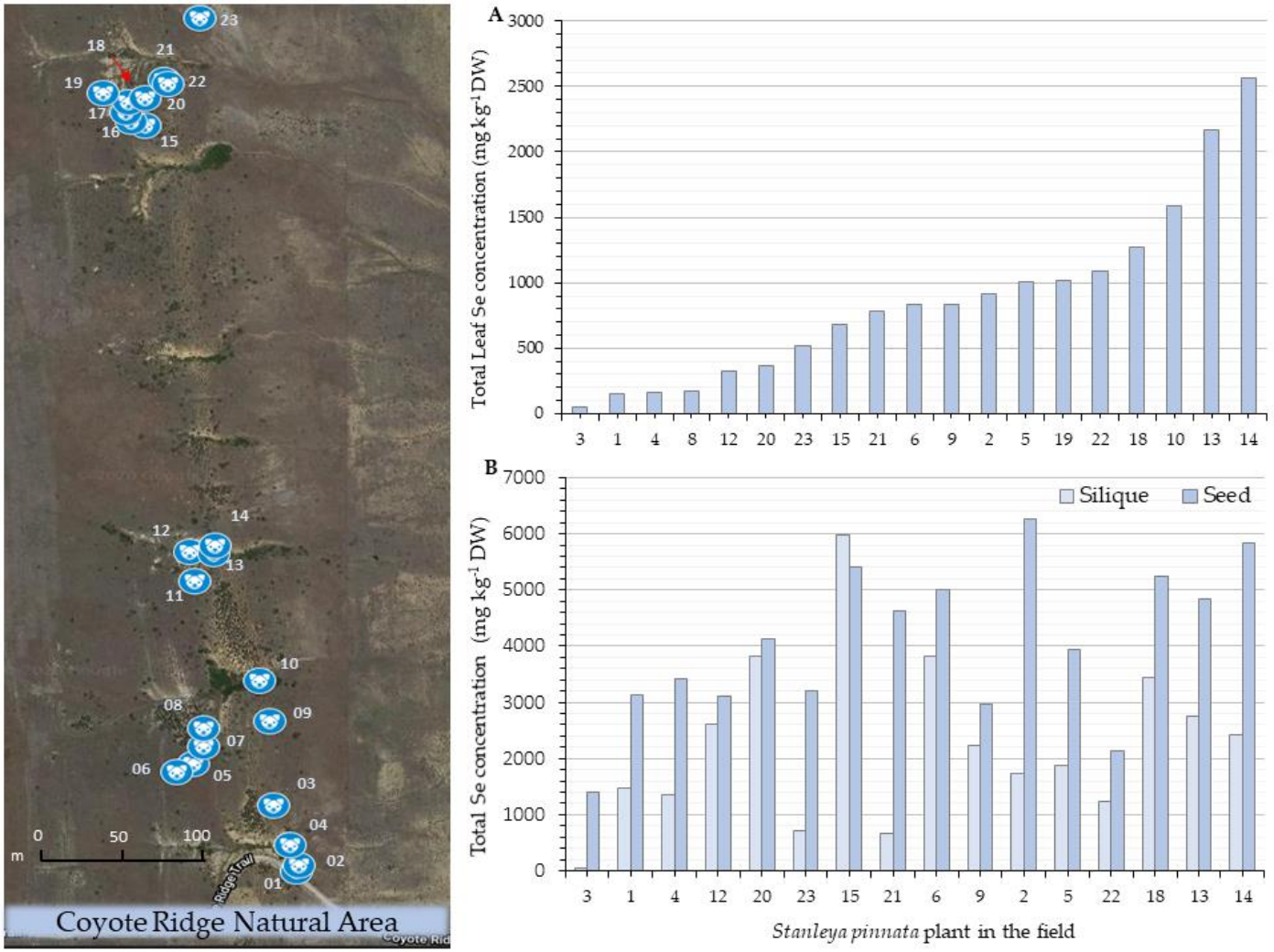

Figure 3-2. *Stanleya pinnata* plant locations at the Coyote Ridge natural area (left) and tissue Se concentrations (right). The numbers on the horizontal axis represent collection numbers of each plant. (A) shows leaf Se concentration of individual plants, ordered according to their Se levels. (B) shows silique and seed Se concentration of the same plants, where the numbering was maintained for consistency. (Lima *et al*., 2022)

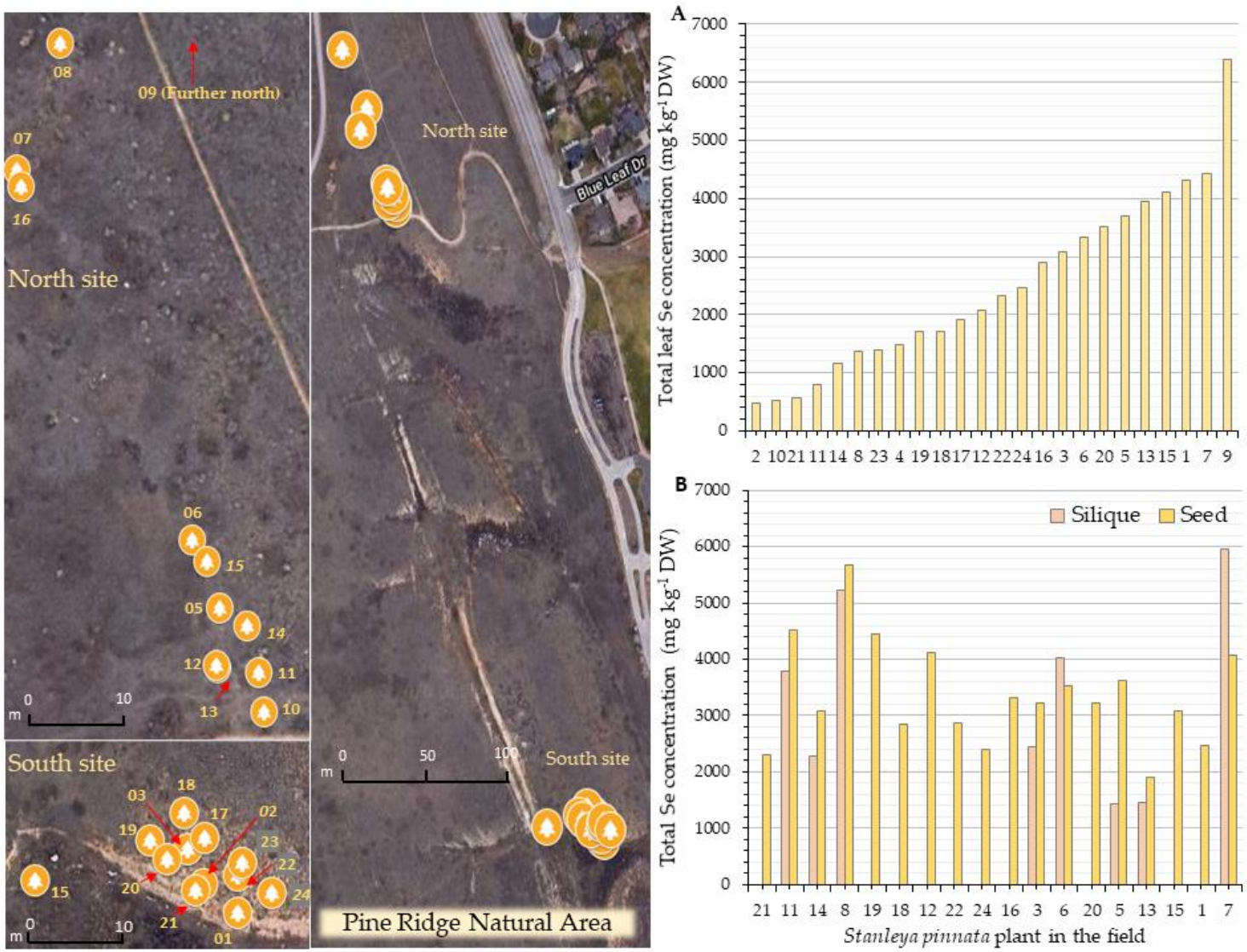

Figure 3-3. *Stanleya pinnata* plant locations at the Pine Ridge natural area (left) and tissue Se concentrations (right). The numbers on the horizontal axis represent collection number for each plant. (A) shows leaf Se concentration ordered according to their Se levels. (B) shows silique and seed Se concentration for the same plants; the numbering was maintained for consistency**.** (Lima *et al*., 2022)

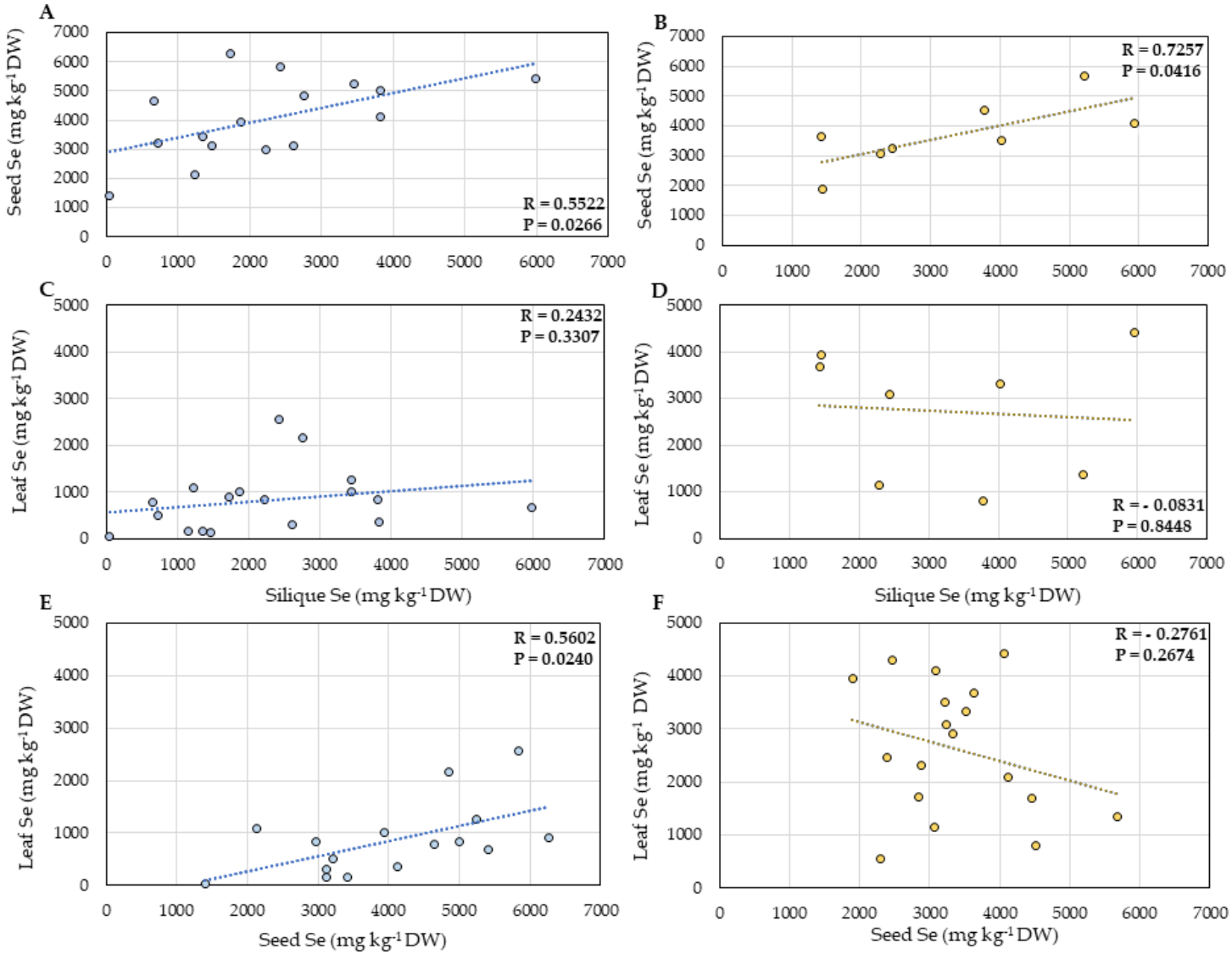

Figure 3-4. Correlation between seed and silique Se concentration (A, B), leaf and silique Se concentration (C, D), leaf and seed Se concentration (E, F). Correlation coefficient R and P-value are shown in each panel. Panels A, C, and E show the Coyote Ridge data. Panels B, D, and F show the Pine Ridge data. (Lima *et al*., 2022)

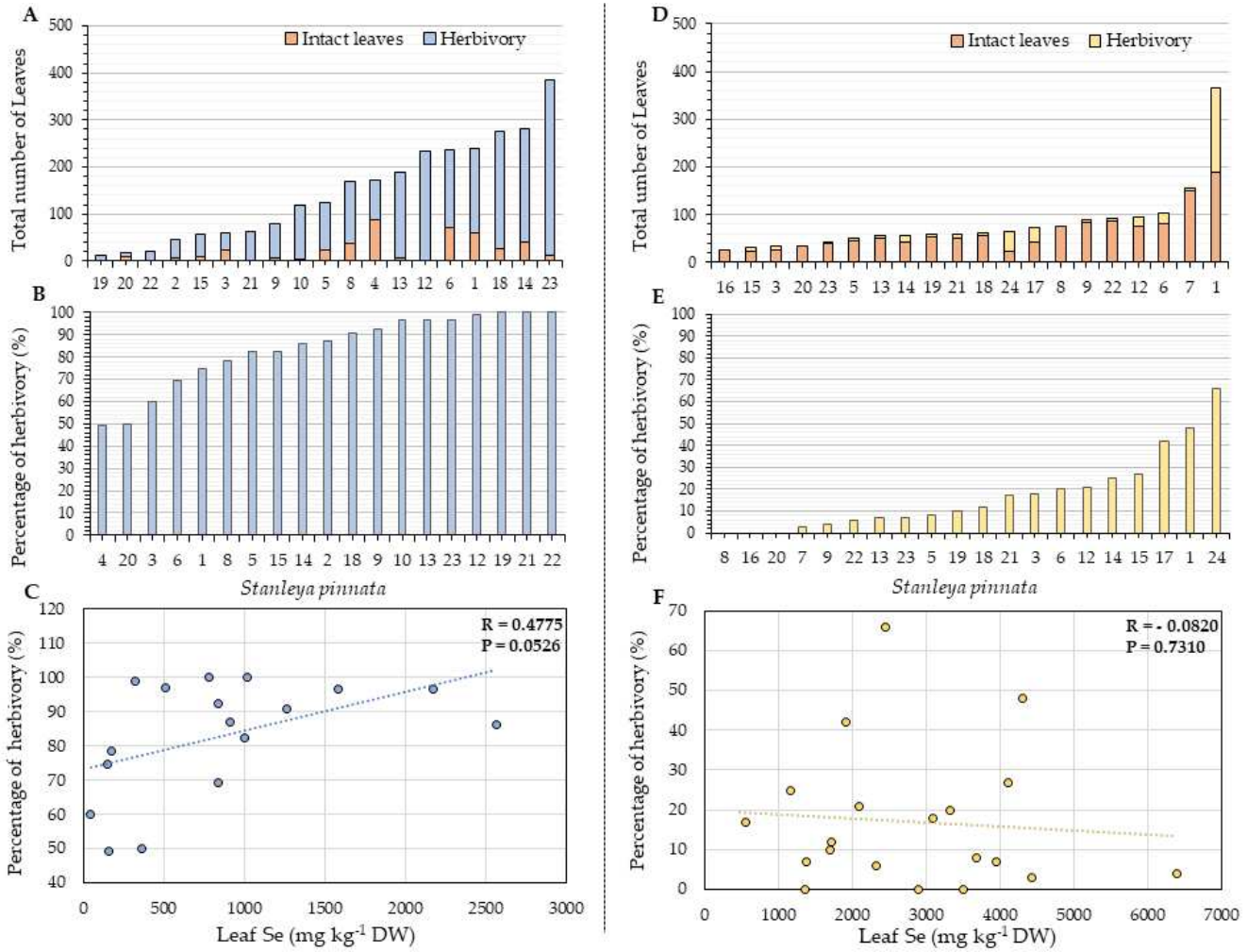

Figure 3-5. Total number of leaves with and without signs of herbivory per plant (A, D). The state of herbivory is represented as the percentage of leaves with herbivory per plant (B, E). Correlation between leaf Se con-centration with herbivory (C, F) for *S. pinnata* plants growing at Coyote Ridge (A-C) and Pine Ridge (D-F). The numbers on the horizontal axis (A, B, D, F) represent collection numbers of individual plants at the locations indicated in Figures 3-2 and 3-3. The graph shows the correlation coefficient R and P-value for panels C and F. Coyote Ridge samples were collected in September Pine Ridge samples in May when herbivory damage was lower. (Lima *et al*., 2022)

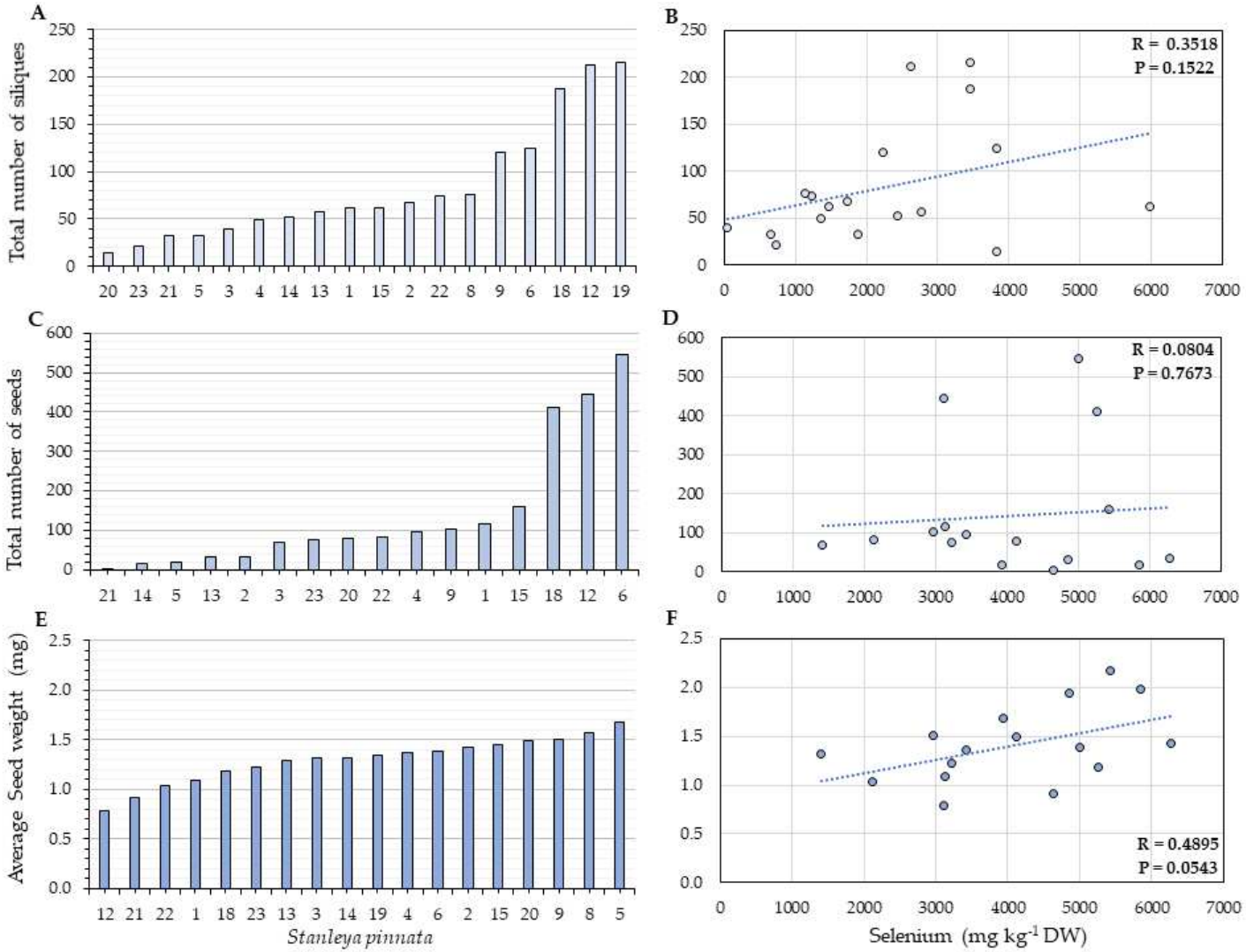

Figure 3-6. Reproductive fitness parameters (A, C, E) and their correlation with silique (B) or seed (D, F) Se concentration for *S. pinnata* plants growing at Coyote Ridge . (A, B) total number of siliques per plant; (C, D) total number of seeds per plant; E, F: average seed weight. The numbers on the horizontal axis (A, C, F) represent collection numbers of individual plants at the locations indicated in Figure 3-2. The graphs show the correlation coefficient R and P-value for panels B, D, and F. (Lima *et al*., 2022)

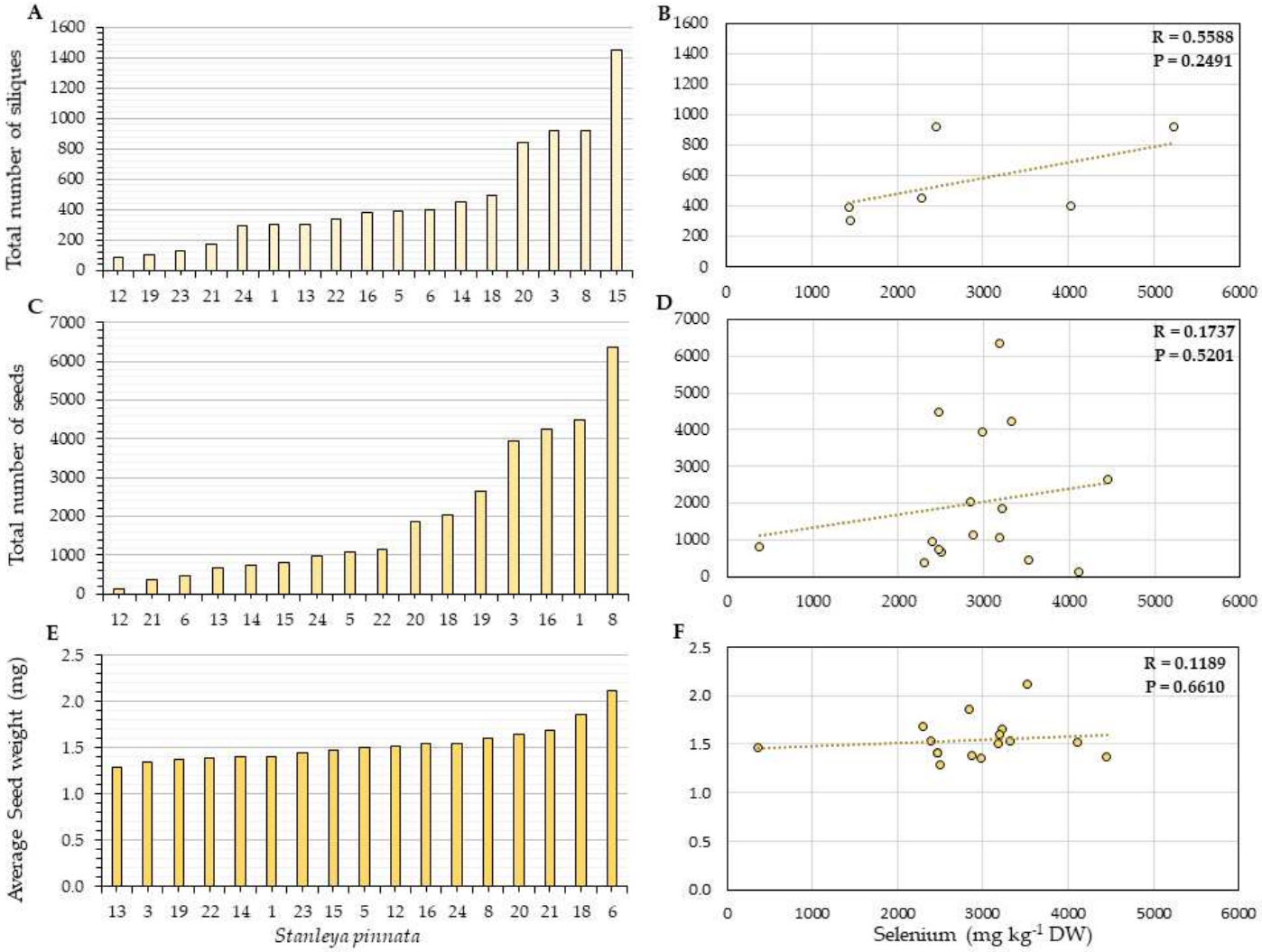

Figure 3-7. Reproductive fitness parameters (A, C, E) and their correlation with silique (B) or seed (D, F) Se concentration for *S. pinnata* plants growing at Pine Ridge. (A, B) total number of siliques per plant; (C, D) total number of seeds per plant; E, F: average seed weight. The numbers on the horizontal axis (A, C, F) represent collection numbers for individual plants at the locations indicated in Figure 3-3. The graphs show the correlation coefficient R and P-value for panels B, D, and F. (Lima *et al*., 2022)

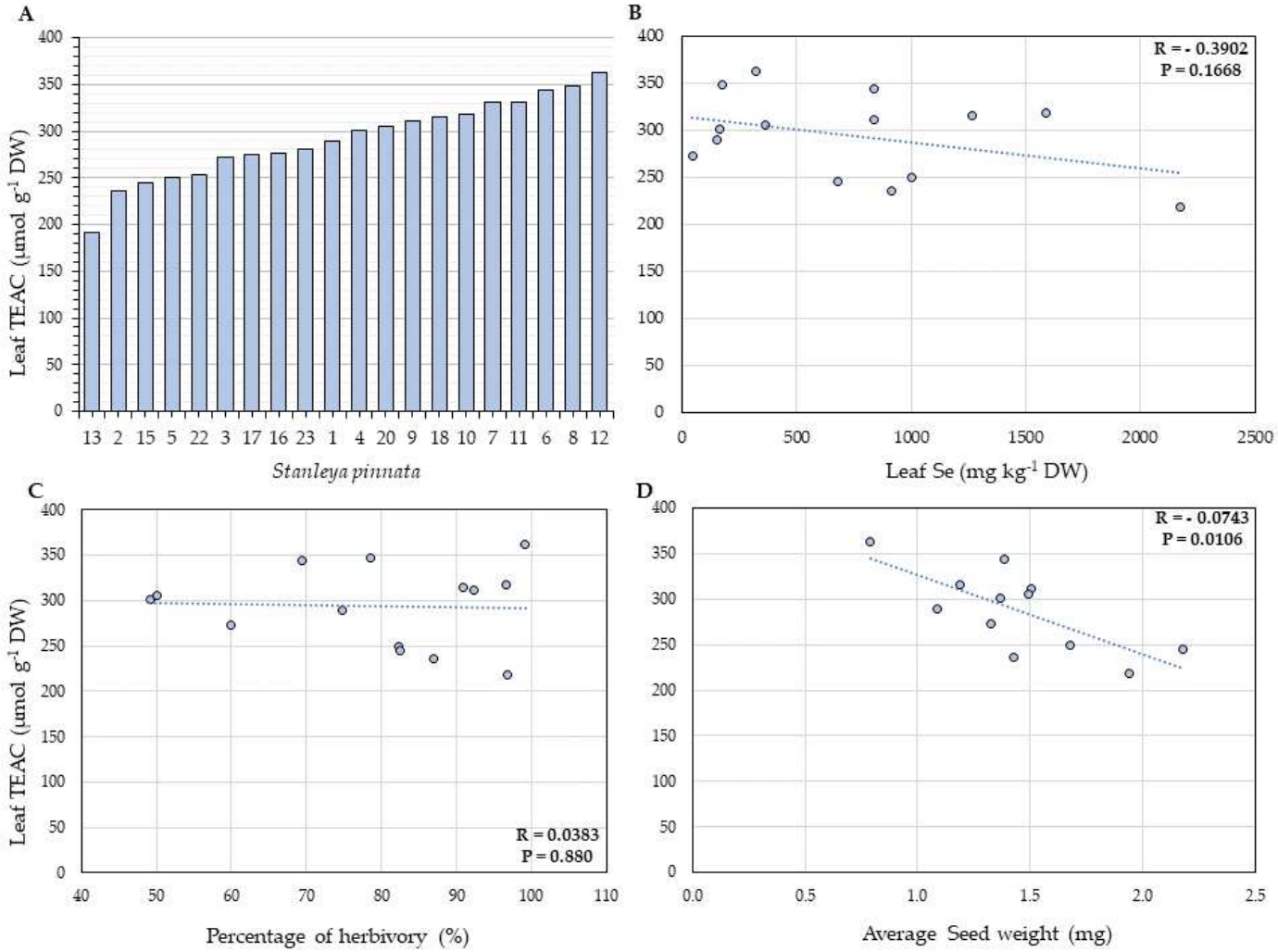

Figure 3-8. A: Total antioxidant capacity in leaves of *S. pinnata* plants (Coyote Ridge), expressed as Trolox (Vitamin E equivalents) (TEAC). The error bars in panel A represent the Standard Deviation of the mean from three technical replicates per plant. B-D: Correlation between leaf TEAC (Vertical axis) with leaf Se concentration (B), percentage of herbivory (C), and average seed weight (D). The numbers on the horizontal axis (A) represent collection numbers for individual plants at the locations indicated in Figure 3-2. The graphs show the correlation coefficient R and P-value for panels B, C, and D. (Lima *et al*., 2022)

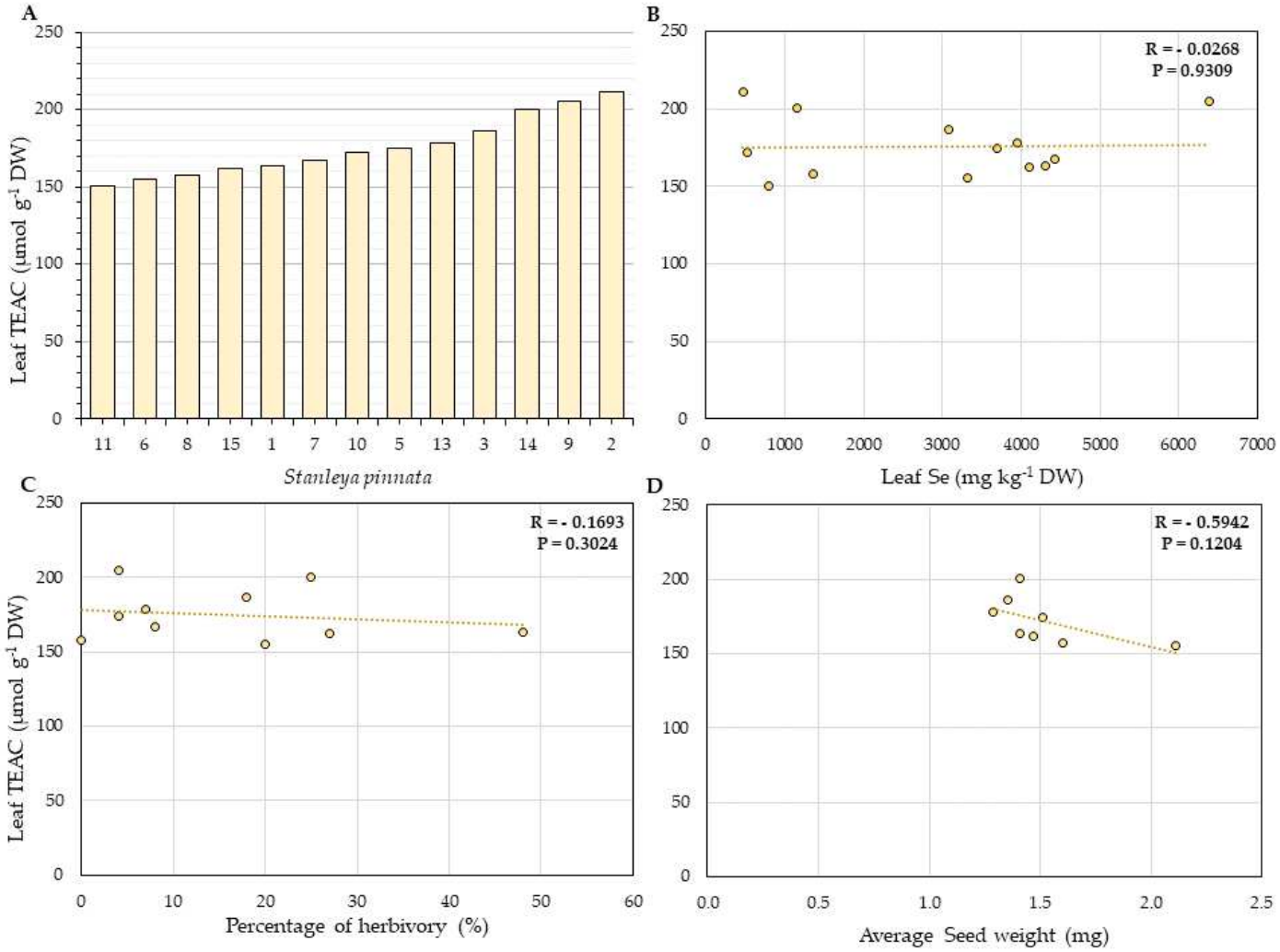

Figure 3-9. A: Total antioxidant capacity in leaves of *S. pinnata* plants (Pine Ridge), expressed as Trolox (Vitamin E equivalents) (TEAC). The error bars in panel A represent the Standard Deviation of the mean from three technical replicates per plant. B-D: Correlation between leaf TEAC (Vertical axis) with leaf Se concentration (B), percentage of herbivory (C), and average seed weight (D). The numbers on the horizontal axis (A) represent collection numbers for individual plants at the locations indicated in Figure 3-3. The graphs show the correlation coefficient R and P-value for panels B, C, and D. (Lima *et al*., 2022)

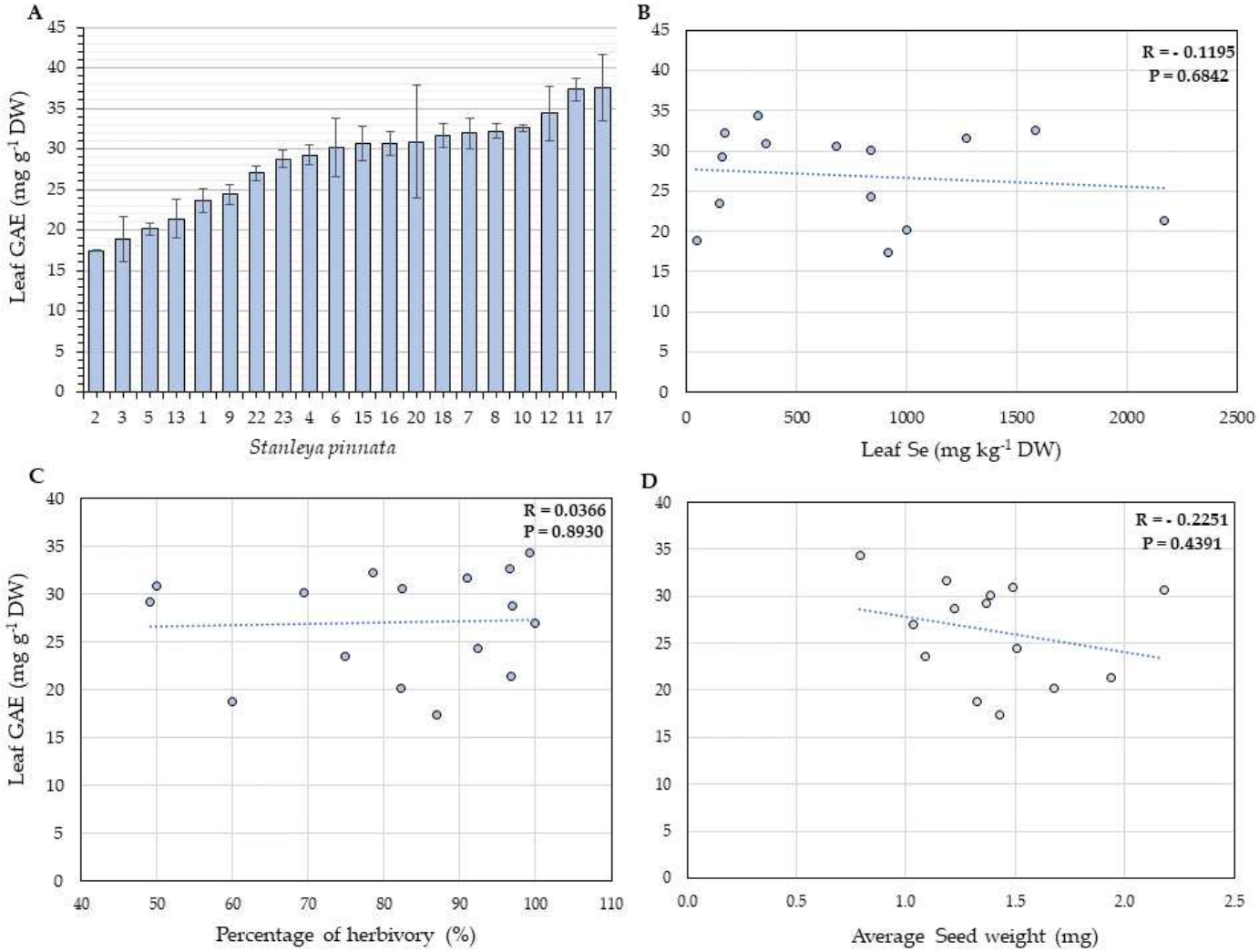

Figure 3-10. A: Total phenolics concentration in leaves of *S. pinnata* plants growing at Coyote Ridge (locations indicated in Fig. 2), expressed as gallic acid equivalents (GAE). (B-D) Correlation between leaf phenolics and leaf Se concentration (B), and number of leaves (C) and between seed phenolics concentration and average seed weight (D) . The numbers on the horizontal axis (A) represent individual plants at the locations indicated in Figures 3-2. Correlation coefficient R and P-value for panels B, C and D are shown in the graphs. (Lima *et al*., 2022)

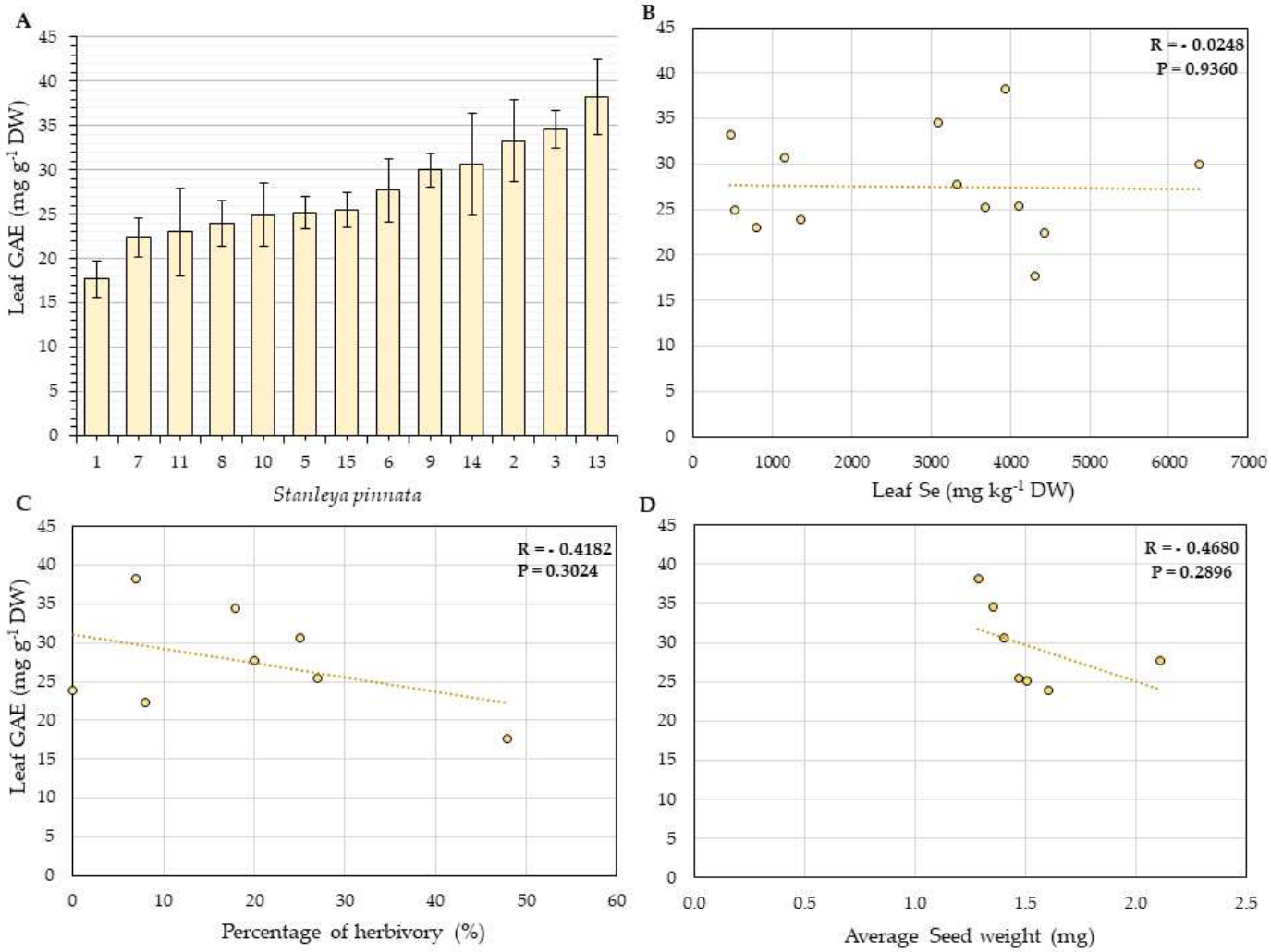

Figure 3-11. A: Total phenolics concentration in leaves of *S. pinnata* plants growing at Pine Ridge (locations indicated in Fig. 3), expressed as gallic acid equivalents (GAE). (B-D) Correlation between leaf phenolics and leaf Se concentration (B), and number of leaves (C) and between seed phenolics concentration and average seed weight (D) . he numbers on the horizontal axis (A) represent individual plants at the locations indicated in Figure 3-3. Correlation coefficient R and P-value for panels B, C and D are shown in the graphs. (Lima *et al*., 2022)

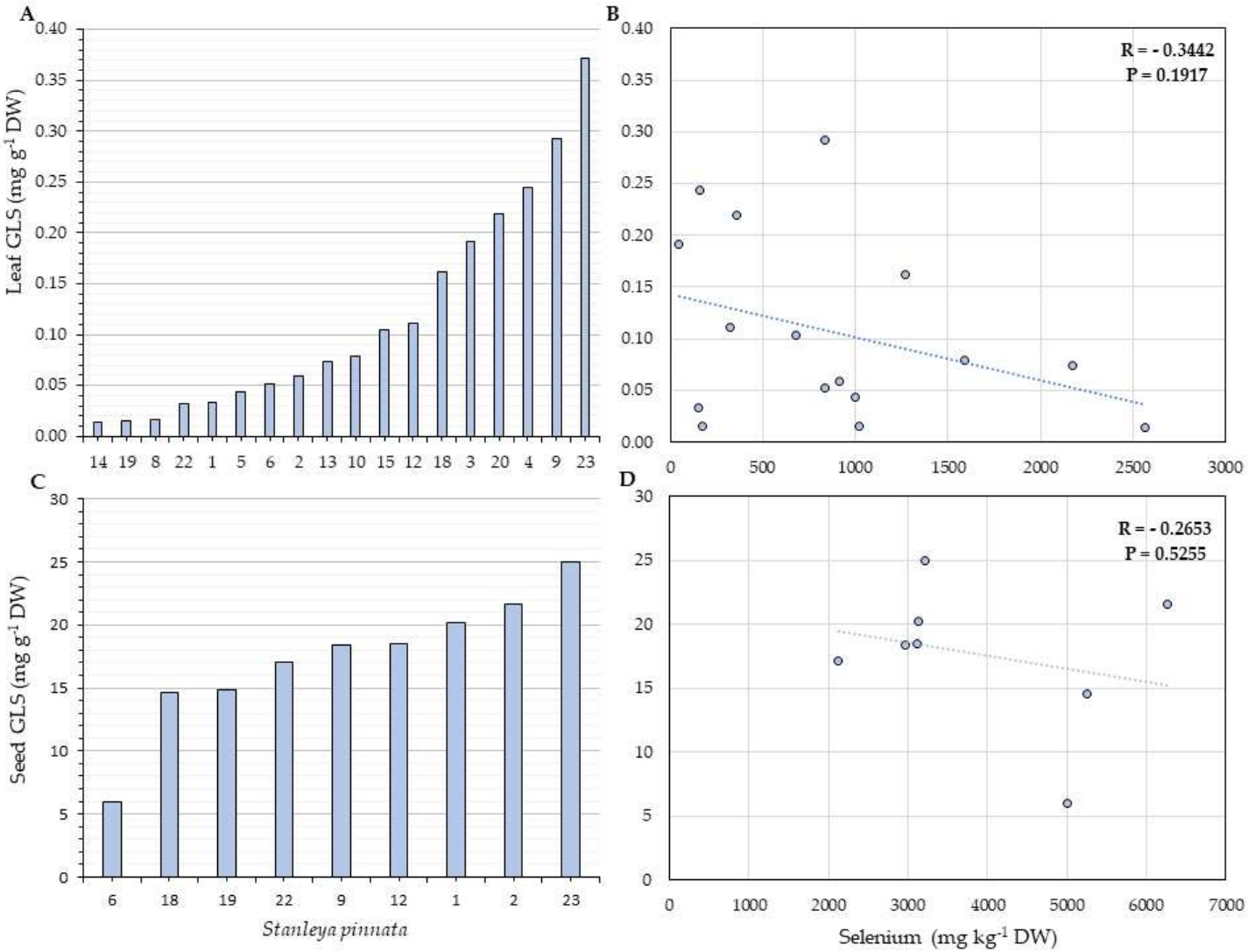

Figure 3-12. Total glucosinolates (GLS) concentration in leaves (A) and seeds (C) and their correlation with leaf (B) and seed (D) Se concentration for *S. pinnata* plants growing at Coyote Ridge (locations shown in Figure 3-2). The numbers on the horizontal axis (A, C) represent collection numbers for individual plants at the locations indicated in Figure 3-2 . The graphs show the correlation coefficient R and P-value for panels B and D. (Lima *et al*., 2022)

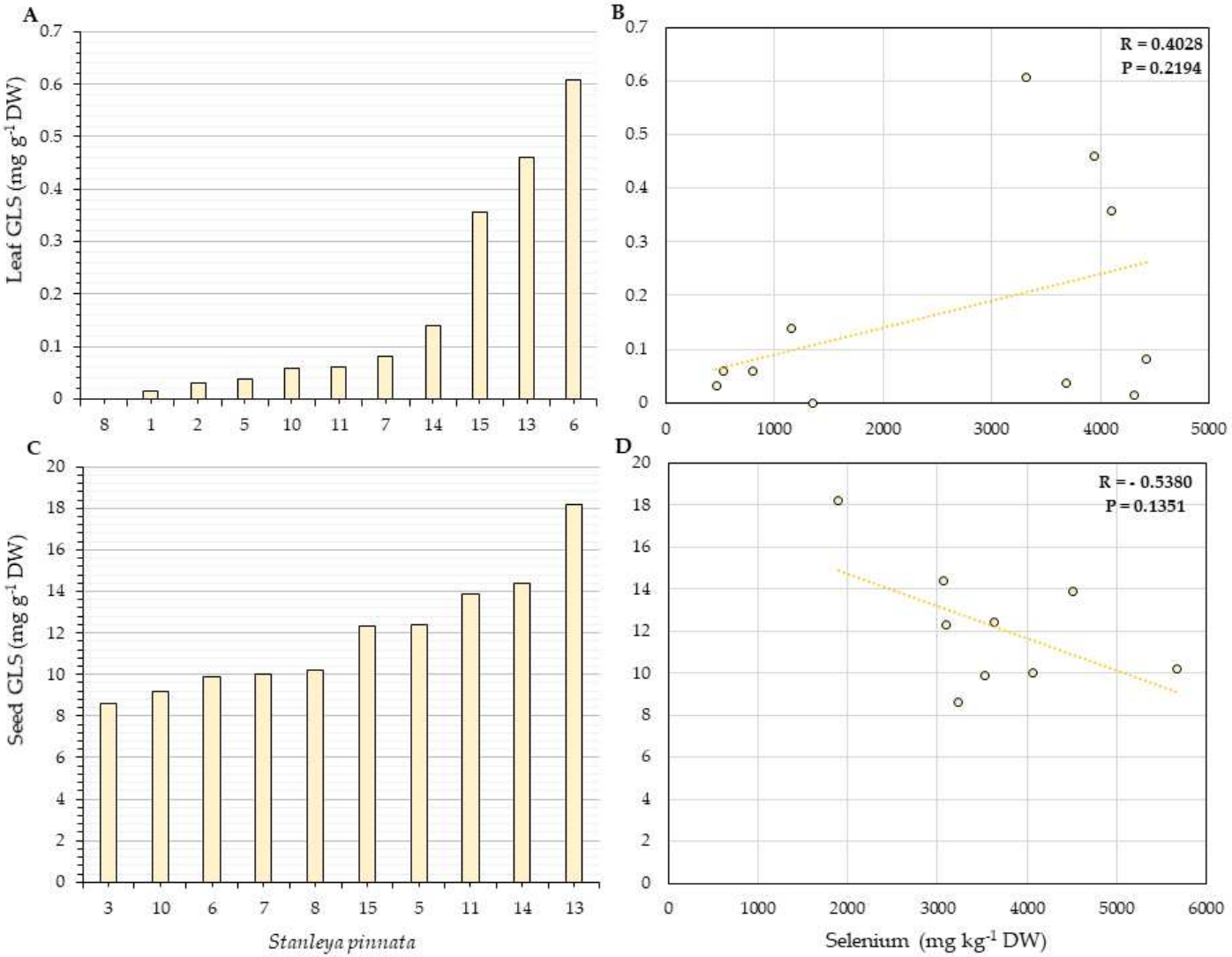

Figure 3-13. Total glucosinolates (GLS) concentration in leaves (A) and seeds (C) and their correlation with leaf (B) and seed (D) Se concentration for *S. pinnata* plants growing at Pine Ridge (locations shown in Figure 3-3). The numbers on the horizontal axis (A, C) represent collection numbers for individual plants at the locations indicated in Figure 3-3. The graphs show the correlation coefficient R and P-value for panels B and D. (Lima *et al*., 2022)

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CHAPTER 4: CHARACTERIZATION OF POTENTIAL SELENATE SPECIFIC TRANSPORTER SpSULTR1;2 FROM *STANLEYA PINNATA* VIA HETEROLOGOUS EXPRESSION IN *ARABIDOPSIS THALIANA*

4.1 INTRODUCTION

Selenium (Se) is necessary for the healthy and balanced metabolism of different prokaryotes, algae, and animals, including humans (Rayman, 2012; Schiavon and Pilon-Smits 2017). This element is found in 25 different human proteins, called selenoproteins, which are responsible for the proper cellular redox state, antioxidant metabolism, hormone synthesis, heart health, and reproduction (Labunkyy *et al*., 2014; Kieliszek, 2019; Qazi *et al*., 2019; Rocca *et al*., 2019; Rayman, 2020; Schomburg, 2020; Xia *et al*., 2021). The amino acid selenocysteine (SeCys) can be incorporated into the antioxidant metabolite glutathione (GSH), and in antioxidant enzymes such as glutathione peroxidase (GSH-Px). While essential at trace levels, recommended 75 µg Se/day for humans, a sustained Se intake higher than 400 µg Se/day can be harmful, and anything above 1,200 µg Se/day can be deadly (IOM, 2000; ATSDR, 2003), mainly due to oxidative stress caused by non-specific substitution of cysteine (Cys) by SeCys in proteins (Van Hoewyk *et al*., 2008; Zhang and Gladyshev, 2009).

Due to the window between Se adequacy and toxicity being quite narrow, between 75 and 400 µg Se/day, both Se deficiency and toxicity are worldwide problems. Despite the potentially devastating effect of Se toxicity, Se deficiency is more threatening in reality, affecting about one billion people around the world, including regions in Asia, South America, Africa, and Europe (Lyons *et al.,* 2003; Lyons *et al*., 2009; Dos Reis *et al.,* 2017). This nutritional deficiency is linked to different health problems in those populations, including the Keshan disease in China (Collipp

and Chen, 1981; Li *et al.,* 1984), hormonal imbalance, heart disease (Lima *et al*., 2021). Different strategies can be used to augment Se levels in deficient populations, including biofortification of food crops (Malagoli *et al.,* 2015; Wu *et al.,* 2015).

While plants do not require Se and lack the system that specifically incorporates Se-amino acids into selenoproteins, they can nonspecifically take up and metabolize Se via sulfur (S) transporters and enzymes. At low levels, these selenocompounds benefit the plant, owing to upregulated defense and antioxidant mechanisms (Pilon-Smits *et al*., 2009). Plant species differ considerably in the extent of their ability to accumulate and tolerate Se and can be organized in three general groups: Se non-accumulators, secondary accumulators, and hyperaccumulators. Most plant species do not accumulate more than 100 μg Se g^{-1} dry weight and are considered non-accumulators; most crops fall in this group. The secondary accumulator plant species are able to accumulate up to 1000 μg Se g^{-1} dry weight, owing to a propensity to accumulate S; several crops are included in this group such as canola (*Brassica napus*) and brown mustard *(Brassica juncea*). The non-accumulators and secondary accumulators are physiologically similar, differing only on their degree of S/Se uptake (El Mehdawi *et al*., 2012). The hyperaccumulators are plant species that can accumulate and withstand extremely high Se levels, above 1,000 μg Se g^{-1} dry weight in all organs, up to 1.5% of their dry weight, and accumulate such levels when growing on naturally high-Se areas (seleniferous soil).

The Se hyperaccumulation trait is relatively rare in the plant kingdom, it has been found in 45 taxa, in 14 genera from 6 dicot plant families (White, 2016), including species from the Fabaceae family, e.g., *Astragalus bisulcatus*, and Brassicaceae, e.g. *Stanleya pinnata* (prince's plume). These hyperaccumulators have mechanisms to prevent the misincorporation of Se into proteins, including the methylation of SeCys to MeSeCys (methylselenocysteine) by the enzyme SeCys

methyltransferase (MST) (Pilon-Smits *et al.,* 2009), and further conversion of MeSeCys into the volatile dimethyldiselenide (DMDSe), thus eliminating excess Se (Pilon-Smits and LeDuc, 2009). Interestingly, the hyperaccumulators show a higher Se/S ratio than other plants, indicating a possible mechanism for the specific uptake of Se over S (Harris *et al*., 2014; El Mehdawi *et al*., 2018). The underlying molecular mechanism remains to be elucidated and may involve a selenate-specific transporter. If such a transporter can be identified, this will not only provide better insight into the key genes responsible for Se hyperaccumulation but could also be helpful when developing and engineering crops that can uptake higher Se levels, also under high-S conditions.

The efficient biofortification of the edible organs of crop species with suitable levels of Se requires a deeper understanding of the Se uptake mechanisms by plants, as well as the Se/S interactions at the root level (Malagoli *et al.,* 2015; Wu *et al.,* 2015; Schiavon and Pilon-Smits 2017). The uptake of Se by plants is mainly controlled by two different factors, its concentration, and phytoavailability in soils. As mentioned, plants generally take up Se inadvertently due to its chemical similarity to S (White *et al*., 2004; White *et al*., 2007; White, 2016). The main chemical forms of Se that can be taken up are inorganic, as selenite (IV), mainly as HSe_3O^{3-} and SeO_3^{2-}, and selenate (VI), as SeO_4^{2-}, which are soluble and can be found at well-drained soil at pH levels between 4 and 9. Selenate is most common; it is normally found in soils with a prominent alkaline and oxidized profile and is taken up by the root system via sulfate transporters, H^+ and S symporters, which will be mentioned henceforward as SULTR (Gigolashvili and Kopriva, 2014; White, 2016; White, 2018), while selenite, which is normally found at soils with high levels of moist and anaerobic conditions, is taken up by phosphate and silicon transporters (Hopper and Parker, 1999; Zhao *et al.,* 2010; Zhang *et al.,* 2014).

The SULTR proteins are members of a family of membrane transporters that are characterized by their specific twelve transmembrane domain structures (Takahashi *et al*., 2011). There are approximately twelve to sixteen reported genes encoding sulfate (SO_4^{2-}) transporters in each plant species (Buchner *et al*., 2004). These proteins can be classified according to their amino acid sequence similarities into four SULTR subfamilies (Buchner *et al*., 2004). Subfamily 1 of SULTR transporters are expressed in the root hairs, cortex, and epidermis (Buchner, 2004, Tripp and Pilon-Smits, 2021). The SULTR 1;1 and SULTR1;2 are high affinity H^+ co-transporters; three protons are taken up for every sulfate/selenate molecule. SULTR1;2 is constitutively expressed and represents the main portal for sulfate/selenate uptake, while SULTR1;1 function as a backup and is upregulated under S starvation (Schiavon and Pilon-Smits, 2017). The SULTR2 group transporters can be found in the xylem and phloem vessels; specifically, SULTR2;1 is expressed in the xylem parenchyma and pericycle, while SULTR2;2 in the phloem and bundle sheath cells (Takahashi *et al*., 2000). The third subfamily, SULTR3;1 to SULTR3;5, are all chloroplast transporters (Cao *et al*., 2013, Tripp and Pilon-Smits, 2021); among these, SULTR3;5 is located at the xylem cortex and can contribute to load of selenate/sulfate to that vessel (Tripp and Pilon-Smits, 2021). Finally, the fourth subfamily of sulfate transporters, consisting of SULTR4;1 and SULTR4;2 are vacuolar efflux transporters found in the tonoplast membrane that can contribute to remobilization out of the cell (Gigolashvili and Kopriva, 2014; Tripp and Pilon-Smits, 2021).

Sulfate transporters were first characterized in the Se-resistant mutants of *Arabidopsis thaliana* carrying mutations in the SULTR1;2 coding sequence, or a T-DNA insertion in the SULTR1;2 promoter (Shibagaki *et al.,* 2002; El kassis *et al.,* 2007). Shibagaki *et al.* (2002) first indicated a role for the SULTR transporters in selenate transmembrane movement and translocation in *A. thaliana*. Further studies showed SULTR1;2 as the main portal for selenate uptake into the plant

roots, where *A. thaliana* SULTR1;2 K.O. mutants were tolerant to selenate when compared to the wild-type plants as well as the SULTR1;1 K.O. mutants (Barberon *et al*., 2008).

Studies on the *S. pinnata* hyperaccumulator species (El Mehdawi *et al*. 2018; Wang et al., 2018;) have demonstrated an elevated expression of different SULTR transporters, which could explain their higher Se levels; however, it does not explain the higher Se/S ratio in their tissues. Recently, El Mehdawi *et al*. (2018) demonstrated that hyperaccumulator *S. pinnata*'s selenate uptake is less inhibited by sulfate, as compared to non-hyperaccumulator relatives *Stanleya elata* and *Brassica juncea*. Selenate uptake in *S. pinnata* was not significantly affected by up to 100-fold higher sulfate concentration in the same medium, indicating a selenate-sulfate discrimination capability in the roots and, possibly, the presence of a selenate-specific transporter. Interestingly, these results were positively correlated with constitutively elevated expression of two sulfate transporters, SULTR1;2 and SULTR2;1 in roots of *S. pinnata*, when compared to non-hyperaccumulator *S. elata*, indicating these transporters might play an important role in the higher and Se-specific uptake. Further studies conducted by our group indicated that *S. pinnata* plants have higher transcript levels of different SULTR1;2, SULTR2;1, and SULTR3;5 transporters in the roots, both in the presence and absence of Se, when compared to the non-hyperaccumulator *S. elata* (Wang *et al*., 2018).

In this study, to investigate the possible selenate specificity of the *S. pinnata* SULTR1;2 transporter, cDNA from the corresponding gene, as well as from non-hyperaccumulator *S. elata* were amplified, sequenced, and compared. Furthermore, the cDNAs were cloned into a plant binary vector and transformed into *A. thaliana* Col-0 wild type and *A. thaliana* SULTR1;2 K.O. plants, using *Agrobacterium tumefaciens*. The transgenic plant lines were bred to homozygosity and compared with untransformed control plants for Se uptake characterization via accumulation

and tolerance experiments. The study also characterized the chemical form and location of Se in different plant tissues using x-ray microprobe analysis. It was hypothesized that plants transformed with the *S. pinnata* SULTR1;2 gene would be characterized by higher Se:S ratios when compared to plants transformed with the non-hyperaccumulator homologue and untransformed control plants. Furthermore, the *S. pinnata* SULTR1;2 transgenic plants were expected to accumulate more Se from selenate, even in the presence of high sulfate levels. Since *A. thaliana* is a Se-sensitive non-accumulator, the transgenic plants overexpressing the SULTR1;2 would be expected to be less tolerant to high Se treatments compared to the untransformed controls.

4.2 RESULTS AND DISCUSSION

SULTR1;2 AMINO ACID SEQUENCES ALIGNMENT AND PREDICTED PROTEIN STRUCTURE.

As a first approach to obtain a better insight into the possible selenate specificity of the hyperaccumulator *Stanleya pinnata's* SULTR1;2 transporter (SpSULTR1;2), the coding sequence of *S. pinnata* was cloned and sequenced, as well as that of related non-accumulator *S. elata* (SeSULTR1;2), from previously amplified root tissue cDNA (El Mehdawi *et al*., 2018). The aligned amino acid sequences of the hyperaccumulator *S. pinnata*, the non-hyperaccumulator *S. elata* (SeSULTR1;2), and the published reference sequence of *Arabidopsis thaliana* (AtSULTR1;2) transporters (Figure 4-1) exhibit a significant degree of amino acid sequence similarity, around 98% of similarity across all proteins. However, interesting differences between the three predicted proteins were found (Figure 4-1) that could be important for the proper protein folding, activity, and substrate specificity of the SpSULTR1;2.

The SULTR1;2 protein possesses twelve transmembrane domains corresponding to the catalytic region of the transporter, connected by a linking region to a cytosolic C-terminus

regulatory domain called STAS (sulfate transport anti-sigma factor antagonist) (Shibagaki and Grossman, 2006, 2010). There are a total of eleven amino acid sequence differences between the SpSULTR1;2 and the SeSULTR1;2 proteins (marked in red in Figure 4-1). Most interestingly, seven unique amino acids can be found in the hyperaccumulator *S. pinnata's* protein compared to the non-accumulator SeSULTR1;2 and the AtSULTR1;2. Furthermore, four residues are found uniquely in the non-hyperaccumulator species *S. elata* when compared to the hyperaccumulator *S. pinnata* and the control *A. thaliana*. Two amino acid residues are different among all three species (represented in red, yellow, and blue highlights in Figure 4-1).

A deeper analysis of the aligned amino acid sequences can narrow down to relevant amino acid changes conferring potential selenate specificity to the transporter in *S. pinnata*, or into its altered expression level. The consequences of amino acid changes to the activity and folding of a protein depend largely on the position of that residue in the protein, and its chemical properties dictated by their specific side chain (-R), their polarity, and hydrophobicity, for example (Betts and Russel, 2003). Natural mutagenesis happens randomly across all life domains, and some of the resulting amino acid changes could potentially have drastic effects on protein activity. The SULTR1;2 of hyperaccumulator *S. pinnata* possesses one unique glutamine (Q) residue in the third transmembrane spanning domain, different from the arginine (R) found in the two non-hyperaccumulator SULTR proteins. Three unique residues are also found in the C-terminal STAS domain of the hyperaccumulator protein sequence: first a glutamine (Q), which is different from the leucine (L) in the SeSULTR1;2, and the histidine (H) in the AtSULTR1;2 proteins; second, a histidine (H) replacing proline (P), and a leucine (L) replacing isoleucine (I), respectively. Glutamine is a polar and hydrophilic amino acid, and it is often involved in proteins' active or binding sites (Betts and Russel, 2003); its replacement by a hydrophobic non-polar amino acid like

leucine, or by a positively charged amino acid like arginine could potentially change the enzyme's conformation, activity, or regulation. Potential conformational differences are visualized on the predicted model of the SpSULTR1;2 when compared to SeSULTR1;2 (Figure 4-2).

Amino acid replacements can also be particularly relevant when located in a regulatory region of the enzyme, not directly affecting the catalytic function of the protein, but its regulation. Two out of the three amino acid differences in the regulatory cytosolic C-terminal STAS domain of the hyperaccumulator protein sequence, which regulates its activity, stability, and localization within the membrane (Shibagaki and Grossman, 2010), correspond to a non-polar to polar amino acid replacement. Interestingly, the amino acids leucine and proline, both non-polar and hydrophobic, were found in the non-hyperaccumulator *S. elata's* protein, where in the hyperaccumulator protein glutamine and histidine were found, which are both polar and hydrophilic. These changes may have regulatory consequences.

The regulatory C-terminal STAS domain is believed to control the activity of the SULTR1;2 in Arabidopsis by interacting with the cytosolic enzyme complex cysteine synthase, also named O-acetylserine (thiol) lyase (OASTL), which catalyzes the formation of Cys or SeCys from O-acetylserine (OAS) and sulfide/selenide, respectively, in plastids. The mode of action of this STAS domain is still unresolved; however, it is known that deletion of this region of the protein results in reduced or completely suppressed transport activity (Wang *et al*., 2021). The proposed mode of regulation of the SULTR1;2 transporter, first described by Shibagaki and Grossman (2010), consists of the activation or repression of the transporter's activity based on its interaction with the OASTL enzyme. At high OAS and relatively low selenide cytosolic levels, the enzyme OASTL binds to the cytosolic C-terminal STAS domain of the SULTR1;2 transporter, inactivating its activity and blocking the influx of more sulfate to the cell. In contrast, the OASTL-STAS

complex favors the activity of the cysteine synthase enzyme, lowering the OAS levels as a consequence of the Cys synthesis. When the Sulfide and OAS levels are balanced, the OASTL-STAS complex dissociates, and the SULTR1;2 transporter becomes active, increasing the influx of selenate. The previously mentioned replacement of the hydrophobic amino acids in the SeSULTR1;2 STAS domain protein, by the polar and hydrophilic amino acids SpSULTR1;2 STAS domain, could potentially increase or decrease its interaction with the enzyme OASTL in the cytosol, altering SULTR1;2 transport activity. However, definitive conclusions cannot be drawn at this time and more experiments are required, also to analyze the effects of these mutations on the transporter's specificity to selenate over sulfate in the hyperaccumulator *S. pinnata*.

SELENIUM LOCALIZATION AND SPECIATION IN FLOWERS, SILIQUES AND LEAVES USING X-RAY MICROPROBE ANALYSIS.

To further characterize the selenium speciation and localization in the transgenic lines, synchrotron micro X-Ray Fluorescence (μXRF) was used. Seedlings of the transgenic lines were treated for 20 days with 20 μM of $NaSeO_4$ (sodium selenate). The Se localization in different samples of flower (Figure 4-5), silique (Figure 4-6) and leaves (Figure 4-7) of *Arabidopsis thaliana* SULTR1;2 K.O. transgenic plants transformed with the SpSULTR1;2, SeSULTR1;2, and the non-recombinant control *Arabidopsis thaliana* Col-0 wildtype (W.T.) are presented. Selenium was evenly distributed in all analyzed flower tissues, for both the transgenics and the control plants (Figure 4-5), and no specific pattern of distribution can be identified. The siliques of both transgenics show high-intensity Se signals, and the accumulation is evenly distributed across the fruit (Figures 4-6C-F). The non-recombinant *A. thaliana* Col-0 W.T. silique (Figures 4-6A and 4-6B) shows a less intense signal, indicating this plant had relatively less Se in comparison to the transgenic individuals tested. Furthermore, the Se is more concentrated in the seeds and in the

septum of the control *A. thaliana* fruit (Figures 4-6A and 6B), when compared to both transgenics (Figures 4-6C-F). The Se distribution in the leaves follows the same pattern for all tissue samples analyzed, where the element can be mostly seen in the veins and midrib in the leaf blade (Figure 4-7).

The hyperaccumulator *S. pinnata* was shown earlier to accumulate its highest Se levels in the reproductive organs, including the flowers, seeds, and fruits (Quinn *et al*., 2011). This possibly serves as a means of protection against herbivory. Evidence that Se in plant tissues can deter different generalist herbivores was previously found in laboratory studies and field surveys (Hanson *et al*., 2003; Freeman *et al*., 2009; Quinn *et al*., 2010; El Mehdawi and Pilon-Smits, 2012). The protection of Se against herbivory was found for all plant species tested, whether they accumulate inorganic Se or, like hyperaccumulator species *S. pinnata*, organic MeSeCys, and both at moderate and at much higher levels. The hyperaccumulator likely translocates Se from the root system to the shoot as inorganic or organic Se via xylem vessels, and from the leaves to the reproductive organs in organic forms via phloem vessels (Freeman *et al*., 2006). The hyperaccumulators are known to store Se mainly in the form of methyl-SeCys near the periphery of the leaf blade, including the edges and tip of the leaf, while non-hyperaccumulators tend to store Se as selenate in the vascular tissues and mesophyll (Freeman *et al*., 2006).

Thus, based on these -limited- studies, no apparent differences in the pattern of redistribution were evident between the different SULTR1;2 transgenics and the control *A. thaliana* plants. More studies would be needed to further investigate whether the expression of the SULTR1;2 affects Se distribution in different parts of mature plants. In any case, it is likely that the hyperaccumulator's unique distribution pattern involves an intricate system composed of multiple transporters, enzymes, and signaling proteins (Freeman *et al*., 2006; Quinn *et al*., 2011).

SULTR1;2 may only play a minor role in bringing about this phenotype. Therefore, it is not surprising to find no differences in the distribution in any of the tissues, even in the leaf of transgenics transformed with the SpSULTR1;2.

The Micro X-ray absorption near-edge structure (μXANES) spectra collected on reproductive organs (flowers and siliques) overall showed a substantial degree of organic Se accumulation for the control plant tissues as well as the transgenic tissues. The organic Se was modeled as C-Se-C compounds, which can be either SeCys, SeMet, MeSeCys, and/or Se-lanthionine, all indistinguishable by μXANES. Among the three plant types, flowers of SpSULTR1;2 transgenics showed the lowest fraction of organic Se (48%), flowers of SeSULTR1;2 transgenics showed the highest fraction (82%) of organic Se, and the control *A. thaliana* plants were intermediate (73% C-Se-C, Figure 4-8, top row). The remainder of the Se in flowers of the control *A. thaliana* and of SpSULTR1;2 transgenics were inorganic selenate (Se(VI), 27% and 52%, respectively), while SeSULTR1;2 flowers accumulated some inorganic selenite (Se(IV), 13%) and elemental Se (Se(0), 5%). Incidentally, the modeling accuracy is +/-10%, therefore percentages below the 10% threshold are unreliable. In the siliques, the fraction of organic Se was also greater than the fraction of inorganic forms for the control *A. thaliana* and for the SpSULTR1;2 transgenics (52% and 59% of total Se, respectively). The SeSultr1;2 transgenics, however, showed only 13% organic Se (Figure 4-8, middle row). The remainder of the Se in siliques from all plants consisted of inorganic Se(VI), and Se(IV), at different fractions. In transgenic SeSULTR1;2 siliques, Se(VI) made up the majority (55%) of total Se, and Se(IV) made up 35%.

Differently from the reproductive tissues analyzed the leaves of all plants accumulated mostly inorganic Se (Figure 4-8, bottom row). Nevertheless, substantial fractions of organic C-

Se-C were also found in leaves from the control *A. thaliana* and the SpSULTR1;2 transgenic (43% and 28%, respectively). The SeSultr1;2 transgenic leaf showed barely 10% organic Se and approximately equal fractions of Se(VI) and Se(IV).

As mentioned, in earlier studies, *A. thaliana* was found to accumulate almost exclusively selenate in its leaves when supplied with selenate (Van Hoewyk *et al*., 2005), as did the related non-hyperaccumulator *B. juncea*. However, in reproductive organs, *B. juncea* was found to accumulate predominantly organic C-Se-C compounds, in addition to selenate (Quinn *et al*., 2011). Compared to these earlier studies, the fractions of organic Se in the leaves of control *A. thaliana* and SpSULTR1;2 transgenics were unexpectedly high, while the results from the SeSULTR1;2 transgenics were as expected. It appears that non-accumulator Brassicaceae are able to convert selenate to organic C-Se-C compounds, similar to Se hyperaccumulators, but at a lower rate. The fraction of organic Se in organs may depend on the rate of influx (regime of Se supply and uptake rate) and the rate of Se assimilation, which may be controlled by one or more enzymes from the sulfate assimilation pathway and by partitioning. The higher fraction of organic Se in reproductive organs may point to a more active sulfate assimilation or to redistribution of organic Se via the phloem. The biggest differences in speciation were found between SeSULTR and the other two (control *A. thaliana* and SpSULTR1;2), and not between the two SULTR transgenics and the control. Therefore, overexpression of a SULTR transporter did not consistently alter Se speciation. Despite a potentially higher selenate influx rate, the genetic background (including other transporters and sulfate assimilation rate) of the transgenics was the same as the control plants, and this appears to be more influential for chemical speciation of Se.

SELENIUM UPTAKE CHARACTERIZATION VIA ACCUMULATION AND TOLERANCE EXPERIMENTS.

To further characterize the possible Se specificity of the SpSULTR1;2, different accumulation and tolerance experiments were carried out at the seedling level, to compare selenate accumulation and tolerance of the SpSULTR1;2 and SeSULTR1;2 transgenics with different control plants, their untransformed counterpart *A. thaliana* SULTR1;2 K.O., and the Col-0 W.T. *A. thaliana*.

Two different experimental setups were used to analyze and compare the uptake of selenate by the seedlings and its possible inhibition by sulfate. First, a 15-day experiment was carried out using vertical plates (Figures 4-9, 4-10) to measure Se accumulation in the shoot as well as the root length and dry weight with or without 25 μM of $NaSeO_4$ (sodium selenate), as a measure of Se tolerance. The root length of the control *A. thaliana* SULTR1;2 K.O. did not show any statistical difference when treated with or without selenate (Figures 4-9, 4-10). Similar results were obtained for the transgenic SeSULTR1;2 (*A. thaliana* SULTR1;2 K.O. expressing the non-hyperaccumulator transporter, SeSULTR1;2) (Figures 4-9, 4-10). The six different SpSULTR1;2 transgenic lines showed more variation in their root length in comparison to the *S. elata* transgenic lines and the *A. thaliana* SULTR1;2 K.O. controls, however no statistical difference in root length was displayed between the treatments with and without Se (Figure 4-10A) and the tolerance index (percentage root length with Se /without Se) was not significantly different (Figure 4-10C). On average, the *A. thaliana* seedlings transformed with the SpSULTR1;2 did show a somewhat more pronounced decrease in root length when treated with Se, in comparison to the untransformed controls: an average decrease of almost 22 % in root length rather than 10% in the K.O. control plants. Higher selenate sensitivity would be in agreement with the hypothesis that the SULTR1;2 genes display selenate transport capacity.

Despite the small differences in root length between the control and the SpSULTR1;2, and SeSULTR1;2 transgenics, 25 μM of $NaSeO_4$ does not seem to be high enough to disrupt root growth and overall seedling development by S uptake inhibition and toxicity, making it hard to draw any conclusions from root length data about Se tolerance.

Seedling dry weight was measured as another parameter for Se tolerance (Figure 4-10B). Overall, no statistical differences were found between plus or minus Se treatments for any of the plant types, despite averages being consistently lower for the +Se treatments. The control *A. thaliana* SULTR1;2 K.O. seedlings showed, on average, around 1.7-fold lower dry weight when treated with Se as compared to the treatment with no Se. The transgenic *A. thaliana* seedlings transformed with the hyperaccumulator SpSULTR1;2 gene, showed, on average, 1.4-fold lower dry weight when treated with Se, while the SeSULTR1;2 transgenics showed, on average, 1.3-fold lower dry weight when treated with Se (Figure 4-10B).

There was substantial shoot Se accumulation (around 500 mg kg^{-1} DW) in the seedlings for all lines, but no significant difference in shoot Se concentration was found between the control and any of the individual transgenic lines (Figure 4-10D). There was variation among the different SpSULTR1;2 lines in this respect. The finding that Se accumulation was not affected by expression of SULTR1;2 from *S. pinnata* or *S. elata* is contrary to expectations, if these genes encode selenate transporters, especially if the endogenous *AtSultr1;2* is knocked out, as it should be in the untransformed control plants. Based on the results available, it is hard to explain these results. Perhaps the plants volatilized excess Se, reducing the Se levels in their tissues. When selenate non-specifically enters the sulfate assimilation pathway and is incorporated into the amino acid SeMet, further processing can take place to convert this amino acid to the volatile compound dimethyl selenide (DMSe), via the enzymatic mechanisms used by the plants to produce the volatile

dimethyl sulfide (DMS), thus reducing Se incorporation into proteins (Tagmount, 2002). Another important step to avoid toxicity by high Se is the breakdown of the amino acid SeCys into alanine and elemental Se, via the enzyme selenocysteine lyase (SL), avoiding the incorporation of SeCys into proteins (Van Hoewyk *et al*., 2005). These mechanisms could be used by the transgenic lines to avoid Se toxicity, thereby reducing the effect of the Se treatment on seedling growth and root development. Finally, it cannot be ruled out that the transgenic SULTR1;2 proteins are not functioning properly in the transgenics. The same tagged gene constructs were tested previously in yeast and significantly accumulation was observed in those cells, so the proteins should be functional (Guignardi, MS thesis, 2017). However, in plants a different promoter was used (CaMV35S promoter), and the protein also has to function in a different cellular environment. Efforts to detect the transporter proteins using antibodies against the Myc-His tag proved unsuccessful due to a high background signal (not shown). It is also possible that the proteins were mistargeted, or that they failed to interact properly with regulatory plant proteins.

The hyperaccumulator *S. pinnata*'s selenate uptake is known to be less inhibited by sulfate, when compared to the non-hyperaccumulator *S. elata* (El Mehdawi *et al*., 2018), at high sulfate levels in the growth medium (5 mM, 100-fold excess over selenate). These previous results were correlated with the constitutively higher expression of the SULTR1;2 transporter in those analyzed plants; other studies indicated that *S. pinnata* plants have indeed higher transcript levels of the SULTR1;2 transporter in the roots, both in the presence or absence of Se, again as compared to the non-hyperaccumulator *S. elata* (Wang *et al*., 2018). To further test the possible Se specificity of this transporter, two other accumulation experiments using horizontal plates were carried out. Three different sulfate levels were used this time, 0.9 mM (basal S level in the medium), 1.8 mM,

and 2.7 mM, in combination with a lower selenate concentration, at 5 µm selenate, to test if the Se uptake by the hyperaccumulator transporter would be inhibited by high S.

The first experiment did not show evidence of selenate accumulation inhibition by 1.8 mM sulfate for the controls *A. thaliana* Col-0 W.T. and *A. thaliana* SULTR1;2 K.O. seedlings, and surprisingly the K.O. had twice as high Se levels as the W.T. (Figure 4-11A). Interestingly, for the transgenic *A. thaliana* lines expressing SpSULTR1;2 or SeSULTR1;2 the accumulation of Se was inhibited up to 2-fold by the high sulfate treatments; for only two lines expressing the hyperaccumulator transporter was the inhibition not significant (Figure 4-11A). Incidentally, the line F(5) that showed the highest values of dry weight per seedling among all lines analyzed (Figure 4-11B), no significant difference was found between the dry weight when treated with high or low sulfate. Interesting to note is that the dry weight of the K.O. was half as large as that of the W.T., and that there was a large variation in dry weight production among SpSULTR1;2 transgenics. The finding that expression of SpSULTR1;2 leads to enhanced sensitivity of selenate accumulation by high sulfate levels is contrary to the hypothesis that SpSULTR1;2 is a selenate-specific transporter.

As an effort to further investigate selenate uptake under normal and high sulfate conditions, one final experiment was carried out using higher sulfate treatment, at 2.7 mM sulfate, while maintaining the same low level of Se, at 5 (Figure 4-12). Fewer transgenic lines were used per construct. All but one of the tested plant lines (SpSULTR1;2 line C(7)) showed significant reduction in selenate accumulation by 2.7 mM sulfate. The other SpSULTR1;2 line tested, D(6), also showed relatively less inhibition (~35%), compared to the SeSULTR1;2 lines and the control K.O and W.T. Arabidopsis (~50%, Figure 4-12). These data may point to less reduction of selenate uptake by sulfate for the SpSULTR1;2 protein, relative to the endogenous AtSULTR1;2 and the

SeSULTR1;2. However, the smaller difference in Se accumulation is due to lower Se levels under normal sulfate conditions rather than higher Se levels under high sulfate conditions (Figure 4-12A). An explanation for the lower Se levels under normal S conditions is not readily apparent.

Worth noting is that all these seedlings can still uptake Se even though the medium contains 500-fold more sulfate than selenate. Even W.T. *A. thaliana* may have a transporter (SULTR1;2 or otherwise) that could be distinguishing selenate and sulfate and preferentially taking up selenate. Alternatively, the internal root concentration of sulfate and selenate may be different due to different rates of their mobilization or assimilation, thus affecting the respective influx rates. In any case, based on the results so far, there is no supporting evidence for the hypothesis that the SpSULTR1;2 transporter from the hyperaccumulator *S. pinnata* is selenate-specific and responsible for the ability of this species to preferentially accumulate Se over S. Nevertheless, SpSULTR1;2 most likely plays an important role in the overall Se hyperaccumulation syndrome, by likely being the main portal for Se into the plant. More experiments are needed to obtain more conclusive evidence that SpSULTR1;2 does not play a role in Se specificity (other experimental conditions, other developmental stages), and to test the importance of other potential transporters or enzymes for Se specificity.

4.3 MATERIALS AND METHODS

PLASMID CONSTRUCT

Previously generated cDNA from root tissue of *S. pinnata* (Western Native Seed, Coaldale, CO), *S. elata* (El Mehdawi *et al*., 2012), and *A. thaliana* (Genbank AB042322) was used for the sequence and ligation into the PYES2 vector, using digested fragments obtained from the previously cloned pCR4-TOPO vector. The restriction digestions were performed using the enzyme EcoRI (New England BioLabs) as follows: 1 µl of the restriction enzyme, 1 µg of DNA,

5 µl (1X) of 10X NEBuffer, in a total reaction volume of 50 µl. The reaction was incubated at 37°C on a VWR Digital Multi Heat Block for 15 minutes. All ligations were performed with T4 Ligase (Thermofisher) at room temperature for 30 minutes as follows: 0.5 µl of T4 ligase enzyme, 2 µl of 10X ligase buffer, 1-3 fold excess of the insert over the vector fragment, to 20 µl volume using distilled H_20.

After each ligation, plasmids were initially transformed into DH5-α competent *E. coli* cells via $CaCl_2$/ heat shock transformation, for plasmid amplification and screening for positive transformants (Taylor *et al*., 1993). The PYES2 vector ligated with the genes of interest (10 µl), were added to 200 µL of thawed competent cells in a 1.5 ml Eppendorf microcentrifuge tube on ice for 30 minutes. Then, the cells were heat shocked at 42°C on a water bath for 45 seconds, and then placed on ice for 5 minutes. 1 ml of pre-warmed liquid LB media was added to the cells, and they were incubated at 37°C for 1 hour. The cells were then plated (200 µl) on LB agar media supplemented with 100 µg / ml ampicillin, overnight. The next morning the resultant colonies were transferred to new LB agar plate with ampicillin for PCR confirmation and *E. coli* transgenic library establishment.

The PCR (Polymerase chain reaction) reactions were carried using a total volume of 25ul as follows: 2.5µl of 10X Buffer without $MgCl_2$, 2.5 µl of free deoxynucleotides (dNTPs, 2 mM each), 1.5 µl of $MgCl_2$ (25 mM), 0.2 µl of the enzyme Taq polymerase (1 unit), 1 µl of each primer, 2 µl of the target, and 14.3 µl of distilled H_2O. All PCR reactions were performed in an Eppendorf Mastercycler gradient thermocycler with the following cycling conditions: Initial denaturation for 2 minutes at 95°C followed by 30 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 10 seconds, and extension 70°C for 40 seconds, with a final extension at 70°C for 10 minutes. To purify restriction fragments and PCR products, samples were loaded onto a 1% agarose TBE

gel and run at 90V for 40 minutes. DNA bands were visualized using a UV light illuminator, excised with a scalpel, and moved to a 1.5 ml Eppendorf microcentrifuge tube. The DNA was then purified from the gel piece using a Qiagen Gel Extraction kit (Qiagen, Hilden, Germany). Plasmids were purified using the Qiagen Plasmid Miniprep kit, and sequence verified via Sanger sequencing (http://www.genewiz.com) using the following primers:

- pYES2_F2 (5'-AACCCCGGATCGGACTACTA-3'),
- pYES2_R2 (5'-CTTTTCGGTTAGAGCGGATG-3'),
- SpinSultr1;2_5FW_EcoRI (5'-TGCAGAATTCACATTTAAGTCACCTACAAACCCA-3'),
- SpinelaSultr1;2_3Rev_EcoRI (5'-TGCAGAATTCATTTCAGACCTCGTCGGAGAG-3'),
- AtSultr1;2_5Fw_EcoRI (5'-GAGCGAATTCATGTCGTCAAGAGCTCACCC-3'),
- AtSultr1;2_3Rev_EcoRI (5'-GCGCGAATTCTCAGACCTCGTTGGAGAG-3'),
- SelaSultr1;2_5FW_EcoRI (5'-TGCAGAATTCACATTTAAGTCACCTACAAATCCA-3'),
- Spin_Sultr1;2_QuarterFw (5'-CGGTTTATATTCGAGTTTTGTTCC-3'),
- Spin_Sultr1;2_QuarterRev (5'-GGAACAAAACTCGAATATAAACC-3'),
- SpinelaSultr1;2_centerFw (5'-CCTTAACAGAAGCTGTAGCGAT-3'),
- SpinelaSultr1;2_centralRev (5'-GAAGAGCAATGTCAAGAGAACG-3'),
- SpinSultr1;2_ThreequarterFw (5'-CCTGAAGCCACTATGGTTCCAG-3'),
- SpinSultr1;2_ThreequarterRev (5'-CCCTGGAACCATAGTGGCTTC-3'),
- AtSultr1;2_centF (5'-GACCTTCCTTCTCACGTCTAAGA-3'),
- AtSultr1;2_centR (5'-CCCTTAGCAAGGTTATCACCAG-3').

After verification of the plasmid sequences and correct orientation, the genes of interest were digested from PYES2 using BstX1 enzyme and further cloned into the plant binary vector pFGC5941 under control of the CaMV35S promoter, following the methods described in the

previous paragraphs. The binary vector was used for the stable expression in *A. thaliana* via *Agrobacterium tumefaciens* (strain GV3101) transformation. The binary plant vector pFGC5941 was first prepared for the genes from PYES2 vector, by insertion of a linker containing the BstX1 restriction site, as well as two flanking sites for the enzymes AscI and Sma1, both sites used for the ligation of the linker into the pFGC5941 plasmid (Oligonucleotide: 5'-GGGCCAGTGTGCTGGCCATCACACTGGGG-3'). The plant binary vector was first digested with the AscI and Sma1 restriction enzymes, and the linker was further ligated into the binary vector using the method previously described. Before cloning the genes of interest into the binary vector, the MycHis tag (Myc AA sequence: GAACAAAAACTCATCTCAGAAGAGGATCTG/ His AA: CATCACCATCACCATCAC) was added to the constructs so as to be able to compare their expression levels using immunoblotting.

AGROBACTERIUM AND PLANT TRANSFORMATION

The pFGC5941 constructs containing the gene of interest of each SULTR1;2 gene were further transformed into the *Agrobacterium tumefaciens* strain GV3101 (containing a C58 chromosomal background with rifampicin resistance and the Ti plasmid pMP90 with gentamicin resistance). The *Agrobacterium* strain was grown overnight at 28°C in 5 ml of LB medium. The next morning 2 ml of the overnight culture was added to 50 ml LB medium in a 250 ml incubation flask and shook at 250 rpm at 28°C until the OD_{600} of 1.0. After this growth period the culture was chilled on ice for 10 minutes. The cell suspension was further centrifuged at 3000g (6000 RPM) for 5 minutes at 4°C. The supernatant was discarded, and the cells were resuspended in 1ml of 20mM $CaCl_2$ solution. Following, 0.1ml of the solution was aliquoted into prechilled Eppendorf test tubes. Around 1µg of plasmid DNA was added to the cells and mixed gently. The cells with the added plasmid were frozen quickly using a Qiagen lyser block from the -80°C freezer. After 5

minutes the cells were heat-shocked at 37°C in a water bath for 5 minutes. After, 1 ml of LB medium was added, and the cells were incubated for 4 hours at 28°C. After incubation, the cells were centrifuged at 6000 RPM using a microcentrifuge. The supernatant was discarded leaving around 0.1 ml in the tube, the cells were then resuspended using the remaining supernatant. The cell suspension was then transferred (0.1 ml) to LB plates containing rifampicin and gentamicin and incubated for 2 or 3 days at 28°C until transformed colonies were visible. The colonies were further grown in LB medium and stored in glycerol at -80°C for further plant transformation.

Arabidopsis thaliana (Col-0 Wild Type and SULTR1;2 K.O.) seeds were vernalized at 4°C in 5 ml of sterile H20. The vernalized seeds were germinated on wet soil containing PRO-MIX HP soil in 4 inch pots, 5 seeds per pot, and grown in a growth room under short-day controlled conditions, 8h of light/16h of dark, 25°C day/night, at a light intensity of 200 µmol m^{-2} s^{-1}. The pots were watered three times per week from the bottom using tap water. The watering solution at week four contained a 1:1000 dilution of Miracle-Gro Liquid All Purpose Plant Food (Scotts Company, Marysville, OH). After four weeks the plants were transferred to long-day conditions for flowering inducing, 16h of light/8h of dark, 25°C day/night, at a light intensity of 200 µmol m^{-2} s^{-1}. The plants were watered daily from the bottom using with ½ cm of tap water.

The day before the plant transformation the Agrobacterium was inoculated in a 100 ml culture using LB and the antibiotics rifampicin and gentamicin. The pots with the plants, 3 pots per construct, were labeled the construct name and date. The soil was saturated with H_2O before the dip. The OD_{600} of the cell cultures was measured, the cells were centrifuged at 4°C for 10 minutes at 8000 RPM using centrifuge bottles. After centrifugation the supernatant was discarded and the cells were resuspended in fresh dip medium, containing sterile H_20 and 5% sucrose (50 grams/liter) and 0.05% Silwet L-77 (500 µl/L), to OD_{600} of 0.8. The dip solution was transferred

to a 1L beaker, before dipping the flowers into the solution siliques were cut from the stem. The stems of each flower were dipped in the solution for 1 minute, 3 pots per construct were dipped in the same solution. After dipping the pots with the plants were transferred to a tray and kept in the dark with high moisture overnight. The plants were watered twice a week until most of the siliques turned brown, at around 3 weeks after dip. At week four no water was given, and the stems were collected in Ziploc bags. The seeds were harvested from the bags and cleaned from plant debris using cheesecloth two weeks after all siliques were dried and opened. Cleaned seeds were stored in labeled microcentrifuge tubes in the fridge, 4°C.

HOMOZYGOUS TRANSGENIC ARABIDOPSIS THALIANA LINES SELECTION

The positively transformed Arabidopsis thaliana seeds from the Agrobacterium tumefaciens floral dipping transformation, henceforward called generation 0 seeds, were selected using BASTA (gluphosinate ammonium, 25mg/L), on MS (Murashige and Skoog) medium. First, the seeds were surface sterilized as follows: in sterile laminar flow hood, 30 mg of seeds (around 900 seeds) were transferred to a sterile 15 ml falcon tube (VWR), 1 ml of 70% ETOH was added to the tube, after mixing, the seeds were let to sit for 1 minute. The seeds were then washed twice using 10 ml of distilled H20. After the first wash, 10 ml of 10% bleach with a drop of Tween-20 was added to the tube, the tube was then vortexed for 30 seconds every minute for a total 10 minutes. The bleach solution was then removed using sterile tips, the seeds were then washed five times using 10 ml of distilled H_20. After the last wash, 5 ml of distilled H_20 was added, and the tube was transferred to the fridge at 4°C for 3 days. After this period all the content of the tube was poured on the surface of a large petri dish, containing 100 ml of selective MS medium, supplemented with BASTA (25mg/L) and cefotaxime (100 Mg/L). The petri dish with the seeds

was then transferred to a growth chamber (8h of light/16h of dark, 25°C day/night, at a light intensity of 200 µmol m^{-2} s^{-1}, HR 70%).

The MS medium plates were prepared using 0.67g of MS salts (Sigma-Aldrich, St Louis, MO), 1g of Sucrose (Sigma-Aldrich) , and 1.2g of PhytoAgar (Research Products International, Mt. Prospect, IL), the solution pH was adjusted to 5.8 using 1M KOH. The solution was then autoclaved for 20 minutes, and after cooling down the selective herbicide BASTA, and the antibiotic cefotaxime was added. The solution was then poured into the petri dishes and let dry inside the laminar flow hood.

The transformed seeds that survived the selection medium were then transferred to pre-wet soil (PRO-MIX HP) in 4-inch pots, 1 seedling per pot. The seedlings were grown in a growth room under short-day controlled conditions, 8h of light/16h of dark, and 25°C day/night, at a light intensity of 200 µmol m^{-2}. s^{-1}. After 3 weeks the pots were transferred to long-day, 16h of light/8h of dark, 25°C day/night, at a light intensity of 200 µmol m^{-2} s^{-1}, and watered 3 times a week. The seeds were then collected in separated Ziploc bags and cleaned from plant debris. These seeds will be henceforward called generation 1, a total of 14 BASTA resistant survivors were obtained for the transgenic lines transformed with the SpSULTR1;2 gene and they were labeled with a letter representing each line, from A to N. For the transgenic lines transformed with SeSULTR1;5 gene, a total of 7 resistant survivors were obtained, lines A to G.

A total of 100 seeds from each generation 1 line were surfaced sterilized, following the procedures described before. Two small Petri dishes containing 50 ml of selective MS medium each, supplemented with selective medium with BASTA (25mg/L) and cefotaxime (100 Mg/L), were used per transgenic line. A total of 50 seeds were plated per petri dish using a sterilized toothpick. The Petri dishes with the seeds were then transferred to a growth chamber (8h of

light/16h of dark, 25°C day/night, at a light intensity of 200 µmol m^{-2} s^{-1}, HR 70%) for 3 weeks. After the growth period, a total of 15 seedlings per line were then transferred to pre-wet soil (PRO-MIX HP) in 4 inch pots, 1 seedling per pot. The plants were grown to seeds as described before. These seeds will be henceforward called generation 2. The 15 BASTA resistant survivors of generation 2 were obtained for the transgenic lines transformed with the SpSULTR1;2 gene were then labeled with the same letter representing the previous generation, and a number representing the current generation, 1 to 15.

Following, 100 seeds from each of these 15 generation 2 lines were surface sterilized and divided into two small petri dishes containing 50 ml of selective MS medium each, supplemented with selective medium with BASTA (25mg/L). At this time, the lines that contained 100% of survivors on both petri dishes (homozygous) were selected to be used for the tolerance and tolerance experiments.

The SULTR1;2 K.O. genotype was checked via PCR utilizing a combination of 3 different primers, according to the Salk information. Primer 1: Salk LB 1,3_tDNA (039) 5'_ATT TTG CCG ATT TCG GAC _3'; Primer 2: = LPSultr1;2 (501) 5'_TGC ATC GTC TAC TAC CTT GCC_3'; Primer 3: RPSultr1;2 (502) 5'_ CGT TGG TGA TAG GCA AGC TAC_3'.The Wild-Type plants should amplify a fragment size of 1124bp, while K.O. plants should only give a small fragment, at around 470bp to 770 bp. The transgenic A. thaliana not showing the K.O. phenotype were discarded and not used for the transformations.

LEAF DNA EXTRACTION AND AMPLIFICATION VIA PCR

Leaf DNA was extracted, amplified with PCR, and separated using gel electrophoresis, from each generation 1 line to confirm the presence of the SULTR1;2 genes. The DNA extraction was performed: fresh leaf material was ground with 600 µl of extraction buffer (EB: 100 mM Tris pH

8, 50 mM EDTA pH 8, 500 mM NaCl) in a 1.5 ml tube. After grinding, an extra 150 µl EB buffer was added and mixed. Further, 50 µl of 20% SDS was added to the solution and incubated at 65°C for 10 minutes. Next, 250 µl of K-acetate (60 ml of 5M KOAc, 11.5 ml acetic acid, 28.5 ml of H_20) was added and mixed, the solution was then incubated in ice for 20 minutes. The tube was centrifuged at top speed for 10 minutes, and the supernatant was then transferred to a new 1.5 ml tube containing 500 µl of isopropanol, mixed, and incubated at -20°C for 20 minutes. After incubation, the samples were centrifuged at top speed for 10 minutes, the supernatant was discarded, and the pellet dried at room temperature. The pellet was then resuspended in 30 µl of TE buffer (10 mM Tris pH, 1 mM EDTA pH 8, 20% SDS, 100% isopropanol, 3M NaOAc, pH 5.2). Further, 30 µl of NaOAc and 1000 µl of 200 proof EtOH were added and mixed. The solution was then transferred overnight to the -80°C freezer. The next day, the samples were thawed in ice and then centrifuged at top speed for 10 minutes. The supernatant was discarded, and 1 ml of 75% ETOH was added. The sample was centrifuged at top speed for 2 minutes. The supernatant was then discarded, and the remaining solution was removed using a 100 µl pipette. The pellet was then resuspended in 50 µl DNase-free H_20.

The extracted DNA was then quantified using Nanodrop 2000 spectrophotometer (ThermoFisher Scientific). The DNA was further amplified via PCR using the pFGC5941_35s_Fw promoter (5'_GGA GAG GAC ACG CTC GAG TAT AAG_3'), and the respective SULTR1;2 promoters, Spin_ela_SULTR1;2_central_Rev (5'_GAA GAG CAA TGT CAA GAG AAC G_3') (Figure 4-3), and Sela_SULTR1;2_quarter_Rev (5'_GGA ACA AAA CTC GAA TAT AAA CC_3'), (Figure 4-4). The PCR fragments were then separated by gel electrophoresis (100 ml of 1X TBE buffer, 1g of agarose, and 10 µl of ethidium bromide).

TOLERANCE AND ACCUMULATION EXPERIMENTS

Transgenic seeds from the previously selected homozygous lines were surface sterilized following the procedure previously described. The tolerance and the uptake experiments were carried out on polystyrene Petri dishes, 3 replicates per transgenic line, either horizontal (uptake) using square dishes, or vertical (tolerance) using round dishes. Each plate was prepared with MS agar medium in a sterile laminar flow hood. For the tolerance experiment a total of 15 sterilized seeds were transferred using a sterile toothpick to the top of each MS agar plate. The plates were incubated horizontally in a growth chamber (8h of light/16h of dark, 25°C day/night, at a light intensity of 200 µmol m^{-2} s^{-1}, HR 70%). The root length of all plates was monitored daily. The experiment was finalized when the first root reached the bottom of a plate (~15 days). The shoot of all seedlings per plate was collected, washed with cold water, and dried for 3 days. After this period, the dry weight per plate was measured, and the material was digested for elemental analyses. The uptake/accumulation experiments utilized small circular Petri dishes with 50 ml of MS agar medium, with 50 sterilized seeds each, 3 plates per treatment. The experiment was carried out for 25 days, and at the end, the shoot of all seedlings per plate was collected, washed in cold water, and dried following the procedure for the tolerance experiments.

SELENIUM LOCALIZATION AND SPECIATION VIA X-RAY MICROPROBE ANALYSIS

Seedlings of the transgenic lines (generation 0) were treated for 20 days with 20 µM of NaSeO4 (sodium selenate). After this period, different tissues were collected and frozen in liquid nitrogen for further µXRF analyzes. Selenium (and calcium) localization and speciation were analyzed in Arabidopsis thaliana's leaf, flower, and silique using X-ray microprobe imaging (Zarcinas et al., 1987). Analyses were performed at beamline 10.3.2 (X-ray Fluorescence

Microprobe) of the Advanced Light Source (ALS), at Lawrence Berkeley National Lab (Berkeley, CA, USA) using a Peltier cooling stage (−25°C). Micro-focused X-ray fluorescence (μXRF) maps were recorded at 13 keV incident energy, using 20 μm × 20 μm pixel size, a beam spot size of 7 μm × 7 μm, using 70 ms dwell time, and 50 ms dwell time. Maps were then deadtime-corrected and decontaminated. Selenium K-edge micro X-ray absorption near-edge structure (μXANES) spectroscopy (in the range 12,500–13,070 eV) was used to analyze Se speciation on eleven different spots on samples, close to areas showing high Se concentration in the μXRF maps. Spectra were energy calibrated using a red amorphous Se standard, with the main peak set at 12,660 eV. Least-square linear combination fitting of the μXANES data was performed in the range of 12,630 to 12,850 eV using a library of 52 standard selenocompounds and procedures described by Fakra (2018). All data were recorded in fluorescence mode using a 7-elements Gesolid-state detector (Canberra, ON) and processed using custom LabVIEW programs available at the beamline.

ELEMENTAL COMPOSITION

Fresh tissue samples were dried at 50°C until constant weight. The plant material was acid-digested as described (Németh *et al*., 2015); in short, 100 mg samples were digested with 1 mL of nitric acid for 2 h at 60°C and then 6 h at 125°C, then diluted to 10 ml with distilled water. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to analyze the digested seed samples' elemental composition (Se), using appropriate standards and quality controls.

STATISTICAL ANALYSIS

The software JMP-IN 13.0.0 (SAS Institute, Cary, NC, USA) was used for statistical data analysis. Student t-test was used to compare different treatments. The different letters above bars in figures 4-10, 4-11, and 4-12, indicate statistically different means among transgenic lines ($P < 0.05$).

4.4 CONCLUSIONS

This study investigated the possible selenate specificity of the hyperaccumulator *S. pinnata* SULTR1;2 transporter. The amino acid sequence and predicted 3-dimensional structure of the hyperaccumulator transporter were compared to its homologue from the non-hyperaccumulator species *S. elata*, and to the sequence of *A. thaliana*. Additionally, the chemical form and location of Se in different plant tissues were characterized using x-ray microprobe analysis. Furthermore, transgenic lines were created and bred to homozygosity for SpSULTR1;2 and SeSULTR1;2 and physiologically compared with untransformed control plants in Se accumulation and tolerance experiments.

It was hypothesized that plants transformed with the *S. pinnata* SULTR1;2 gene would be characterized by a higher Se:S ratio when compared to plants transformed with the non-hyperaccumulator homologue and to untransformed control plants. The transgenic plants transformed with the *S. pinnata* SULTR1;2 gene were expected to accumulate more Se from selenate, even in the presence of high sulfate levels, as a result of the putative Se specificity of that transporter. The transgenic plants overexpressing either of the SULTR1;2 proteins would be expected to be less tolerant to high Se treatments compared to the non-recombinant controls, due to a higher uptake and accumulation of toxic levels of Se.

My data show that the Se hyperaccumulator *S. pinnata* possesses a SULTR1;2 transporter with several unique amino acids in its protein structure compared to homologues from related non-accumulators, *S. elata* and the published sequence from the model plant *A. thaliana*. Three unique amino acid residues are present in the C-terminal STAS regulatory domain of the hyperaccumulator protein. This domain is believed to be involved in the regulation of the transporter's activity by its interaction with the enzyme complex cysteine synthase. Most notably, the hyperaccumulator protein contains two unique polar and hydrophilic amino acids, glutamine, and histidine, as compared to the non-polar hydrophobic amino acids leucine and proline, found in the non-hyperaccumulator *S. elata*. The replacement of hydrophobic to hydrophilic amino acids in the hyperaccumulator could potentially alter the hyperaccumulator protein's folding and activity, and with that the protein's activity and potentially its specificity to Se.

Transgenic *A. thaliana* plants expressing SpSULTR1;2 or SeSULTR1;2 showed similar Se tissue distribution and chemical speciation as untransformed control plants, as judged from x-ray microprobe analysis. Physiological experiments comparing transgenics and wild-type controls at the seedling level found no clear and consistent differences in selenate tolerance or accumulation. My main hypothesis, that SpSULTR1;2 is a selenate-specific transporter and thus that selenate uptake by SpSULTR1;2 transgenics would be less inhibited by sulfate was not consistently supported by the data; only when seedlings were supplied with very high sulfate levels (2.7 mM) and very low (5 μM) selenate levels, *A. thaliana* transformed with the *S. pinnata* SULTR1;2 showed less reduction in Se accumulation between the control and the high sulfate treatments than the *S. elata* SULTR1;2 controls and the W.T and K.O. Arabidopsis controls. However, this was due to their Se levels being lower under the low-S conditions. Thus, overall, no conclusive evidence for Se specificity of this hyperaccumulator transporter was found. Nevertheless, more

experiments could still be done with the transgenics under different treatment conditions and other plant developmental stages. Additionally, alternative mechanisms for Se-specific uptake and accumulation may be envisioned and explored. Previous studies indicated that other SULTR transporters in the root system are also overexpressed in the hyperaccumulator *S. pinnata*, as compared to the non-hyperaccumulator *S. elata*, for instance the SULTR2;1 and the SULTR3;5 (Wang *et al*., 2018). These two sulfate transporters mediate the selenate/sulfate loading into the xylem vessels in the root system; specific Se uptake could potentially be happening at the level of the xylem parenchyma or pericycle by means of one of these transporters. The SULTR1;2 transporter might be responsible for the unspecific Se uptake in the root cortex, root hairs, and epidermis, loading high levels of selenate and sulfate to the symplast and apoplast. The SULTR2;1 and/or SULTR3;5 transporters could potentially select selenate over sulfate before its loading to the xylem. It is also possible that the selenate specificity happens at metabolic level, during the process of activation and reduction by ATP sulfurylase or APS reductase. These hypotheses may be further investigated. alternative mechanisms for Se-specific uptake and accumulation may be envisioned and explored.

4.5 FIGURES

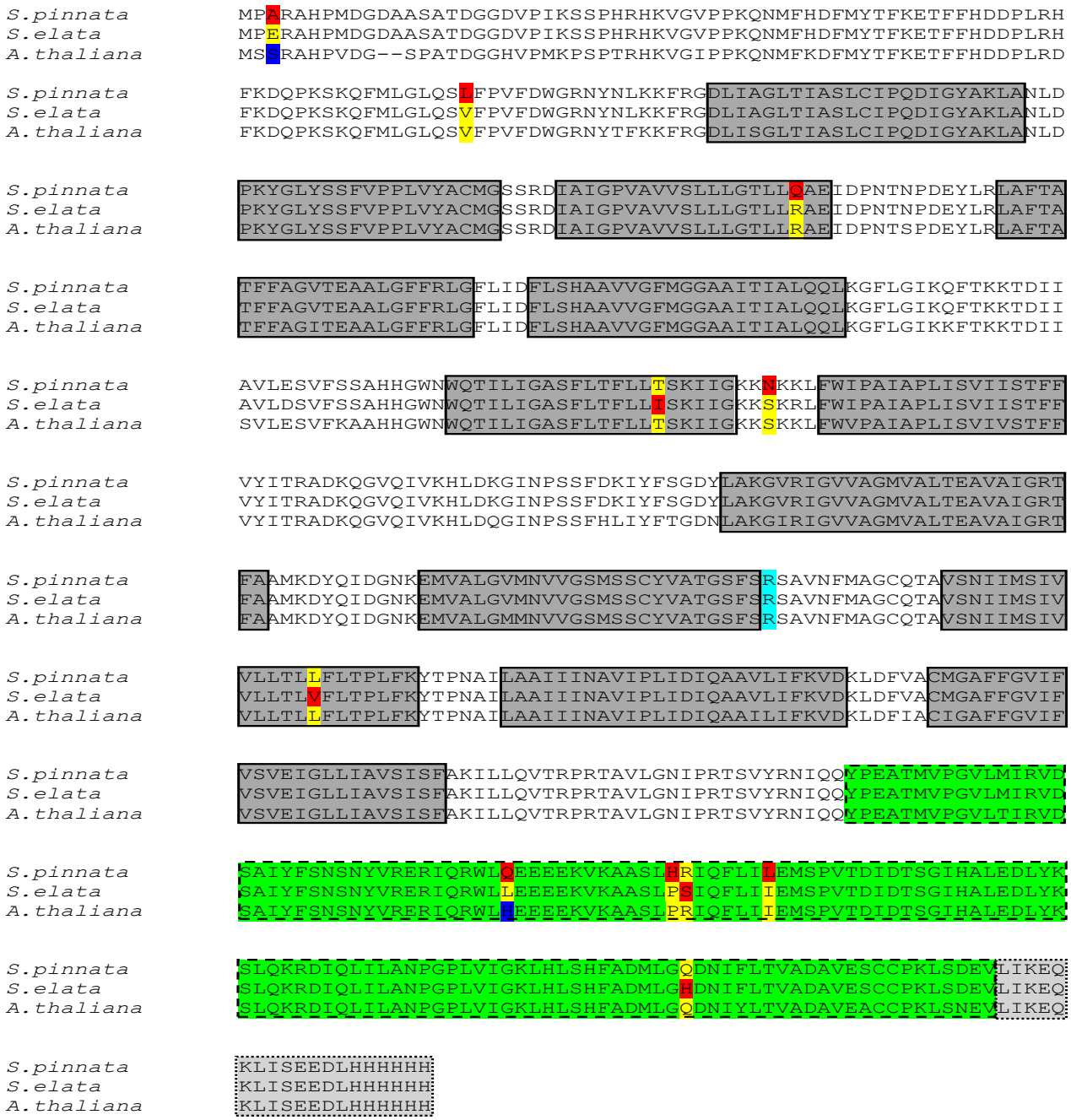

Figure 4-1. Amino acid sequence alignment (MAFFT alignment tool) of SULTR1:2 protein from *S. pinnata*, *S. elata* and *A. thaliana*. Yellow, red, and blue highlights represent amino acid differences between the *S. pinnata*, *S. elata*, and *A. thaliana* proteins. Dark gray represents the twelve membrane spanning domains (MSD). Blue "R" after MSD9 represents the conserved Arg (Arginine) reportedly involved in substrate binding. Green amino acids correspond to the C-terminal regulatory STAS domain. The light gray box represents the MycHis tag.

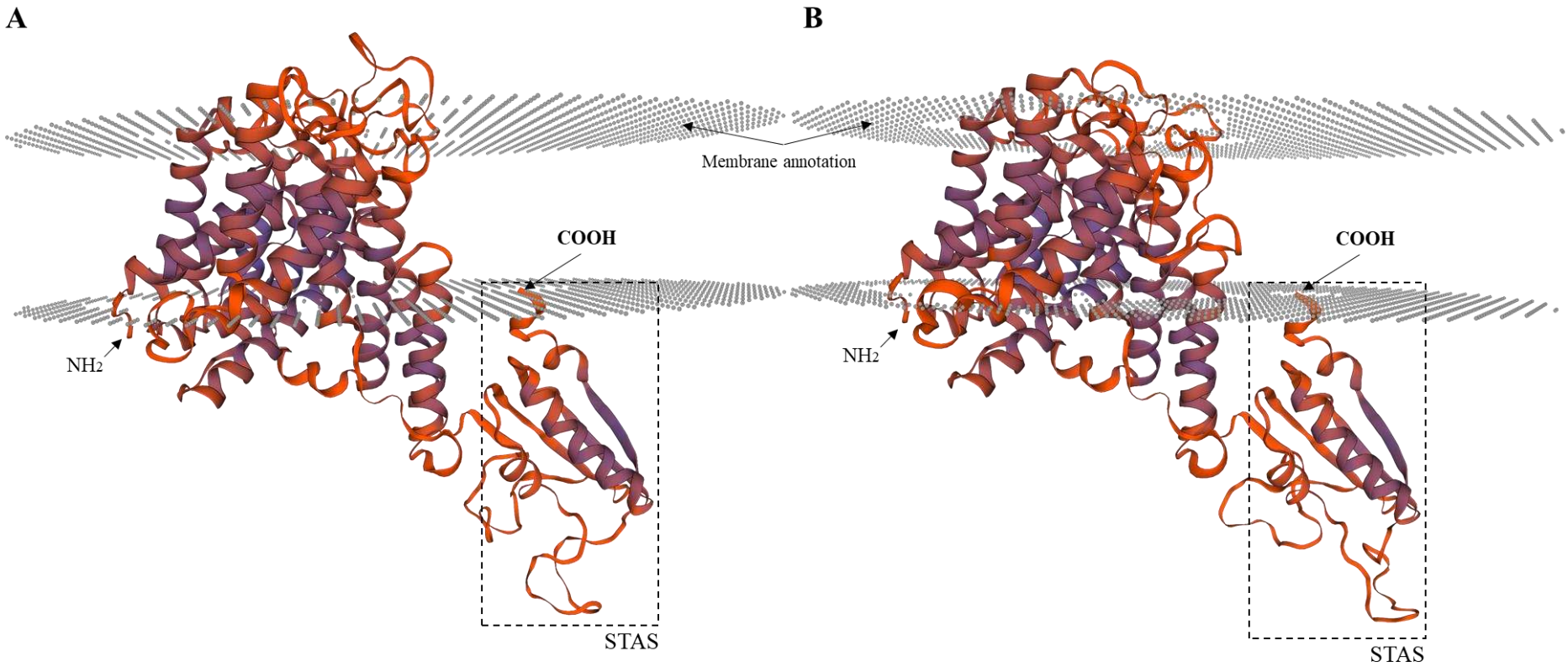

Figure 4-2. Predicted structures of SULTR1;2 from *S. pinnata* (A) and *S. elata* (B). The N-terminus (NH2), C-terminus (COOH), and the C-terminal STAS domain are indicated. the SLC26 sulfate transporter was used as a template.

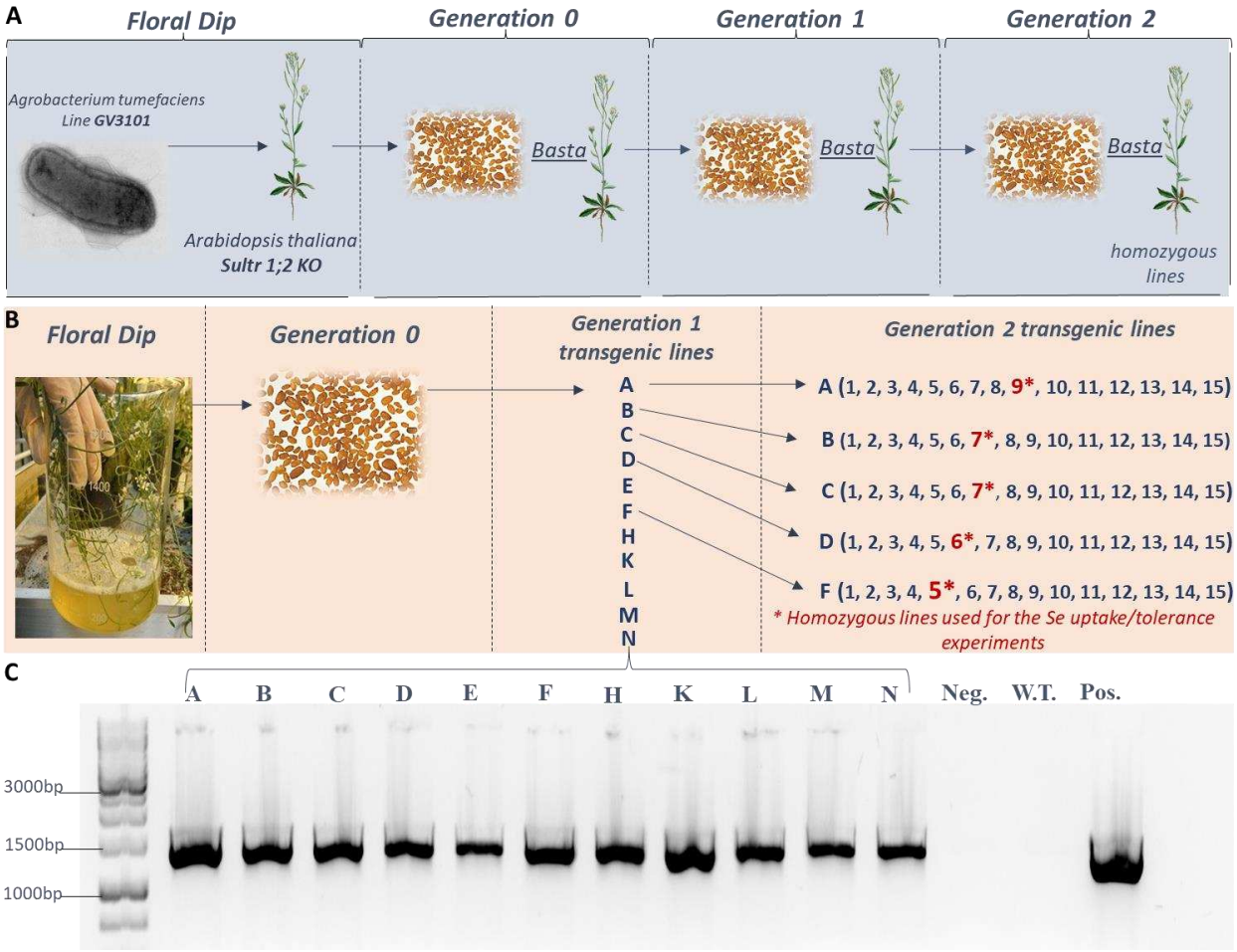

Figure 4-3. Schematic representation of the genetic transformation of *Arabidopsis thaliana* Sultr1;2 K.O. plants with the *Stanleya pinnata* SULTR1;2 gene, via floral dipping using *Agrobacterium tumefaciens.* Panel A shows the generations of plants selected via BASTA resistance until homozygosity. Panel B shows the transgenic lines for the first and second generations, the homozygous lines highlighted in red with asterisks (generation 2) were used for the Selenium tolerance and uptake experiments. A total 900 seeds (~30mg) from generation 0 mother plant were grown on BASTA. The resistant seedlings (generation 1, lines A to N), were selected and propagated to the next generations. DNA was extracted from each line, and PCR using a 35S promoter forward primer and a *S. pinnata/S. elata* SULTR1;2 specific reverse primer was used to confirm the genetic transformation (Panel C). A total of 15 seedlings per line were propagated and bred to homozygosity.

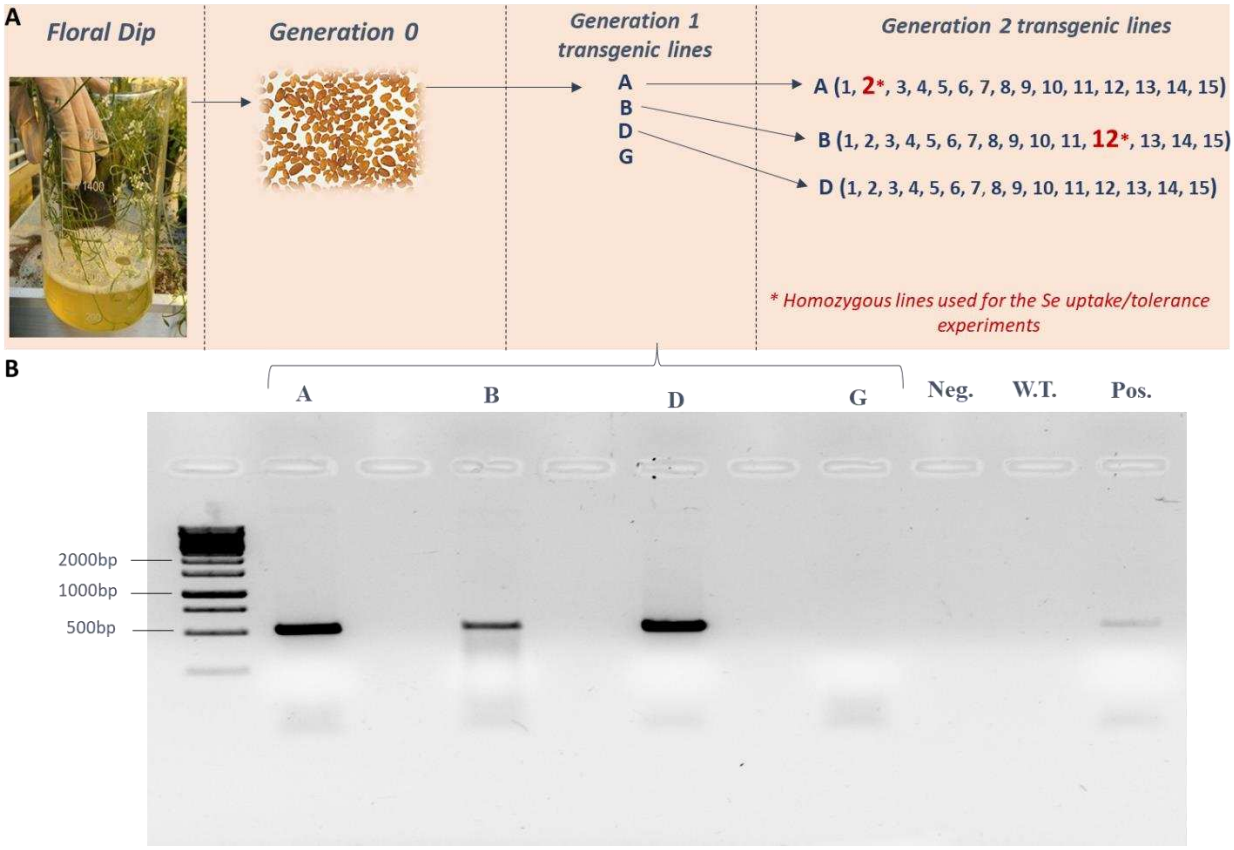

Figure 4-4. Schematic representation of the genetic transformation of *Arabidopsis thaliana* Sultr1;2 K.O. plants with the *Stanleya elata* SULTR1;2 gene, via floral dipping using *Agrobacterium tumefaciens*. Panel A shows the transgenic lines for the first and second generations, the homozygous lines highlighted in red with asterisks (generation 2) were used for the Selenium tolerance and uptake experiments. A total of 900 seeds (~30mg) from generation 0 mother plant were grown on BASTA. The resistant seedlings (generation 1, lines A, B, and D) were selected and propagated to the next generations. DNA was extracted from each line, and PCR using a 35S promoter forward primer and a *S. pinnata/S. elata* SULTR1;2 specific reverse primer was used to confirm the genetic transformation (Panel B). A total of 15 seedlings per line were propagated and tested for homozygosity.

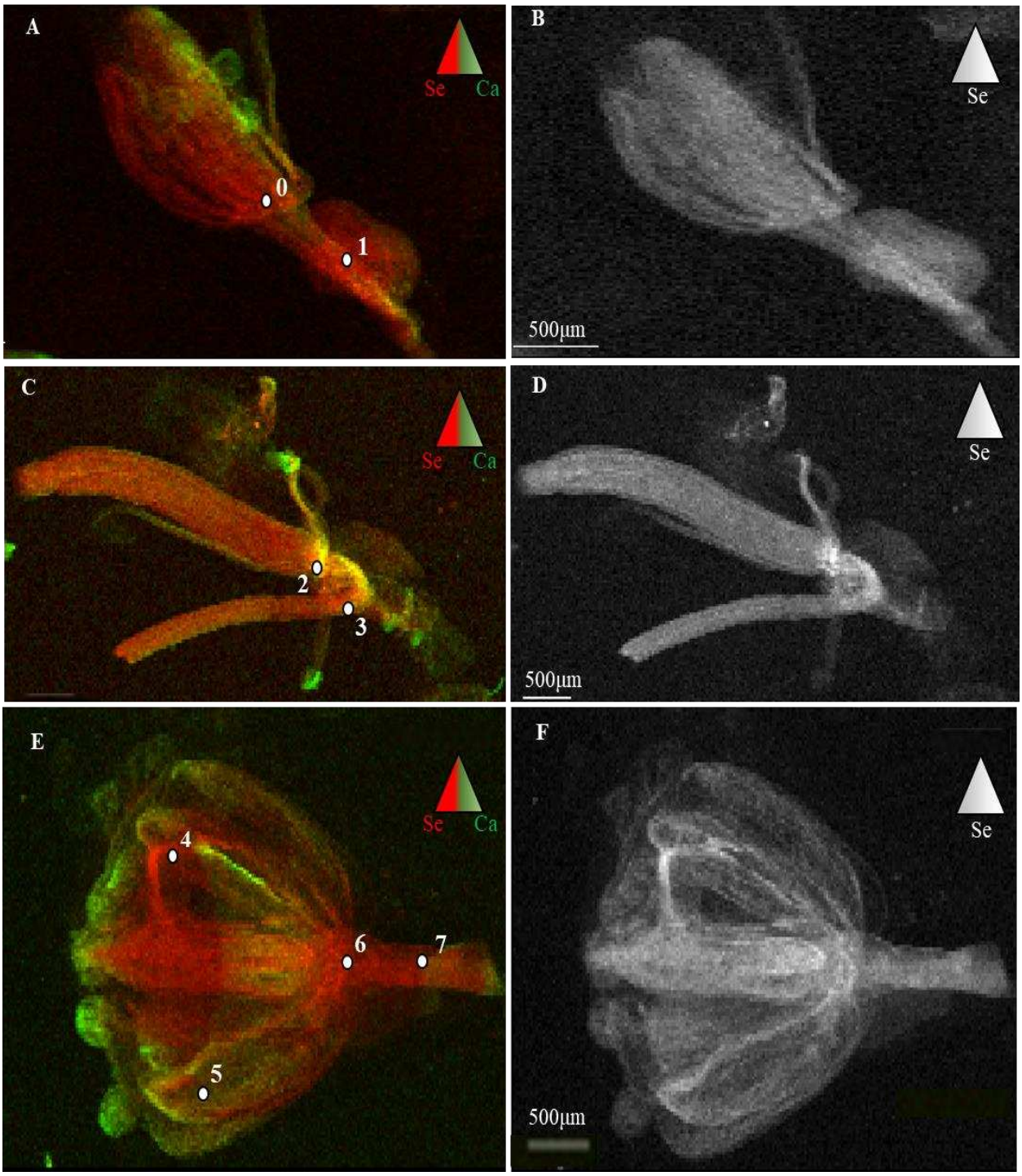

Figure 4-5. Micro X-ray fluorescence (µXRF) elemental distribution maps of *Arabidopsis thaliana* flower samples treated with 20 um Selenate for 7 days. Selenium is shown in red or white, and Calcium in green. Panel A and B shows untransformed *A. thaliana Col-W.T.* flowers; Panels C and D shows *A. thaliana* SULTR1;2 *K.O.* flowers transformed with *S. pinnata* SULTR1;2 gene; Panels E and F shows *A. thaliana* SULTR1;2 *K.O.* flowers transformed with *S. elata* SULTR1;2 gene. Micro X-ray absorption near-edge structure spot locations are shown as numbered white circles; speciation results are shown in Figure 12.

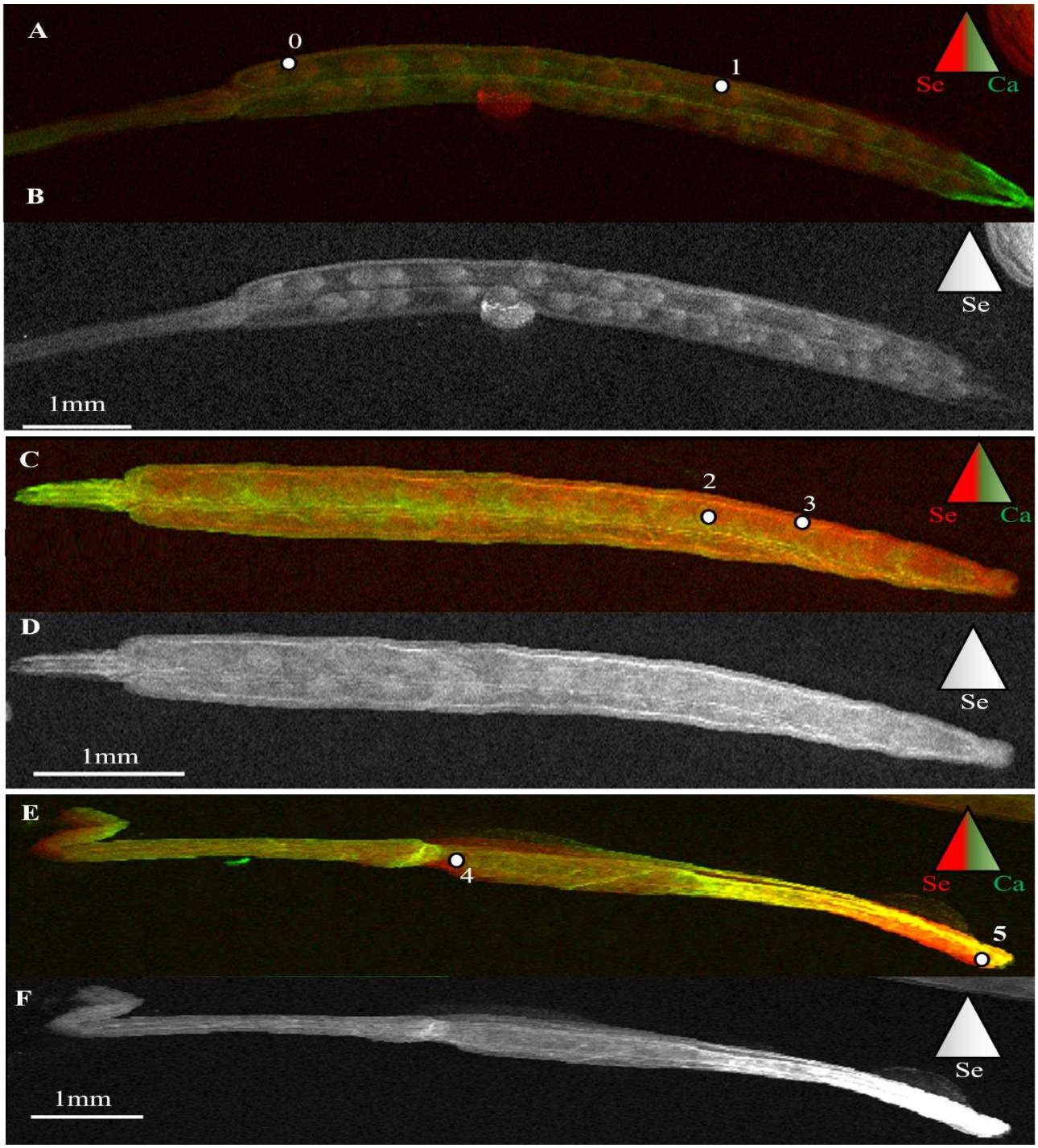

Figure 4-6. Micro X-ray fluorescence (μXRF) elemental distribution maps of *Arabidopsis thaliana* silique samples treated with 20 μM Selenate for 7 days. Selenium is shown in red or white, and Calcium in green Panel A and B shows untransformed *A. thaliana Col-W.T. silique*; Panels C and D shows *A. thaliana* SULTR1;2 *K.O.* silique transformed with *S. pinnata* SULTR1;2 gene; Panels E and F shows D shows *A. thaliana* SULTR1;2 *K.O.* silique transformed with *S. elata* SULTR1;2 gene. Micro X-ray absorption near-edge structure spot locations are shown as numbered white circles; speciation results are shown in Figure 8.

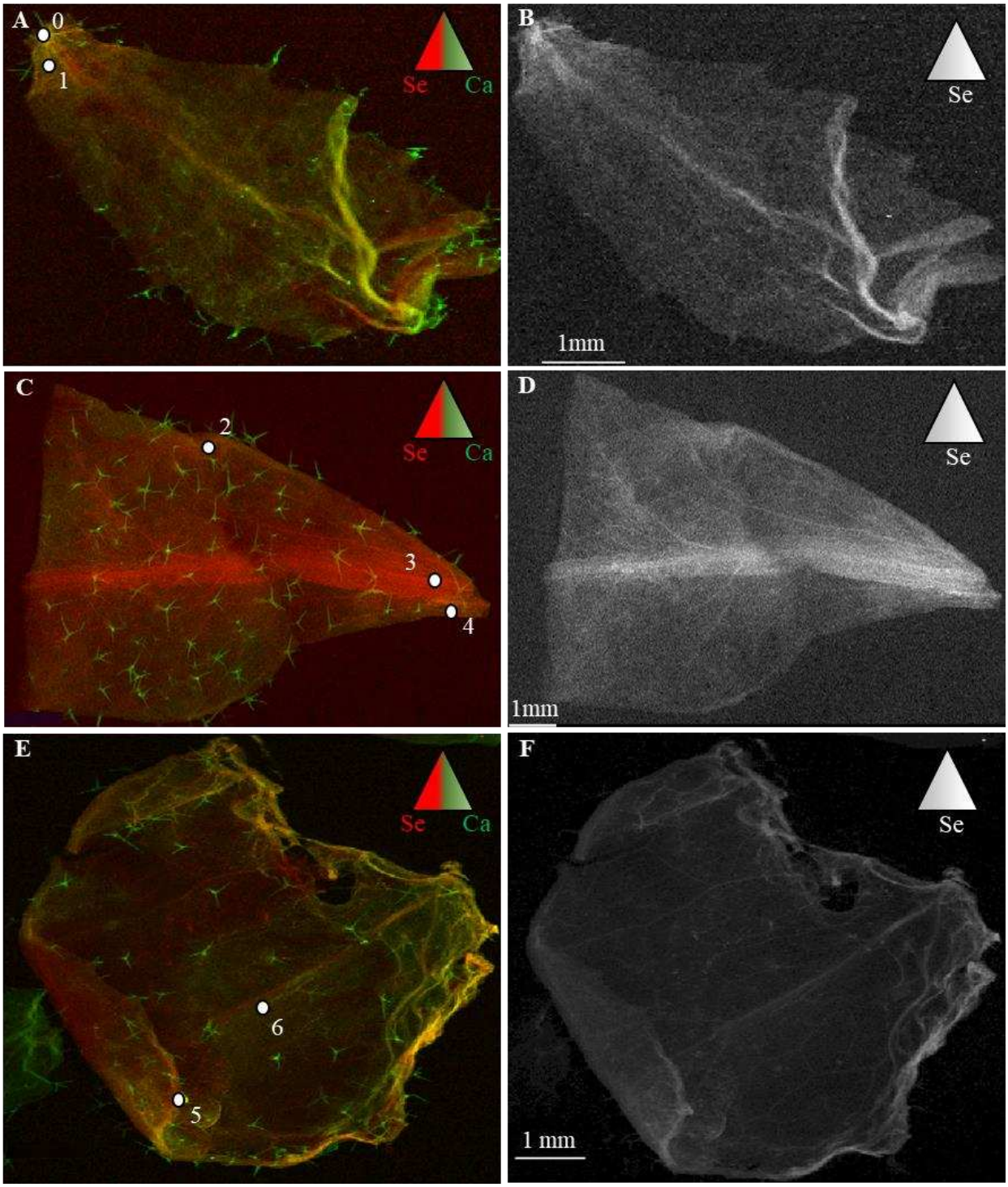

Figure 4-7. Micro X-ray fluorescence (μXRF) elemental distribution maps of *Arabidopsis thaliana* treated with 20 μm selenate for 7 days. Selenium is shown in red or white, and calcium in Panel A and B shows non-recombinant *A .thaliana Col-W.T. silique*; Panels C and D shows *A. thaliana* SULTR1;2 *K.O.* leaf transformed with *S. pinnata* SULTR1;2 gene; Panels E and F shows D shows *A .thaliana* SULTR1;2 *K.O.* leaf transformed with *S. elata* SULTR1;2 gene. Micro X-ray Absorption Near-Edge Structure (XANES) spot locations are shown as numbered white circles; speciation results are shown in Figure 8.

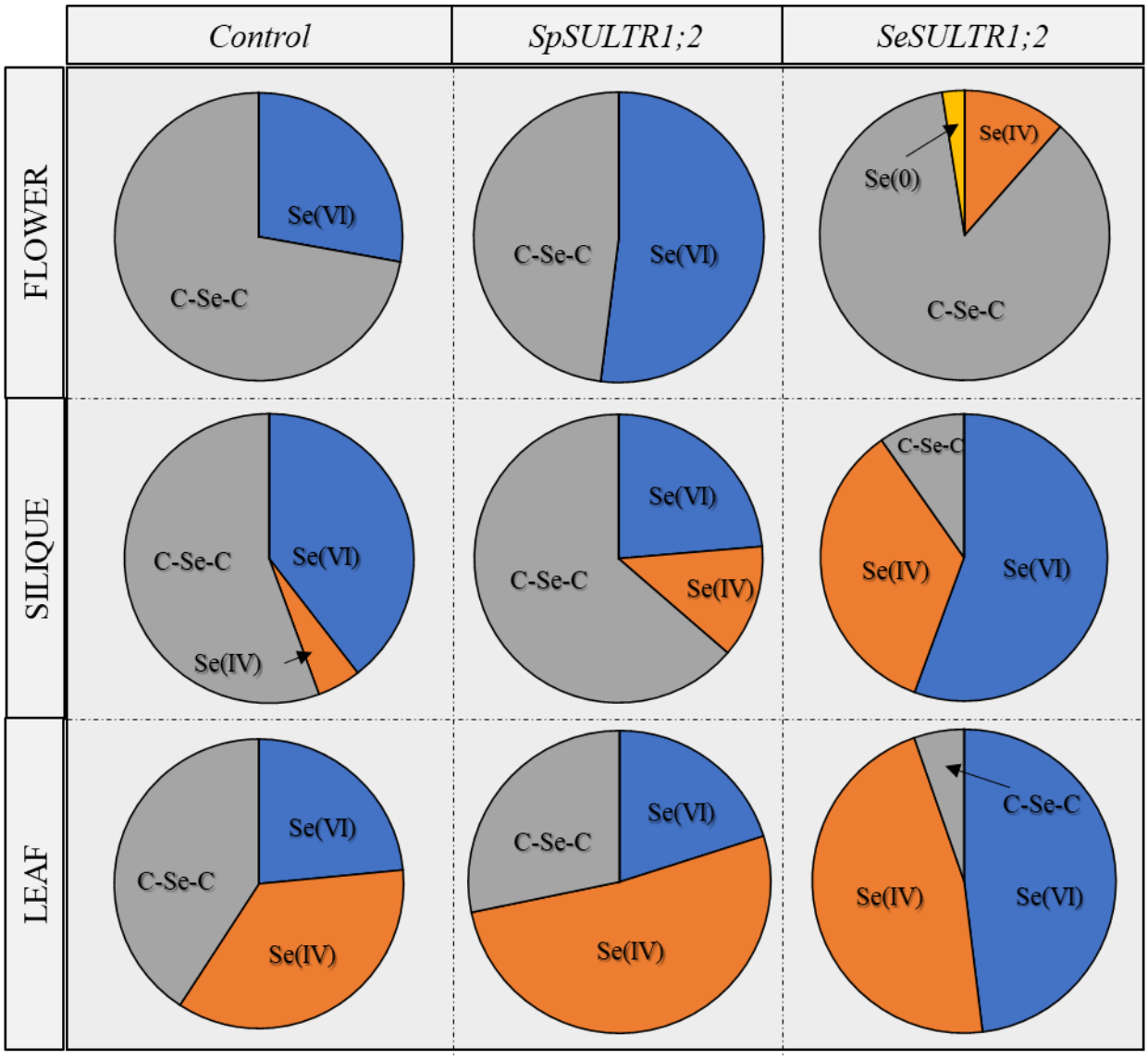

Figure 4-8. Selenium speciation as determined by least-square linear combination fitting of the Micro X-ray absorption near-edge structure (µXANES) spectra collected at the marked spots in the XRF maps (Figures 5, 6 and 7). C-Se-C may correspond to the organic forms SeCys, SeMet, MeSeCys and/or Se-lanthionine, which are indistinguishable by µXANES. Inorganic Se is represented as orange for Selenite (Se IV), and blue for Selenate (Se VI). Errors on fits are +/−10%. The non-recombinant *A. thaliana Col-W.T.* tissues are shown on the first column, followed by *A. thaliana* SULTR1;2 K.O plants transformed with the *Stanleya pinnata* SULTR1;2 in the middle column, and the *A. thaliana* SULTR1;2 K.O plants transformed with the *Stanleya elata* SULTR1;2 in the far right column.

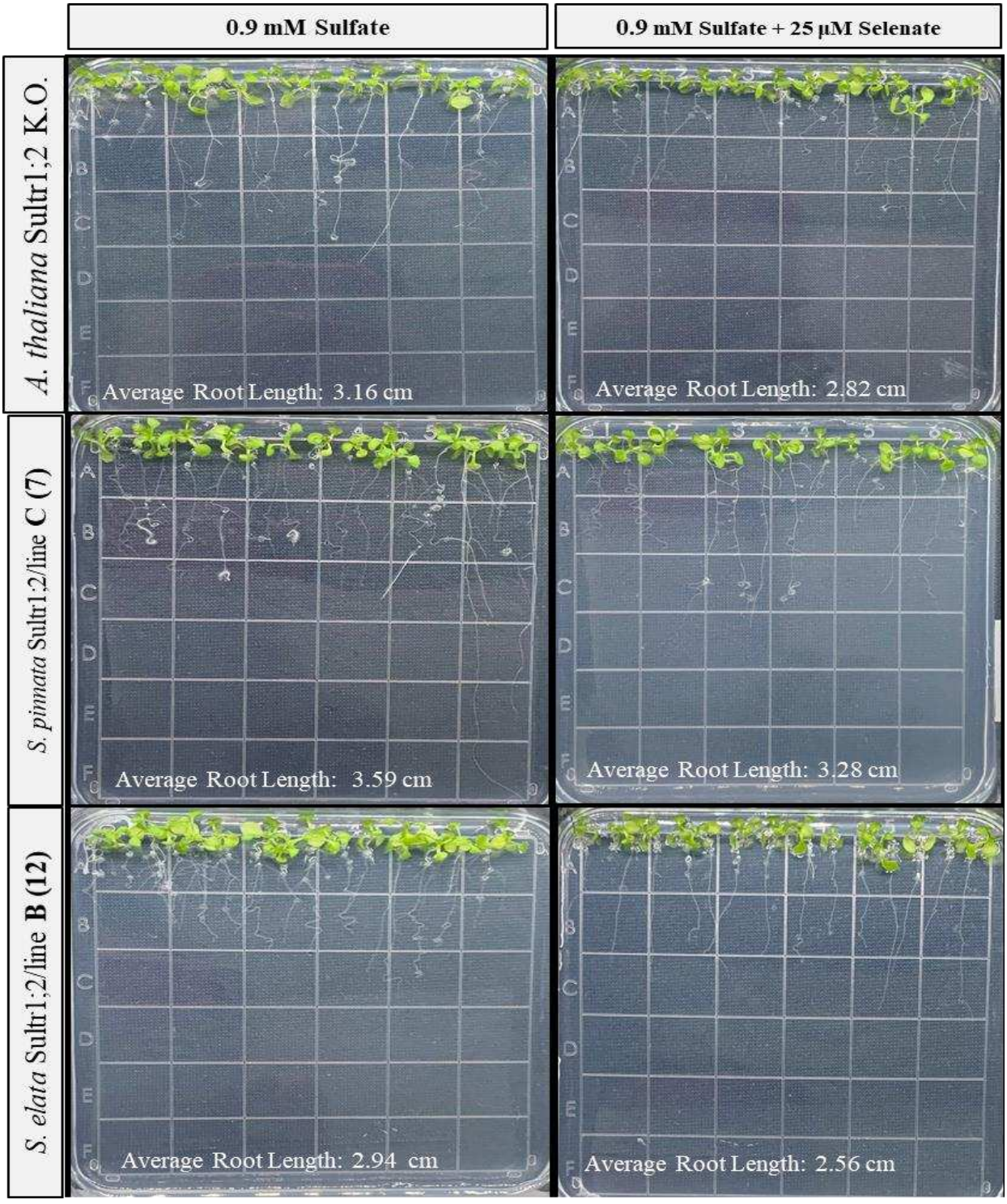

Figure 4-9. Seedling fifteen-days Se tolerance and accumulation experiment on vertical 0.5MS agar plates with or without 25 µM sodium selenate. Visual representation of the data presented in figure 9A (Root length).Values shown in each panel corresponds to the averaged root length (cm) of three different plates per treatment. The transgenics have the *Arabidopsis thaliana* SULTR1;2 K.O. background.

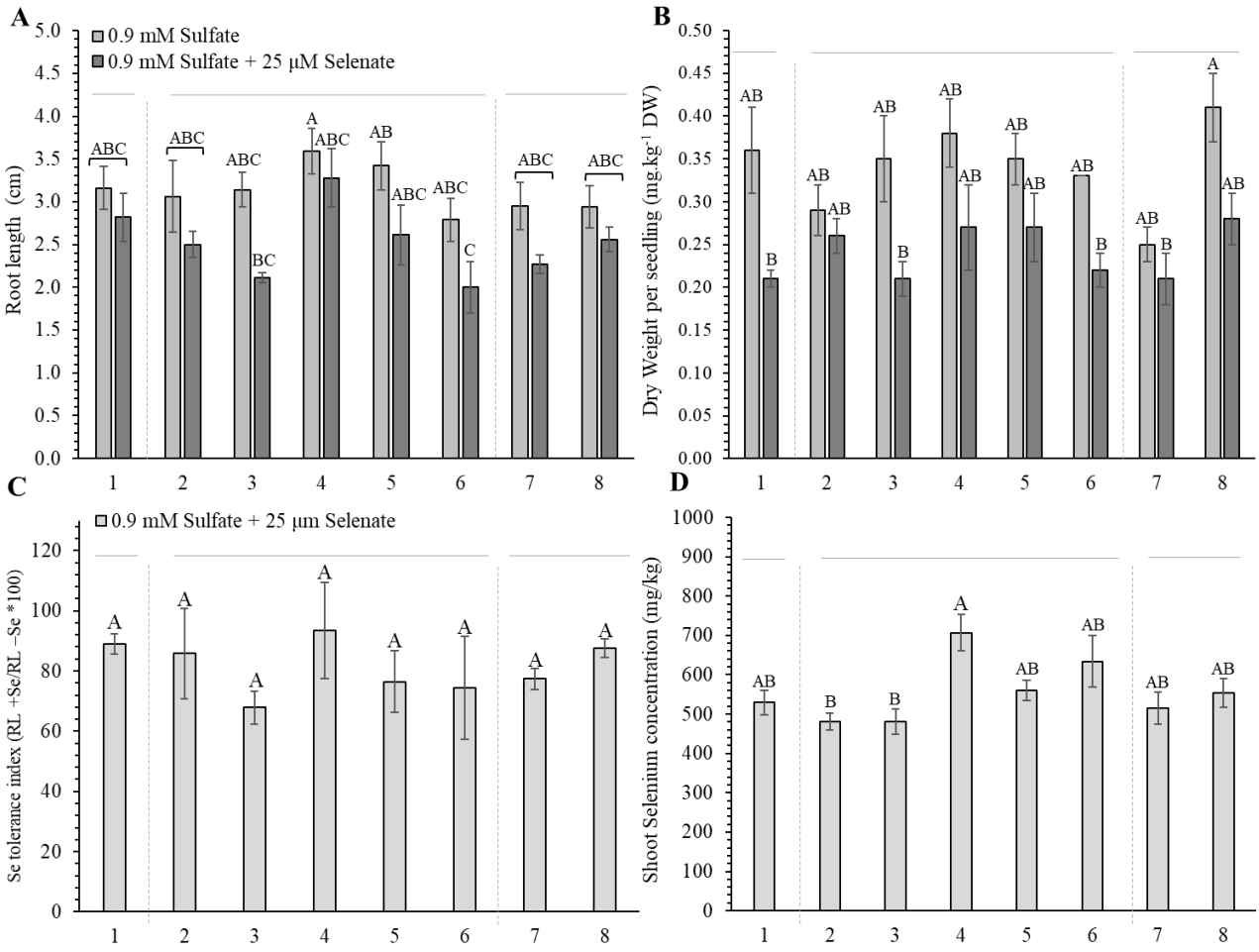

Figure 4-10. Seedling fifteen-days Se tolerance and accumulation experiment on vertical 0.5MS agar plates with or without 25 µM sodium selenate. Panel A: Root length; B: dry weight; C: Selenium tolerance index (Root length at +Se/ Root length at -Se * 100); RL=Root Length. D: shoot Se concentration. X-axis numbers represent different plant lines. **1=** Control *A. thaliana* SULTR1;2 **K.O.**; **2=** *S. pinnata* SULTR1;2 line **A(9)**; **3=** *S. pinnata* Sultr1;2 line **B(7)**; **4=** *S. pinnata* SULTR1;2 line **C(7)**; **5=** *S. pinnata* SULTR1;2 line **D(6)**; **6=** *S. pinnata* Sultr1;2 line **F(5)**; **7=** *S. elata* SULTR1;2 line **A(2)**; **8=** *S. elata* SULTR1;2 line **B(12)**. Transgenics all have the K.O. background. Different letters above bars indicate statistically different means among transgenic lines ($P < 0.05$).

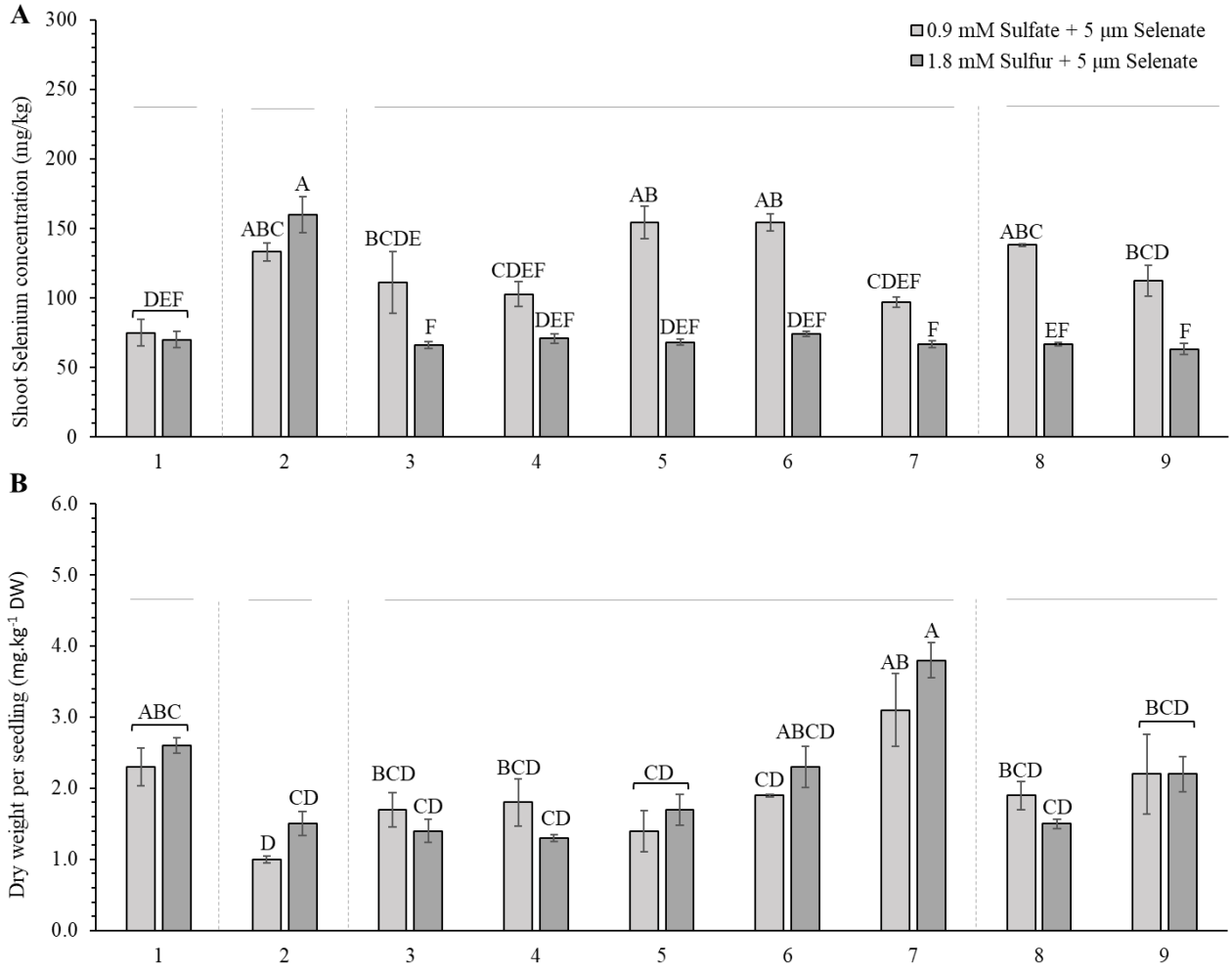

Figure 4-11. Twenty-five days Se accumulation experiment on horizontal 0.5MS agar plates with 5 µM sodium selenate, with regular (0.9 mM) or double (1.8 mM) sulfate supply. A: Shoot Se concentration; B: dry weight per seedling. X-axis numbers represent different plant lines. **1=** *A. thaliana* Col-0 WT; **2=** Control *A. thaliana* SULTR1;2 **K.O.**; **3=** *S. pinnata* SULTR1;2 line **A**; **4=** *S. pinnata* SULTR1;2 line **B(7)**; **5=** *S. pinnata* SULTR1;2 line **C(7)**; **6=** *S. pinnata* SULTR1;2 line **D(6); 7=** *S. pinnata* SULTR1;2 line **F(5)**; **8=** *S. elata* SULTR1;2 line **A(2)**; **9=** *S. elata* SULTR1;2 line **B(12)**. Transgenics all have the K.O. background. Different letters above bars indicate statistically different means among transgenic lines ($P < 0.05$).

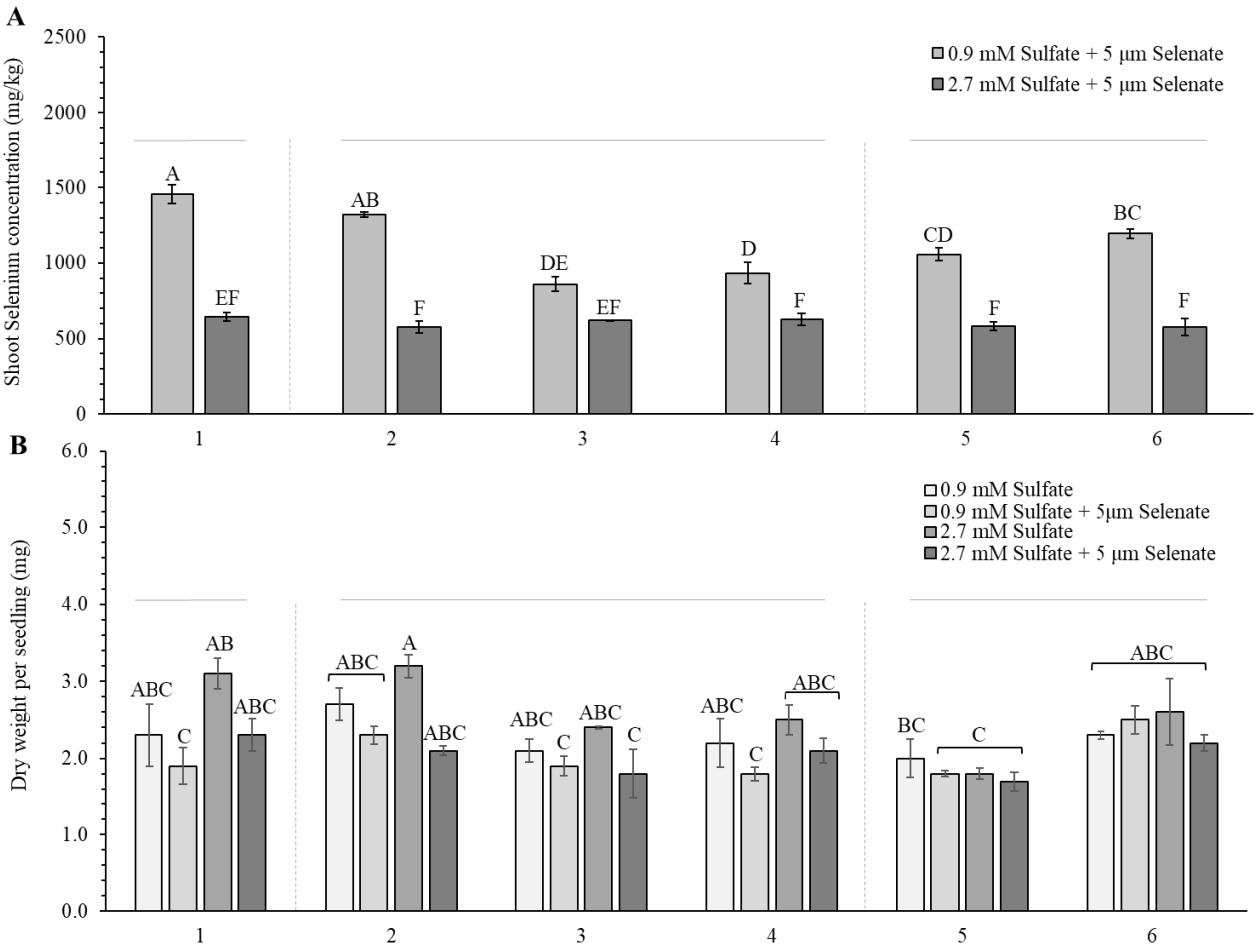

Figure 4-12. Twenty-five days Se accumulation experiment on horizontal 0.5MS agar plates + or - 5 µM sodium selenate, at regular (0.9 mM) or triple (2.7 mM) sulfate supply. A: Shoot Se concentration; B: dry weight per seedling. X-axis numbers represent different plant lines. **1=** *A. thaliana* Col-0 WT; **2=** Control *A. thaliana* SULTR1;2 **K.O.**; **3=** *S. pinnata* SULTR1;2 line **C(7)**; **4=** *S. pinnata* SULTR1;2 line **D(6); 5=** *S. elata* SULTR1;2 line **A(2)**; **6=** *S. elata* SULTR1;2 line **B(12)**. Transgenics all have the K.O. background. Different letters above Standard Error bars indicate statistically different means among transgenic lines ($P < 0.05$).

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CHAPTER 5: CHARACTERIZATION OF ATP SULFURYLASE 2, A POTENTIAL KEY SELENIUM HYPERACCUMULATION ENZYME FROM *STANLEYA PINNATA* VIA HETEROLOGOUS EXPRESSION IN *ARABIDOPSIS THALIANA*

5.1 INTRODUCTION

This study investigates the importance of the *Stanleya pinnata*'s ATPS2 (ATP sulfurylase 2) enzyme for the Se hyperaccumulation syndrome. Sulfur and Se are chemically very similar elements and transported and metabolized by the same proteins. As background for the research presented here, I start with a short summary of sulfur (S) metabolism and the role of ATPS. Sulfur (S) is a crucial macronutrient to plants and many other life forms. It is an integral component of the amino acids cysteine (Cys), and methionine (Met) and therefore is part of many different proteins, vitamins, and secondary metabolites (Mazid *et al*., 2011). This nutrient has a multitude of roles in the plant's metabolism, including structural, catalytic, and regulatory functions. Furthermore, S is an important substrate/reductant in antioxidant metabolism and stress defense mechanisms in plant cells. Glutathione (GSH), a tripeptide formed by the amino acids glutamate (Glu), cysteine, and glycine (Gly), for example, is the major non-protein S source in plants, and plays important roles in the enzymatic antioxidant metabolism and non-enzymatic abiotic stress defense mechanisms, specifically against heavy metal contamination via phytochelatins (Kopriva and Rennenberg 2004; Ghelfi *et al*., 2011; Rennenberg and Herschbach 2012; Seth *et al*., 2012).

Sulfur can be found under different oxidation states in the soil, however, the most common form taken up by plants is sulfate, SO_4^{2-}. Plants can also take up S as sulfite, SO_3^{2-}, and organic S in the form of different S-containing amino acids. These processes are generally energy-dependent.

Sulfate uptake is mediated by specific transmembrane sulfate transporters called SULTR (please see chapters 1 and 4 for more information on these transporters). After its uptake -and usually translocation to the shoot- sulfate is activated by the enzyme ATP sulfurylase and enters an intrinsic chemical pathway where it will be reduced and incorporated into the amino acid Cys, and further to the amino acid Met. Due to the similarity with S, Se metabolism in plants is essentially as described for S, as described in more detail below.

Sulfur and Se can be found in the oxygen family (chalcogens) in the periodic table, so their redox potentials and electronegativity are similar (Wessjohann *et al.,* 2007). Plants inadvertently take up Se, mainly as selenate, via the sulfate transporters and metabolize it via the S assimilation pathway into the amino acids selenocysteine (SeCys) and selenomethionine (SeMet), analogs of Cys and Met. The non-specific incorporation of these seleno-amino acids into proteins, replacing Cys and Met, causes the disruption of their structure and negatively affects their function; in addition, the accumulation of inorganic forms of Se results in strong oxidative stress, (Van Hoewyk, 2013).

In order to contextualize the research presented here, it is important to understand why we care about the element Se. Selenium is an interesting and important element because of its dual facet: it is required at low levels as a nutrient for many species including mammals, but also toxic at an elevated level, and the threshold between adequacy and toxicity is narrow. For plants, Se is not considered a nutrient, but at suitable trace levels, it can confer beneficial responses such as improved growth, productivity, and enhanced antioxidant metabolism (Hartikainen, 2005; Pilon-Smits *et al.,* 2009; Feng *et al*., 2013; Ashraf *et al.,* 2017).

As mentioned, Se is a nutrient for mammals and is known to be part of 25 different human proteins. Due to the redox properties of Se, selenoproteins exhibit excellent antioxidant activity in

biological systems. However, one billion people worldwide suffer from Se deficiency (Lyons *et al.,* 2003). Plants are usually the main portal for Se into the human diet, and different strategies can be utilized to enrich crops with Se to fight the worldwide deficiency issue (Malagoli *et al.,* 2015; Wu *et al.,* 2015). Therefore, a deeper understanding of the Se assimilation mechanisms by plants might increase our chance of successfully biofortify crops with this element (Malagoli *et al.,* 2015; Wu *et al.,* 2015; Schiavon and Pilon-Smits 2017). An interesting group of plants, both for their intrinsic and applied value, are Se hyperaccumulator plant species, which can accumulate over 1,000 μg Se g^{-1} dry weight in all organs when growing on seleniferous soils or when supplied with Se under cultivation (Schiavon and Pilon-Smits 2017). While most plants show a Se:S ratio reflective of that in their environment, hyperaccumulators appear to preferentially take up Se over S and have an elevated Se:S ratio (White *et al*., 2004).

The sulfate/selenate assimilation happens in part in the plastid, the sulfate transporter SULTR3;1 is the main protein responsible to load these elements from the cytosol to the stroma of the organelle (Cao *et al.,* 2013). The first step in selenate reduction is mediated by the enzyme ATP sulfurylase or ATPS, which couples selenate (or sulfate) to ATP (adenosine 5′-triphosphate), producing the activated form of Se/S, the adenosine 5′-phosphosulfate/selenate (APS/APSe) (Sors *et al.,* 2005a; Sors *et al*., 2005b; Pilon-Smits *et al.,* 2009; Schiavon *et al.,* 2015). Four ATPS isoforms have been identified in *A. thaliana*, three localized in the plastid (ATPS1, 3, and 4). The isoform ATPS2 shows a dual localization, being active in the cytosol and plastids (Takahashi *et al.,* 2011; Anjum *et al.,* 2015; Bohrer *et al.,* 2015). According to published studies with *Brassica juncea* expressing this enzyme from *A. thaliana* (ATPS1, specifically), this first activation step in the pathway seems to be rate-limiting for Se assimilation (Pilon-Smits *et al.,* 1999). Transgenic

APS plants overexpressing ATPS1 showed enhanced Se tolerance and accumulation and accumulated organic Se rather than selenate when supplied with selenate.

After its activation by the enzyme ATPS, APS/APSe is further reduced to selenite in a process catalyzed by the enzyme APS reductase (APR), this crucial step is also reported to be rate-limiting to Se assimilation (Sors *et al.,* 2005a; Suter *et al.,* 2000). Different enzymes in the S/Se assimilation pathway were previously investigated, and evidence supporting the role of APR in Se tolerance comes from studies on *A. thaliana* apr2-1 mutants. These plants were shown to contain a high concentration of selenate and lower amounts of selenite (Grant *et al.,* 2011; Chao *et al.,* 2014), indicating the important role of APR in the reduction of inorganic selenate (Chao *et al.,* 2014). In the next step of Se assimilation, selenite is further enzymatically converted to selenide (Se^{2-}), by the enzyme sulfite reductase (SiR) (Yarmolinsky *et al.,* 2012; White, 2016), or non-enzymatically via glutathione mediated reduction, with the formation of selenodiglutathione (GSSeSG) and selenopersulfide (GSSeH) as intermediates, and superoxide as a byproduct (Terry *et al.,* 2000; Anderson *et al.,* 2001). GSSeH is then converted to selenide by the enzyme glutathione reductase (GR) (Hsieh and Ganther, 1975).

Ultimately, selenide is incorporated into SeCys by the enzyme complex cysteine synthase, which catalyzes the formation of SeCys from O-acetylserine (OAS) and selenide (Sors *et al.,* 2005a; Terry *et al.,* 2000; White, 2016). As a mechanism to counteract the deleterious effect of Se on the cell, the amino acid SeCys can be further reduced to SeMet and the volatile DMSe (in non-hyperaccumulator plant species) or DMDSe (in hyperaccumulator plant species), in a multi-enzymatic process (Huysen *et al.,* 2003; Sors *et al.,* 2005a). However, the described process for S/Se assimilation cannot start without the activation of sulfate/selenate by the enzyme ATPS.

Studying Se hyperaccumulator plant species can give us a better insight into the Se tolerance and accumulation mechanisms evolved by these species. A transcriptome study comparing hyperaccumulator *S. pinnata* (Brassicaceae) to the non-hyperaccumulator *Stanleya elata* showed the expression of the gene encoding the enzyme ATPS2 was 120-fold higher in the roots, and 2 to 4-fold higher in leaves of the hyperaccumulator as compared to the non-hyperaccumulator (Schiavon *et al.,* 2015; Wang *et al.,* 2018). This higher expression level of ATPS2 could be partially responsible for the tolerance to the extreme high Se levels found in the hyperaccumulator since this enzyme is the first step in the reduction of sulfate/selenate and the formation of organic and less toxic forms of Se. If the *S. pinnata* ATPS2 enzyme could target Se over S, this could further contribute to the higher assimilation of Se, elevated reduction to organic and less toxic forms, and result in Se:S enrichment. Therefore, the *S. pinnata* ATPS2 is an interesting target for genetic engineering to develop plant species with elevated Se uptake, accumulation, and tolerance capacity to use in both biofortification and phytoremediation technologies.

This study investigates the role of the *Stanleya pinnata's* ATPS2 enzyme on the formation of organic and less toxic forms of Se, the overall plant's tolerance against toxic levels of Se, and ultimately its contribution to the Se hyperaccumulation syndrome. The cDNA from the corresponding gene, as well as from the non-hyperaccumulator *S. elata* were amplified, sequenced, and compared. Furthermore, the cDNAs were cloned into a plant binary vector and transformed into *A. thaliana* Col-0 wild-type plants, using *Agrobacterium tumefaciens*. The transgenic plant lines were bred to homozygosity and physiologically characterized. After treatment with selenate, the chemical form and location of Se in different plant tissues were investigated using x-ray microprobe analysis. Furthermore, the homozygous transgenic lines were compared with the non-recombinant control plants for Se uptake characterization via accumulation and tolerance

experiments. Plants transformed with the *S. pinnata* or *S. elata* ATPS2 gene would express a higher tolerance to elevated levels of Se, as compared to the untransformed controls, because assimilation of selenate to organic forms would alleviate the oxidative stress caused by inorganic Se. Furthermore, the plants expressing either version of ATPS2 were expected to accumulate more organic forms of Se, while selenate was expected in the controls. Finally, if ATPS2 from *S. pinnata* would be Se specific, plants expressing the *S. pinnata* ATPS2 gene would show a lower degree of Se assimilation inhibition by high S treatments, as compared to the controls. Other differences between the *S. pinnata* and *S. elata* ATPS forms may also lead to physiological differences between the respective transgenics.

5.2 RESULTS AND DISCUSSION

ATPS2 AMINO ACID SEQUENCES ALIGNMENT.

The first approach to understanding the role of the *Stanleya pinnata's* ATPS2 (SpATPS2) enzyme on the formation of less toxic forms of Se, and its contribution to the plant's tolerance against toxic levels of Se, was to clone and sequence the specific gene of interest. Further, the amino acid (AA) sequence of *S. pinnata* was aligned with that of the related non-accumulator *S. elata* (SeATPS2), from previously amplified root tissue cDNA (Jiang *et al*., 2018), as well as the model plant species *Arabidopsis thaliana* (AtATPS2). The AA coding sequences of the hyperaccumulator SpATPS2, and the non-hyperaccumulator SeATPS2, (Figure 5-1), showed around 95% of AA similarities across both proteins (15 amino acid differences). Interestingly, the published reference AA sequence of the AtATPS2 enzyme showed an overall lower degree of similarity with both *Stanleya* species, at around 91% (27 amino acid differences).

The AA differences in the hyperaccumulator protein, SpATPS2, in comparison to the other plant species can pinpoint specific mutations in the protein structure which could alter the enzyme

activity. Previously, the transcription level of different isoforms of the enzyme ATP sulfurylase was found to be higher in comparison with *Brassica juncea* and *S. elata* in the root system (Schiavon *et al*., 2015; Jiang *et al*., 2018), indicating that the reduction of the inorganic selenate to organic seleno-amino acids may be more efficient in the hyperaccumulator, which offers a possible mechanism for higher tolerance against the extreme Se concentration found in the hyperaccumulator.

The *S. pinnata* ATPS2 protein shows a total of 15 AA differences when compared to the non-hyperaccumulators, either *S. elata* or *A. thaliana* (Figure 5-1). Among the pool of differences between all proteins, 13 AA can be pointed out that are uniquely found in the hyperaccumulator, while conserved between the two non-hyperaccumulator species. The consequences of amino acid changes to the activity and the proper folding of a protein depend on the position of that residue in the protein, the specific side chain (-R), their polarity, and hydrophobicity (Betts and Russel, 2003). Most of the unique AA found in the hyperaccumulator enzyme are replacing either a hydrophobic to a hydrophobic AA or a hydrophilic to a hydrophilic AA, however, further investigation is required to investigate the overall effect of those changes on the protein's conformation and activity.

Interestingly, the hyperaccumulator *S. pinnata* ATPS2 enzyme is predicted to have a STOP codon, represented by a red x (Figure 5-1), in the chloroplast transit peptide (dashed box in Figure 5-1). There is a second translation initiation Met residue located downstream of this stop codon in each of these species. The stop codon, therefore, results in a uniquely cytosolic activity of ATPS2 in the hyperaccumulator *S. pinnata* species, as opposed to dual localization of ATPS2 in the other species. The unique cytosolic localization as well as the overexpression of the ATPS2 in the roots of the *S. pinnata* species may constitute a Se tolerance mechanism, whereby the plant readily

reduces selenate in the cytosol of the root cells, lowering the concentration of toxic inorganic selenate in roots. In this context, it is interesting to reiterate that the sulfate transporter SULTR1;2 was found to be highly expressed in the *S. pinnata's* root system (see chapter 4 for more details), likely causing a large influx of selenate into the root. The metabolic fate of the APSe (adenosine phosphoselenate) product of the ATPS2 in different plant organs was further investigated using x-ray microprobe analysis.

SELENIUM LOCALIZATION AND SPECIATION USING X-RAY MICROPROBE ANALYSIS

To further characterize the Se speciation and localization in the transgenic lines, synchrotron micro X-Ray Fluorescence (μXRF) was used. Seedlings of the transgenic lines were treated for 20 days with 20 μM of $NaSeO_4$ (sodium selenate). Figure 5-5 shows the Se localization in different samples of seedlings of the *Arabidopsis thaliana* col-0 (W.T.) transgenic plants transformed with the SpATPS2, SeATPS2, and the untransformed control. Selenium was distributed across all tissues in all analyzed leaf samples, in both the transgenics and the control plants (Figure 5-5), with the strongest Se signal in the veins and midrib in the leaf blade. Interestingly, the *Arabidopsis thaliana* col-0 (W.T.) transgenic plants transformed with the SpATPS2 showed a less intense signal for Se (Figures 5-5C and 5-5D), indicating this specific plant accumulated relatively less Se in comparison to the other transgenic samples tested.

The enzyme ATPS2 enzyme is not directly responsible for the translocation or redistribution of Se, therefore, no specific pattern of accumulation was expected for the transgenic plants transformed with either SpATPS2 or SeATPS2. However, the transformed plants were expected to accumulate more organic Se. As mentioned before, this enzyme initiates the reduction of Se and represents one of the key mechanisms in the Se tolerance and hyperaccumulation syndrome in the hyperaccumulator *S. pinnata*. Previous data indicate that the enzyme ATP sulfurylase can

metabolize Se as well as S and is a rate-limiting step for selenate assimilation and uptake in *B. juncea*; the overexpression of ATPS1 isoform from *A. thaliana* enhanced *B. juncea* tolerance to Se and changed the predominant form of accumulated Se from selenate to organic C-Se-C compounds (Pilon Smits *et al*., 1999). Interestingly, the main forms of Se found in hyperaccumulators like *S. pinnata*, either in tissues analyzed from plants collected in natural areas or from plants supplied with Se in a controlled environment, were also organic C-Se-C compounds, either the methylated form of the amino acid SeCys, methylselenocysteine (MeSeCys) or selenocystathionine (Freeman *et al*., 2006).

In the current study, the non-recombinant *A. thaliana* plants accumulated only selenate in their leaf tissues, while the plants expressing ATPS2 from either of the two *Stanleya* spp. were able to accumulate 31% (SpAPTPS2), and 39% (SeATPS2) of organic C-Se-C compounds (Figure 5-6), indistinguishable from either SeMet or MeSeCys by x-ray microprobe analysis. Therefore, the results presented here are similar to what was described before in the literature: the overexpression of the ATPS enzyme correlates with increased accumulation of organic Se (Pilon Smits *et al*., 1999). Assuming that the intracellular localization of the SpAPS2 was purely cytosolic (due to the predicted stop codon), while that of the SeAPS2 was both cytosolic and plastidic, it appears that this difference in localization did not affect Se speciation.

The fact that the SpAPS2 and SeAPS2 transgenics accumulated organic Se to an equal extent may indicate that the overexpression of the enzyme is most likely responsible for the resulting Se tolerance by the hyperaccumulator species. Despite all the differences in protein structure shown in the previous section, no AA changes were found in the predicted active site of the SpATPS2 enzyme in comparison to the other two proteins (bold AA in Figure 5-1), which may indicate the ATPS2 enzyme activity is not affected by those AA mutations shown. This enzyme

represents one of the key mechanisms of hyperaccumulation, by initiating the conversion of toxic selenate to less toxic organic Se species. The mechanism of tolerance against the toxic levels of Se could be solely related to the overexpression of ATPS, or also to enhanced selenate specificity over sulfate. *In vitro S. pinnata* ATPS activity assays published previously indicated that this enzyme can utilize selenate as a substrate (Jiang *et al*., 2018). Next, I investigated the Se-S interactions in the transgenics and control plants.

SELENIUM UPTAKE CHARACTERIZATION VIA ACCUMULATION AND TOLERANCE EXPERIMENTS.

The further characterization of the SpATPS2 role in the tolerance against high levels of Se was performed via different accumulation and tolerance experiments at the seedling level, to compare shoot selenate levels and Se tolerance of the SpATPS2 and SeATPS2 transgenics with the *A. thaliana* Col-0 W.T. control.

The experiments performed here followed the same design as in the previous chapter, where vertical plates (Figures 5-7 and 5-9), containing 15 seedlings each, were maintained in a growth chamber for 15 days, on average, following the measurement of the Se levels in the shoot, as well as the root length and the seedling dry weight (tolerance). The first experimental design utilized the basal S level of 0.9 mM sulfate, as found in the 0.5 strength M.S. plant medium. To test for Se tolerance, the Se level was maintained relatively high, at 45 μM selenate. The root length of all plants analyzed showed statistical differences between the treatments with and without selenate (Figures 5-8A, 5-10A), where the high Se treatment reduced the root length considerably. The average root length of the SpATPS2 transgenics was 4.1 cm without Se and 1.8 cm with 45 μM selenate, a 2.2-fold reduction. Similarly, the SeATPS2 transgenics showed a 1.8-fold reduction in the root length average, from 3.4 cm without Se to 1.9 cm with Se. The untransformed control

plants showed a slightly more pronounced reduction in root length by Se, where the treatment without Se showed an average root length of 4.4 cm and the Se-treated plants 1.7 cm, a 2.5-fold reduction. Thus, both transgenics appear to be more tolerant to selenate than the W.T. control. This may be explained by the finding that the transgenics contained in part organic C-Se-C Se, while the W.T. accumulated purely selenate, which is more toxic. Overall, these results are in line with earlier reports where the enzyme ATPS was a target of genetic engineering (Leustek *et al*., 1994; Pilon-Smits *et al*., 1999, Hatzfeld *et al*., 2000; Van Huysen, 2004; Banuelos *et al*., 2005; Freeman *et al*., 2010), and higher tolerance to selenate was reported in ATPS-overexpressing transgenics.

Leaf dry weight was also measured as an indicative parameter for Se tolerance (Figure 5-8B and 5-10B). Overall, no statistical differences were found between plus or minus Se treatments for any of the plant types, despite averages being consistently lower for most transgenics for the +Se treatments. Two different SpATPS2 transgenic lines showed a higher dry weight on average on the treatment without Se, when compared to the other plants, (Figure 5-8B, plants 2 and 5), however, no difference was found for these plants when Se was applied. The control *A. thaliana* Col-0 (W.T.) seedlings showed, on average, around 1.4-fold lower dry weight when treated with Se, as compared to the treatment with no Se. The transgenic *A. thaliana* seedlings transformed with the hyperaccumulator SpATPS2 gene, showed, on average, 1.45-fold lower dry weight when treated with Se, while the SeATPS2 transgenics showed, on average, 1.23-fold lower dry weight when treated with Se (Figure 5-8B).

When the Se concentration in the shoot was analyzed, the seedlings for all lines accumulated substitution Se levels (Figure 5-8D). The control *A. thaliana* Col-0 (W.T.) accumulated 1.4-fold higher levels of Se (12144 mg/kg) when compared to the SpATPS2 transgenics (8257 mg/kg, on average) and 1.8-fold higher levels of Se, as compared to the SeATPS2 transgenics (6574 mg/kg,

on average). The results showing higher levels of Se in the untransformed control than in the transgenic APS2 lines are contrary to expectations, based on earlier studies with APS1-expressing *B. juncea* (Pilon-Smits *et al*., 1999). However, plants can utilize different processes to eliminate an excess of Se, and perhaps the transgenic lines overexpressing the ATPS2 enzyme are using the process of Se volatilization more efficiently than the control, as Se volatilization happens much faster from organic Se than from inorganic Se, however, further investigation is required to understand the volatilization of Se by these transgenic plants (Terry *et al*., 2000). Further methylation steps can convert the amino acid SeMet to the volatile compound dimethylselenide (DMSe), reducing Se accumulation in tissues and its incorporation into proteins (Tagmount, 2002). Further experiments would need to be carried out to investigate this hypothesis. An argument against this hypothesis is that in earlier studies expressing APS1 from *A. thaliana* in *B. juncea* enhanced its capability to reduce and accumulate organic Se and actually led to 2-3 fold higher levels of Se in plant tissues (Pilon-Smits *et al*., 1999; Van Huysen *et al*., 2004; Banuelos *et al*., 2005).

The positive effect in the transgenic plants overexpressing ATPS2 is further illustrated by the Se tolerance index, calculated as the percentage of the root length +Se/root length -Se (Figure 5-8C): despite having higher Se concentration in its tissues than the transgenics, the control *A. thaliana* Col-0 (W.T.) demonstrated one of the lowest Se tolerance indexes, at around 39%. The average Se tolerance index for the transgenic plants overexpressing the SpATPS2 was 1.2-fold higher than the control, at 46%. This can be explained by the data shown in Figure 5-6, where the control plants accumulated a relatively more toxic form of Se in their tissues than the transgenics (selenate rather than organic Se). Interestingly, the SeATPS2 transgenics showed the highest tolerance index of 58%, on average 1.5-fold higher than the control, and 1.2-fold higher than the

SpATPS2. Thus, *A. thaliana* plants transformed with the non-hyperaccumulator species *S. elata* ATPS2 showed a slightly higher tolerance against Se in comparison to the transgenics expressing the hyperaccumulator *S. pinnata* ATPS2. Thus, the involvement of SpATPS2 in Se hypertolerance of this hyperaccumulator species may be due solely to the overexpression of the ATPS enzyme (Schiavon *et al*., 2015; Wang *et al*., 2018), and not to a unique hyperaccumulator protein property such as enhanced specificity to selenate over sulfate.

A final experiment was conducted using two different S concentrations, 0.9 mM, and 1.8 mM sulfate, two transgenic lines per construct, and 35 µm selenate. The aims were to compare the inhibitory effect of sulfate on Se accumulation from selenate, and Se tolerance to a lower concentration (Figures 5-9, 5-10, and 5-11). No statistical differences in root length were found (Figure 5-9, 5-10A) between the control and the transgenics for any of the treatments. There were also no distinct differences between treatments for the transgenic plants; however, there was a significant decrease in root length for the *A. thaliana* Col-0 (W.T.) control plants treated with 35 µm selenate, in comparison to the -Se 1.8 mM sulfate treatment.

Dry weight was analyzed (Figure 5-10B) as another measurement for Se tolerance and overall seedling development. Overall, no clear difference between the treatments with Se was noticeable. Despite no statistical difference between most of the results, from all treatments and transgenics, the treatment with high S demonstrated the largest biomass values for the control plants and most of the SeATPS2 transgenics. Moreover, the higher sulfate treatment showed a trend to lower the shoot Se concentration in all plant types analyzed, but only for the control plants were the results statistically different (Figure 5-10D). This inhibition of Se uptake and accumulation by sulfate was expected because of the competition between selenate and sulfate for the SULTR transporters in the root system (See chapter 4). No statistical difference in tissue Se concentration was observed

between the high and normal S treatments among all the transgenic lines, however, this is not an indication these plants can accumulate Se in the presence of relatively high S, instead, these plants showed relatively lower Se levels in the shoot in the lower S treatment, in comparison to the control plants. The reason for these lower Se levels in the APS2 transgenics is not clear but may have to do with Se volatilization being enhanced, as discussed earlier.

Interestingly, the untransformed control plants demonstrated a lower Se tolerance index for the treatment with high sulfate and selenate, while no difference in Se tolerance index between the corresponding +Se and -Se treatments was noticed for the SpATPS2 and SeAPS2 transgenic plants (Figure 5-10C). These findings are in agreement with the results from the first experiment, where the transgenics showed a relatively higher Se tolerance in comparison to the controls. Again, the explanation for this tolerance might lie in the ability of the APS2 transgenic plants to accumulate more organic Se, as shown in Figure 5-6; the APS2 enzyme mediates the reduction of selenate to selenite and thus facilitates the reductive assimilation of selenate to organic Se.

The inhibition of Se uptake and accumulation by S was calculated as the sulfur inhibition factor (Figure 5-11), by the equation [1- (Se at 1.8 mM S/ Se at 0.9 mM S)]. Interestingly, sulfur inhibition was less pronounced in the transgenic lines expressing the non-hyperaccumulator enzyme SeATPS2, in comparison to the hyperaccumulator SpATPS2 and especially to the untransformed control. This may indicate that transgenic plants overexpressing the SeATPS2 enzyme could benefit from accumulating organic Se, and potentially be able to take up Se better at high S levels. SpATPS transgenics also accumulate organic Se, but do not show better Se uptake under high S conditions. Perhaps this difference is due to the different intracellular localization of the APS2 protein for SpAPS2 (cytosolic only) and SeAPS2 (both chloroplastic and cytosolic).

More experiments are needed to further investigate and compare the physiological properties of these transgenic lines.

5.3 MATERIALS AND METHODS

PLASMID CONSTRUCT

Previously generated ATPS2 cDNA from root tissue of *S. pinnata S. elata* (Jiang *et al*., 2012), and the publicly available *A. thaliana* was used for the sequence and ligation into the PYES2 vector, using digested fragments obtained from the previously cloned pCR4-TOPO vector (Guignardi, 2017; Jiang *et al*., 2018). The restriction digestions were performed using the enzyme EcoRI (New England BioLabs) as follows: 1 µl of the restriction enzyme, 1 µg of DNA, 5 µl (1X) of 10X NEBuffer, in a total reaction volume of 50 µl. The reaction was incubated at 37°C on a VWR Digital Multi Heat Block for 15 minutes. All ligations were performed with T4 Ligase (Thermofisher) at room temperature for 30 minutes as follows: 0.5 µl of T4 ligase enzyme, 2 µl of 10X ligase buffer, 1-3 fold excess of the insert over the vector fragment, to 20 µl volume using distilled H_20.

After each ligation, plasmids were initially transformed into DH5-α competent *E. coli* cells via $CaCl_2$/ heat shock transformation, for plasmid amplification and screening for positive transformants (Taylor *et al*., 1993). The PYES2 vector ligated with the genes of interest (10 µl), was added to 200 µL of thawed competent cells in a 1.5 ml Eppendorf microcentrifuge tube on ice for 30 minutes. Then, the cells were heat-shocked at 42°C on a water bath for 45 seconds and then placed on ice for 5 minutes. 1 ml of pre-warmed liquid LB media was added to the cells, and they were incubated at 37°C for 1 hour. The cells were then plated (200 µl) on LB agar media supplemented with 100 µg / ml ampicillin, overnight. The next morning the resultant colonies were

transferred to a new LB agar plate with ampicillin for PCR confirmation and *E. coli* transgenic library establishment.

The PCR (Polymerase chain reaction) reactions were carried out using a total volume of 25ul as follows: 2.5µl of 10X Buffer without $MgCl_2$, 2.5 µl of free deoxynucleotides (dNTPs, 2 mM each), 1.5 µl of $MgCl_2$ (25 mM), 0.2 µl of the enzyme Taq polymerase (1 unit), 1 µl of each primer, 2 µl of the target, and 14.3 µl of distilled H_2O. All PCR reactions were performed in an Eppendorf Mastercycler gradient thermocycler with the following cycling conditions: Initial denaturation for 2 minutes at 95°C followed by 30 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 10 seconds, and extension of 70°C for 40 seconds, with a final extension at 70°C for 10 minutes. To purify restriction fragments and PCR products, samples were loaded onto a 1% agarose TBE gel and run at 90V for 40 minutes. DNA bands were visualized using a UV light illuminator, excised with a scalpel, and moved to a 1.5 ml Eppendorf microcentrifuge tube. The DNA was then purified from the gel piece using a Qiagen Gel Extraction kit (Qiagen, Hilden, Germany). Plasmids were purified using the Qiagen Plasmid Miniprep kit, and sequence-verified via Sanger sequencing (http://www.genewiz.com) using the following primers:

- SpinSela/ATPS2_Fw (5'-CATCAAGAGGAACATCATCAGC-3'),
- SpinSela/ATPS2_Re (5'-TTACAGGCTATCTCCAAAACAGC-3'),
- Spin_Fw (5'-GGAATTCCATATGCAATCTGTCACTTCCTCTT- 3'),
- Spin_Re (5'-CGCGGGATCCTTAAGGCTTATCACTTTCTTGCA-3'),
- pYES2_R2 (5'-CTTTTCGGTTAGAGCGGATG-3'),

After verification of the plasmid sequences and orientation, the genes of interest were digested from PYES2 using the BstX1 enzyme and further cloned into the plant binary vector pFGC5941 under the control of the CaMV35S promoter, following the methods described in the

previous paragraphs. The binary vector was used for the stable expression in *A. thaliana* via *Agrobacterium tumefaciens* (strain GV3101) transformation. The binary plant vector pFGC5941 was first prepared for the genes from the PYES2 vector, by insertion of a linker containing the BstX1 restriction site, as well as two flanking sites for the enzymes Ascl and Sma1, both sites used for the ligation of the linker into the pFGC5941 plasmid (Oligonucleotide: 5'-GGGCCAGTGTGCTGGCCATCACACTGGGG-3'). The plant binary vector was first digested with the Ascl and Sma1 restriction enzymes, and the linker was further ligated into the binary vector using the method previously described.

AGROBACTERIUM AND PLANT TRANSFORMATION

The pFGC5941 constructs containing the gene of interest of each SULTR1;2 genes were further transformed into the *A. tumefaciens* strain GV3101 (containing a C58 chromosomal background with rifampicin resistance and the Ti plasmid pMP90 with gentamicin resistance). The *Agrobacterium* strain was grown overnight at 28°C in 5 ml of LB buffer. The next morning 2 ml of the overnight culture was added to a 50 ml LB medium in a 250 ml incubation flask and shook at 250 rpm at 28°C until the OD_{600} of 1.0. After this growth period, the culture was chilled on ice for 10 minutes. The cell suspension was further centrifuged at 3000g (6000 RPM) for 5 minutes at 4°C. The supernatant was discarded, and the cells were resuspended in 1ml of 20mM $CaCl_2$ solution. Following, 0.1ml of the solution was aliquoted into prechilled Eppendorf test tubes. Around 1µg of plasmid DNA was added to the cells and mixed gently. The cells with the added plasmid were frozen quickly using a Qiagen lyser block from the -80°C freezer. After 5 minutes the cells were heat-shocked at 37°C in a water bath for 5 minutes. After, 1 ml of LB medium was added, and the cells were incubated for 4 hours at 28°C. After incubation, the cells were centrifuged at 6000 RPM using a microcentrifuge. The supernatant was discarded leaving around

0.1 ml in the tube, the cells were then resuspended using the remaining supernatant. The cell suspension was then transferred (0.1 ml) to LB plates containing rifampicin and gentamicin and incubated for 2 or 3 days at 28°C until transformed colonies were visible. The colonies were further grown in LB medium and stored in glycerol at -80°C for further plant transformation.

Arabidopsis thaliana (Col-0 Wild Type and SULTR1;2 K.O.) seeds were vernalized at 4°C in 5 ml of sterile H_20. The vernalized seeds were germinated on wet soil containing PRO-MIX HP soil in 4-inch pots, 5 seeds per pot, and grown in a growth room under short-day controlled conditions, 8h of light/16h of dark, 25°C day/night, at a light intensity of 200 µmol m^{-2} s^{-1}. The pots were watered three times per week from the bottom using tap water. The watering solution at week four contained a 1:1000 dilution of Miracle-Gro Liquid All Purpose Plant Food (Scotts Company, Marysville, OH). After four weeks the plants were transferred to long-day conditions for flowering inducing, 16h of light/8h of dark, 25°C day/night, at a light intensity of 200 µmol m^{-2} s^{-1}. The plants were watered daily from the bottom using ½ cm of tap water.

The day before the plant transformation the Agrobacterium was inoculated in a 100 ml culture using LB and the antibiotics rifampicin and gentamicin. The pots with the plants, 3 pots per construct, were labeled with the construct name and date. The soil was saturated with H_2O before the dip. The OD_{600} of the cell cultures was measured, and the cells were centrifuged at 4°C for 10 minutes at 8000 RPM using centrifuge bottles. After centrifugation, the supernatant was discarded and the cells were resuspended in a fresh dip medium, containing sterile H_2O and 5% sucrose (50 g/liter) and 0.05% Silwet L-77 (500 µl/L), to OD_{600} of 0.8. The dip solution was transferred to a 1L beaker, before dipping the flowers into the solution siliques were cut from the stem. The stems of each flower were dipped in the solution for 1 minute, and 3 pots per construct were dipped in the same solution. After dipping the pots containing the plants were transferred to

a tray and kept in the dark with high moisture overnight. The plants were watered twice a week until most of the siliques turned brown, at around 3 weeks after the dip. At week four no water was given, and the stems were collected in Ziploc bags. The seeds were harvested from the bags and cleaned from plant debris using cheesecloth two weeks after all siliques were dried and opened. Cleaned seeds were stored in labeled microcentrifuge tubes in the fridge, at 4°C.

HOMOZYGOUS TRANSGENIC ARABIDOPSIS THALIANA LINES SELECTION

The positively transformed A. thaliana seeds from the A. tumefaciens floral dipping transformation, henceforward called generation 0 seeds, were selected using BASTA (gluphosinate ammonium, 25 mg/L), on MS (Murashige and Skoog) medium. First, the seeds were surface sterilized as follows: in a sterile laminar flow hood, 30 mg of seeds (around 900 seeds) were transferred to a sterile 15 ml conical tube (VWR), and 1 ml of 70% EtOH was added to the tube, after mixing, the seeds were let to sit for 1 minute. The seeds were then washed twice using 10 ml of distilled H2O. After the first wash, 10 ml of 10% bleach with a drop of Tween-20 was added to the tube, the tube was then vortexed for 30 seconds every minute for a total of 10 minutes. The bleach solution was then removed using sterile tips, the seeds were then washed five times using 10 ml of distilled H_2O. After the last wash, 5 ml of distilled H_2O was added, and the tube was transferred to the fridge at 4°C for 3 days. After this period all the content of the tube was poured onto the surface of a large petri dish, containing 100 ml of selective MS medium, supplemented with selective medium with BASTA (25 mg/L) and cefotaxime (100 mg/L). The petri dish with the seeds was then transferred to a growth chamber (8h of light/16h of dark, 25°C day/night, at a light intensity of 200 $\mu mol\ m^{-2}\ s^{-1}$, RH 70%).

The MS medium plates were prepared using 0.67 g of MS salts (Sigma-Aldrich, St Louis, MO), 1 g of sucrose (Sigma-Aldrich), and 1.2 g of PhytoAgar (Research Products International,

Mt. Prospect, IL), the solution pH was adjusted to 5.8 using 1M KOH. The solution was then autoclaved for 20 minutes, and after cooling down the selective herbicide BASTA, the antibiotic cefotaxime was added. The solution was then poured into the Petri dishes and let dry inside the laminar flow hood.

The transformed seeds that survived the selection medium were then transferred to pre-wet soil (PRO-MIX HP) in 4-inch pots, 1 seedling per pot. The seedlings were grown in a growth room under short-day controlled conditions, 8h of light/16h of dark, and 25°C day/night, at a light intensity of 200 µmol m^{-2}. s^{-1}. After 3 weeks the pots were transferred to long-day, 16h of light/8h of dark, 25°C day/night, at a light intensity of 200 µmol m^{-2} s^{-1}, and watered 3 times a week. The seeds were then collected in separated Ziploc bags and cleaned from plant debris. These seeds will be henceforward called generation 1, a total of 14 BASTA resistant survivors were obtained for the transgenic lines transformed with the SpSULTR1;2 genes and they were labeled with a letter representing each line, from A to N. For the transgenic lines transformed with SeSULTR1;5 genes, a total of 7 resistant survivors were obtained, lines A to G.

A total of 100 seeds from each generation 1 line were surfaced sterilized, following the procedures described before. Two small Petri dishes containing 50 ml of selective MS medium each, supplemented with selective medium with BASTA (25 mg/L) and cefotaxime (100 mg/L), were used per transgenic line (Figure 5-4). A total of 50 seeds were plated per petri dish using a sterilized toothpick. The Petri dishes with the seeds were then transferred to a growth chamber (8h of light/16h of dark, 25°C day/night, at a light intensity of 200 µmol m^{-2} s^{-1}, RH 70%) for 3 weeks. After the growth period, a total of 15 seedlings per line were then transferred to pre-wet soil (PRO-MIX HP) in 4-inch pots, 1 seedling per pot. The plants were grown to seeds as described before. These seeds will be henceforward called generation 2. The 15 BASTA resistant survivors of

generation 2 were obtained for the transgenic lines transformed with the SpSULTR1;2 genes were then labeled with the same letter representing the previous generation, and a number representing the current generation, 1 to 15.

Following, 100 seeds from each of these 15 generations 2 lines were surface-sterilized and divided into two small Petri dishes containing 50 mL of selective MS medium each, supplemented with selective medium with BASTA (25mg/L). At this time, the lines that contained 100% of survivors on both Petri dishes (homozygous) (Figure 5-4) were selected to be used for the tolerance and tolerance experiments.

LEAF DNA EXTRACTION AND AMPLIFICATION VIA PCR

Leaf DNA was extracted, amplified with PCR, and separated using gel electrophoresis, from each generation 1 line to confirm the presence of the ATPS2 genes. The DNA extraction was performed: fresh leaf material was ground with 600 µl of extraction buffer (EB: 100 mM Tris pH 8, 50 mM EDTA pH 8, 500 mM NaCl) in a 1.5 ml tube. After grinding, an extra 150 µl of EB buffer was added and mixed. Further, 50 µl of 20% SDS was added to the solution and incubated at 65°C for 10 minutes. Next, 250 µl of K-acetate (60 ml of 5M KOAc, 11.5 ml acetic acid, and 28.5 ml of H_2O) was added and mixed, the solution was then incubated in ice for 20 minutes. The tube was centrifuged at top speed for 10 minutes, and the supernatant was then transferred to a new 1.5 ml tube containing 500 µl of isopropanol, mixed, and incubated at -20°C for 20 minutes. After incubation, the samples were centrifuged at top speed for 10 minutes, the supernatant was discarded, and the pellet dried at room temperature. The pellet was then resuspended in 30 µl of TE buffer (10 mM Tris pH, 1 mM EDTA pH 8, 20% SDS, 100% isopropanol, 3M NaOAc, pH 5.2). Further, 30 µl of NaOAc and 1000 µl of 200 proof EtOH were added and mixed. The solution was then transferred overnight to the -80°C freezer. The next day, the samples were thawed in ice

and then centrifuged at top speed for 10 minutes. The supernatant was discarded, and 1 ml of 75% EtOH was added. The sample was centrifuged at top speed for 2 minutes. The supernatant was then discarded, and the remaining solution was removed using a 100 µl pipette. The pellet was then resuspended in 50 µl DNase-free H_2O.

The extracted DNA was then quantified using Nanodrop 2000 spectrophotometer (ThermoFisher Scientific). The DNA was further amplified via PCR using the pFGC5941_35s_Fw promoter (5'_GGA GAG GAC ACG CTC GAG TAT AAG_3'), and the respective ATPS2 promoter Internal Spin_Sela_APS_REV (5'_CCT GTA ATG ATC AAG CCC GTC_3') (Figures 5-2 and 5-3). The PCR fragments were then separated by gel electrophoresis (100 ml of 1X TBE buffer, 1g of agarose, and 10 µl of ethidium bromide).

TOLERANCE AND ACCUMULATION EXPERIMENTS

Transgenic seeds from the previously selected homozygous lines were surface-sterilized following the procedure previously described. The tolerance and the uptake experiments were carried out on polystyrene Petri dishes, 3 replicates per transgenic line, using vertical (tolerance) square dishes (Figures 5-7 and 5-9). Each plate was prepared with MS agar medium in a sterile laminar flow hood. For the tolerance experiment, a total of 15 sterilized seeds were transferred using a sterile toothpick to the top of each MS agar plate. The plates were incubated horizontally in a growth chamber (8h of light/16h of dark, 25°C day/night, at a light intensity of 200 µmol m^{-2} s^{-1}, RH 70%). The root length of all plates was monitored daily. The experiment was finalized when the first root reached the bottom of a plate (~15 days). The shoot of all seedlings per plate was collected, wash with cold water, and dried for 3 days. After this period, the dry weight per plate was measured, and the material was digested for elemental analyses.

SELENIUM LOCALIZATION AND SPECIATION VIA X-RAY MICROPROBE ANALYSIS

Seedlings of the transgenic lines (generation 0) were treated for 20 days with 20 µM of $NaSeO_4$ (sodium selenate). After this period, different tissues were collected and frozen in liquid nitrogen for further µXRF analyzes. Selenium (and calcium) localization and speciation were analyzed in *A. thaliana's* leaf, flower, and silique using X-ray microprobe imaging (Zarcinas *et al*., 1987). Analyses were performed at beamline 10.3.2 (X-ray Fluorescence Microprobe) of the Advanced Light Source (ALS), at Lawrence Berkeley National Lab (Berkeley, CA, USA) using a Peltier cooling stage (−25˚C). Micro-focused X-ray fluorescence (µXRF) maps were recorded at 13 keV incident energy, using 20 µm × 20 µm pixel size, a beam spot size of 7 µm × 7 µm, using 70 ms dwell time, and 50 ms dwell time. Maps were then deadtime-corrected and decontaminated. Selenium K-edge micro X-ray absorption near-edge structure (µXANES). spectroscopy (in the range 12,500–13,070 eV) was used to analyze Se speciation on eleven different spots on samples, close to areas showing high Se concentration in the µXRF maps. Spectra were energy calibrated using a red amorphous Se standard, with the main peak set at 12,660 eV. Least-square linear combination fitting of the µXANES data was performed in the range of 12,630 to 12,850 eV using a library of 52 standard selenocompounds and procedures described by Fakra (2018). All data were recorded in fluorescence mode using a 7-elements Ge solid-state detector (Canberra, ON) and processed using custom LabVIEW programs available at the beamline.

ELEMENTAL COMPOSITION

Fresh tissue samples were dried at 50˚C until constant weight. Approximately 100 mg of samples were then digested with 1 mL of nitric acid as described (Németh *et al*., 2015); in short, samples were heated for 2 h at 60˚C and 6 h at 125˚C, then diluted to 10 mL with distilled water.

Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to analyze the digested seed samples' elemental composition (Se).

STATISTICAL ANALYSIS

The software JMP-IN 13.0.0 (SAS Institute, Cary, NC, USA) was used for statistical data analysis. Student t-test was used to compare different treatments. The different letters above bars in figures 4-10, 4-11, and 4-12, indicate statistically different means among transgenic lines ($P < 0.05$).

5.4 CONCLUSIONS

This study aimed to investigate the importance of the Se hyperaccumulator *Stanleya pinnata's* ATPS2 (SpATPS2) enzyme for the Se hyperaccumulation syndrome, via expression in non-accumulator *A. thaliana*. The predicted amino acid (AA) sequence of the SpATPS2 enzyme from the hyperaccumulator species was aligned and compared to the AA sequence of the non-hyperaccumulator species from the same genus *Stanleya elata*, SeATPS2, and the non-recombinant control *A. thaliana* Col-0 (W.T.). Furthermore, the chemical form and location of Se in leaves of transgenic plants were investigated using x-ray microprobe analysis. The homozygous transgenic lines were compared with the non-recombinant control plants for Se uptake characterization via accumulation and tolerance experiments.

The predicted protein sequence of the hyperaccumulator enzyme SpATPS2 has a total of 15 AA differences in comparison with the non-accumulator proteins SeATPS2 and *A. thaliana* ATPS2. The unique AA sequences of the hyperaccumulator enzyme could alter its activity; however, data so far indicate this enzyme has no specificity to Se over S. Most interestingly, the hyperaccumulator SpATPS2 enzyme has a STOP codon in the chloroplast transit peptide, resulting

in a putative uniquely cytosolic localization of ATPS2 in this species. The uniquely cytosolic activity of this enzyme in *S. pinnata* may be a possible mechanism for enhanced tolerance against the high influx of Se into the root system mediated by high SULTR1;2 expression. It is feasible that the hyperaccumulator plant readily activates the incoming selenate in the cytosol of the root cells, initiating the cascade of reduction events to produce organic Se, lowering the concentration of toxic inorganic selenate in the roots. Indeed, plants expressing *SpATPS2* and *SeATPS2* accumulated more organic Se (around 30% C-Se-C), while the control plants accumulated 100% selenate.

As hypothesized, plants transformed with the *S. pinnata* ATPS2 gene showed higher Se tolerance, as compared to the untransformed controls; transgenic plants expressing the ATPS2 from non-hyperaccumulator *S. elata* also showed enhanced Se tolerance. The ATPS enzyme is the key enzyme of the sulfate/selenate assimilation pathway, which converts inorganic Se into less toxic, organic forms. Plants expressing the *S. elata* ATPS2 gene showed relatively less inhibition of Se assimilation by high S treatment than untransformed plants; *S. pinnata* ATPS2 transgenics were intermediate in this respect. Overall, there was no evidence that the enzyme SpATPS2 is selenate specific; it does not seem to be an important factor for the earlier observed enrichment of Se over S in the hyperaccumulator. Nevertheless, SpATPS2 likely plays an important role in Se hyperaccumulation *in S. pinnata*. The constitutive overexpression of selenate transporter SULTR1;2 and SpATPS2 in the roots of this hyperaccumulator together mediate rapid selenate influx and efficient reduction of selenate to selenite, quickly initiating the conversion of the toxic inorganic Se to less harmful organic forms. This efficient reduction process, coupled with other uniquely evolved processes to methylate and volatilize Se, to sequester C-Se-C compounds in specific tissues an enhanced antioxidant system ultimately contributes to an extreme overall

tolerance to the high levels of Se found in the hyperaccumulator. Elucidation of the mechanisms that underlie the apparent Se-specific uptake and the resulting higher Se/S ratio in hyperaccumulator tissues requires further investigation. Identification of a Se-specific transporter would be a first of its kind, and would also have applications in Se biofortification, which is often hampered by high sulfate levels.

A challenging barrier in phytoremediation of Se is to find strategies to overcome the inevitable oxidative stress disrupted by the increased Se levels in tissues of non-hyperaccumulator species. As found here and published before by other authors, plants overexpressing the ATPS demonstrate an enhanced Se tolerance due to their enhanced ability to reduce selenate and accumulate organic Se (Pilon-Smits *et al*., 1999, Banuelos *et al*., 2005). Therefore, overexpressing the enzyme ATPS is a promising approach for more efficient phytoremediation strategies; because organic C-Se-C is the best form of dietary Se, the resulting plant material also has interesting biofortification applications.

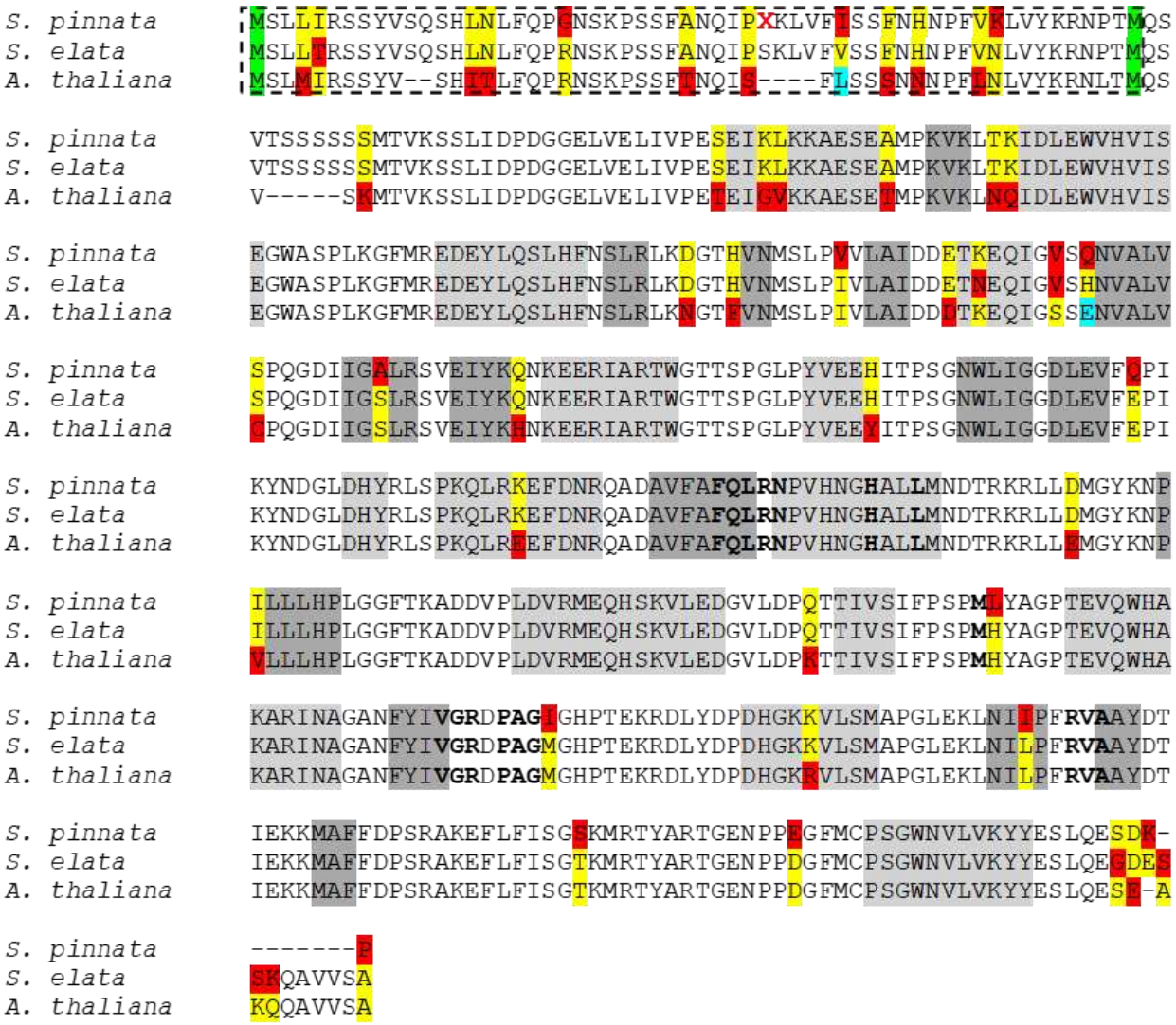

Figure 5-1. Alignment (MAFFT alignment tool) of the predicted amino acid sequence of APS2 from *S. pinnata*, *S. elata,* and *A. thaliana*. The first and second methionine residues flanking the transit peptides are highlighted in green. The dashed box represents the chloroplast transit peptide. The red bold X in the *S. pinnata* APS2 sequence indicates a stop codon in the transit peptide. Red, blight blue, and Yellow highlights represent the amino acid differences between the proteins. Light gray and dark boxes show the predicted alpha-helix and beta-sheet strands, respectively. Bold amino acids represent the predicted fragments of the enzyme's active site. Modified from Jiang *et al*., 2018.

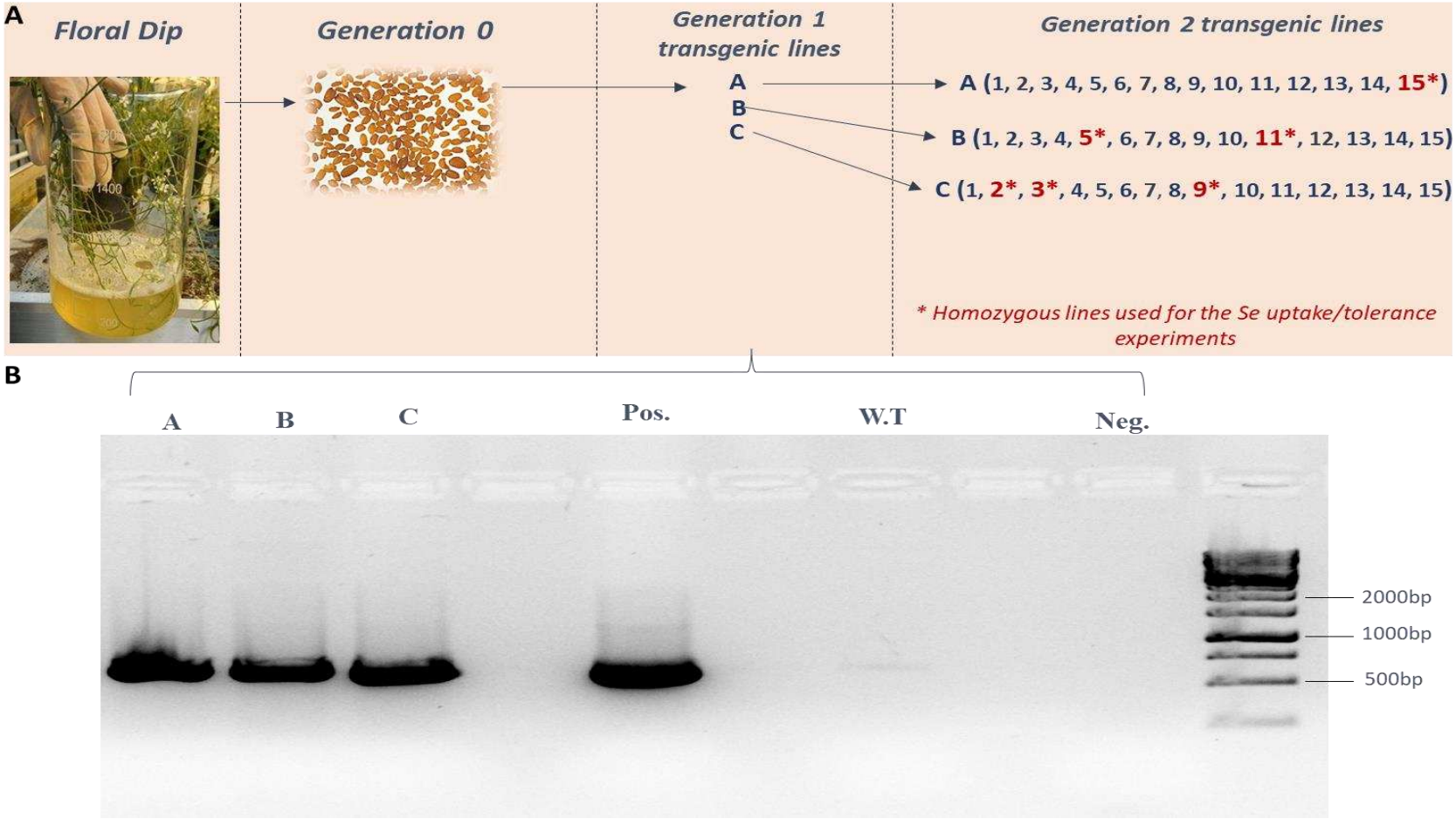

Figure 5-2. Schematic representation of the genetic transformation of *Arabidopsis thaliana* Col-0 W.T. plants with the *Stanleya pinnata* APS2 gene, via floral dipping using *Agrobacterium tumefaciens.* Panel A shows the transgenic lines for the first and second generations, the homozygous lines highlighted in red with asterisks (generation 2) were used for the Selenium tolerance and uptake experiments. A total of 900 seeds (~30 mg) from the generation 0 mother plant were grown on BASTA. The resistant seedlings (generation 1, lines A, B, and C) were selected and propagated to the next generations. DNA was extracted from each line, and PCR using primers specific for the 35S promoter (forward) and *S. pinnata/S. elata APS2* (reverse) was used to confirm the genetic transformation (Panel B). A total of 15 seedlings per line were propagated and tested for homozygosity.

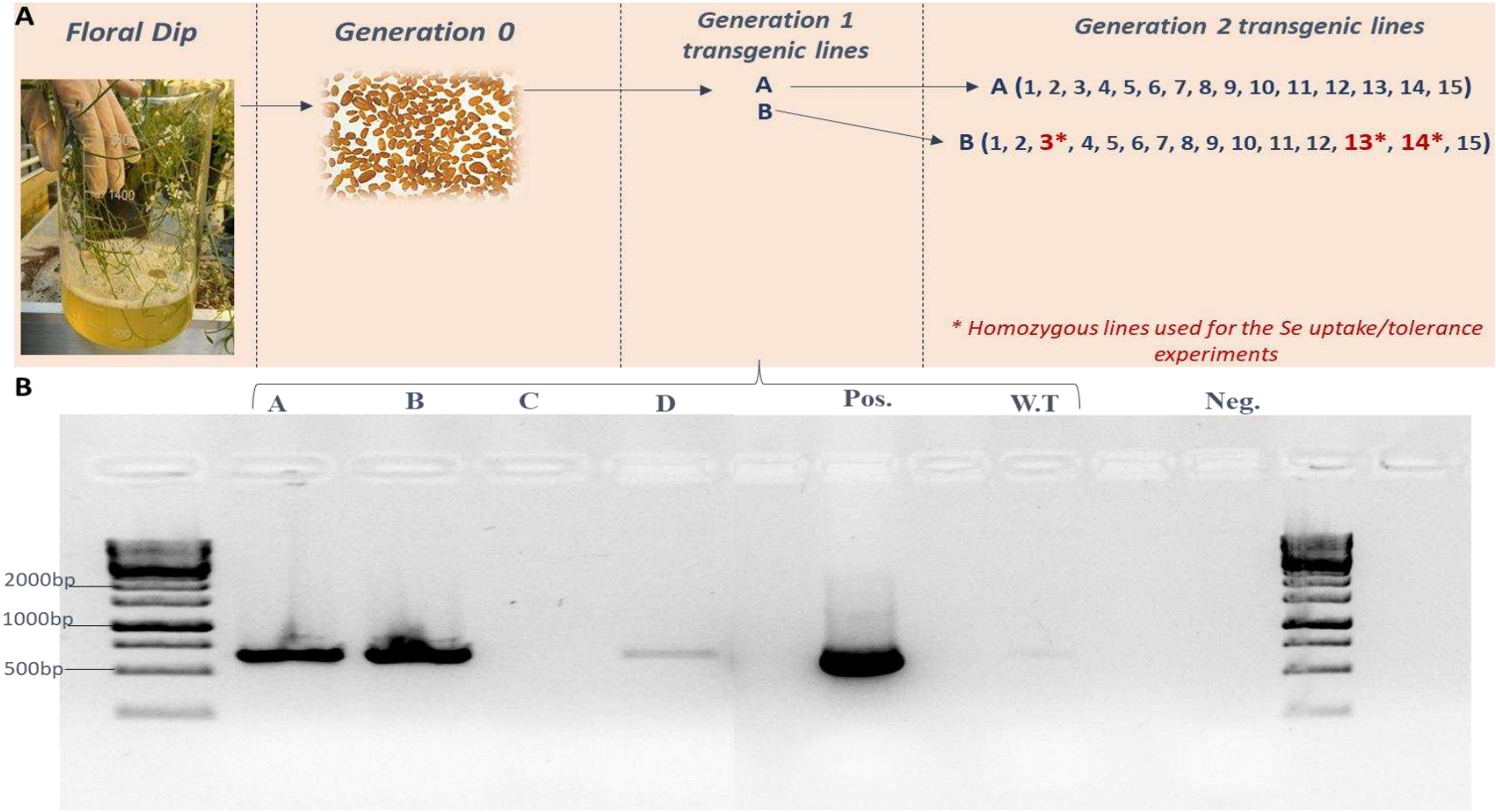

Figure 5-3. Schematic representation of the genetic transformation of *Arabidopsis thaliana* Col-0 W.T. plants with the *Stanleya elata* APS2 gene, via floral dipping using *Agrobacterium tumefaciens.* Panel A shows the transgenic lines for the first and second generations, the homozygous lines highlighted in red with asterisks (generation 2) were used for the Selenium tolerance and uptake experiments. A total of 900 seeds (~30 mg) from the generation 0 mother plant were grown on BASTA. The resistant seedlings (generation 1, lines A and B) were selected and propagated to the next generations. DNA was extracted from each line, and PCR using primers specific for the 35S promoter (forward) and *S. pinnata/S. elata APS2* (reverse) was used to confirm the genetic transformation (Panel B). A total of 15 seedlings per line were propagated and tested for homozygosity.

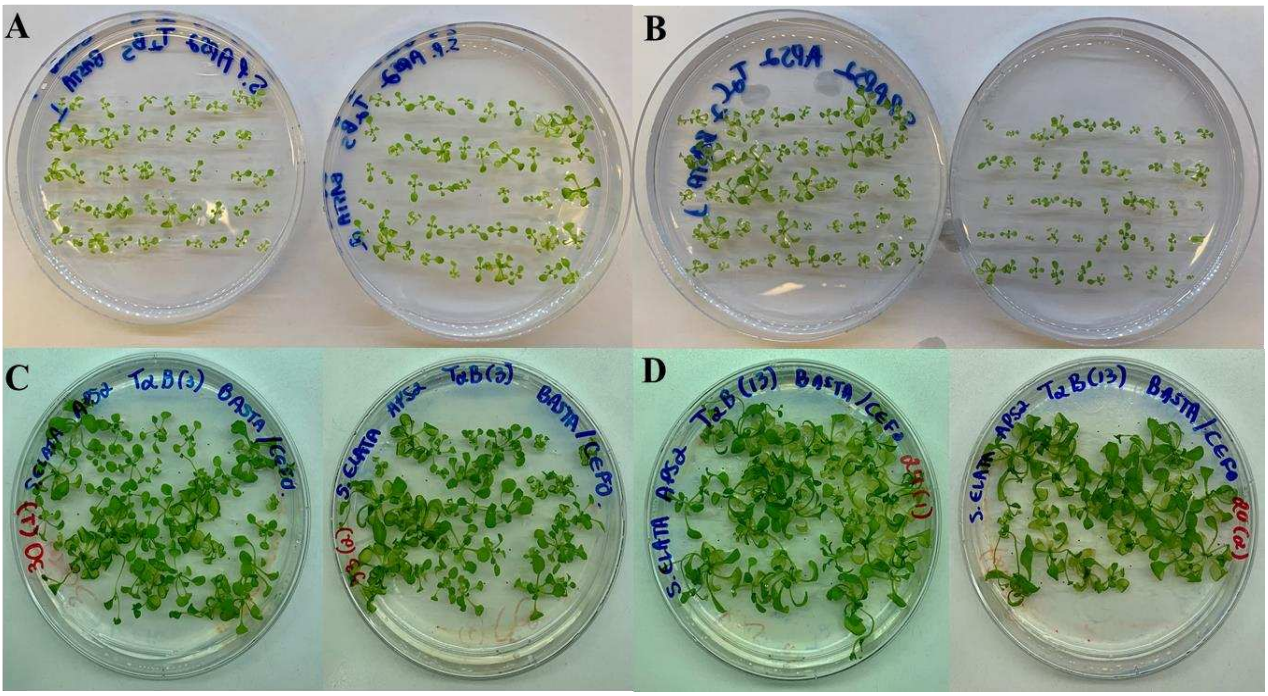

Figure 5-4. Example of homozygous lines selection on horizontal MS agar plates containing BASTA (glufosinate ammonium). A total of 100 seedlings (50 per plate) from each of the 15 generation 2 lines were grown on agar. The transgenic lines with 100% of survivors (homozygous, indicated in red in Figures 5-2 and 5-3), were further propagated and used for uptake and tolerance experiments. Panel A and B show the *A. thaliana* Col-0 WT transformed with the *S. pinnata* APS2 lines **B(5)** and **C(2)**, respectively. Panels C and D, show the *A. thaliana* Col-0 WT transformed with the *S. elata* APS2 lines **B(3)** and **B(13)**, respectively. Seedlings on plates A and B are nine days old. Plates C and D are 3 weeks old and ready to be transplanted into the soil for propagation.

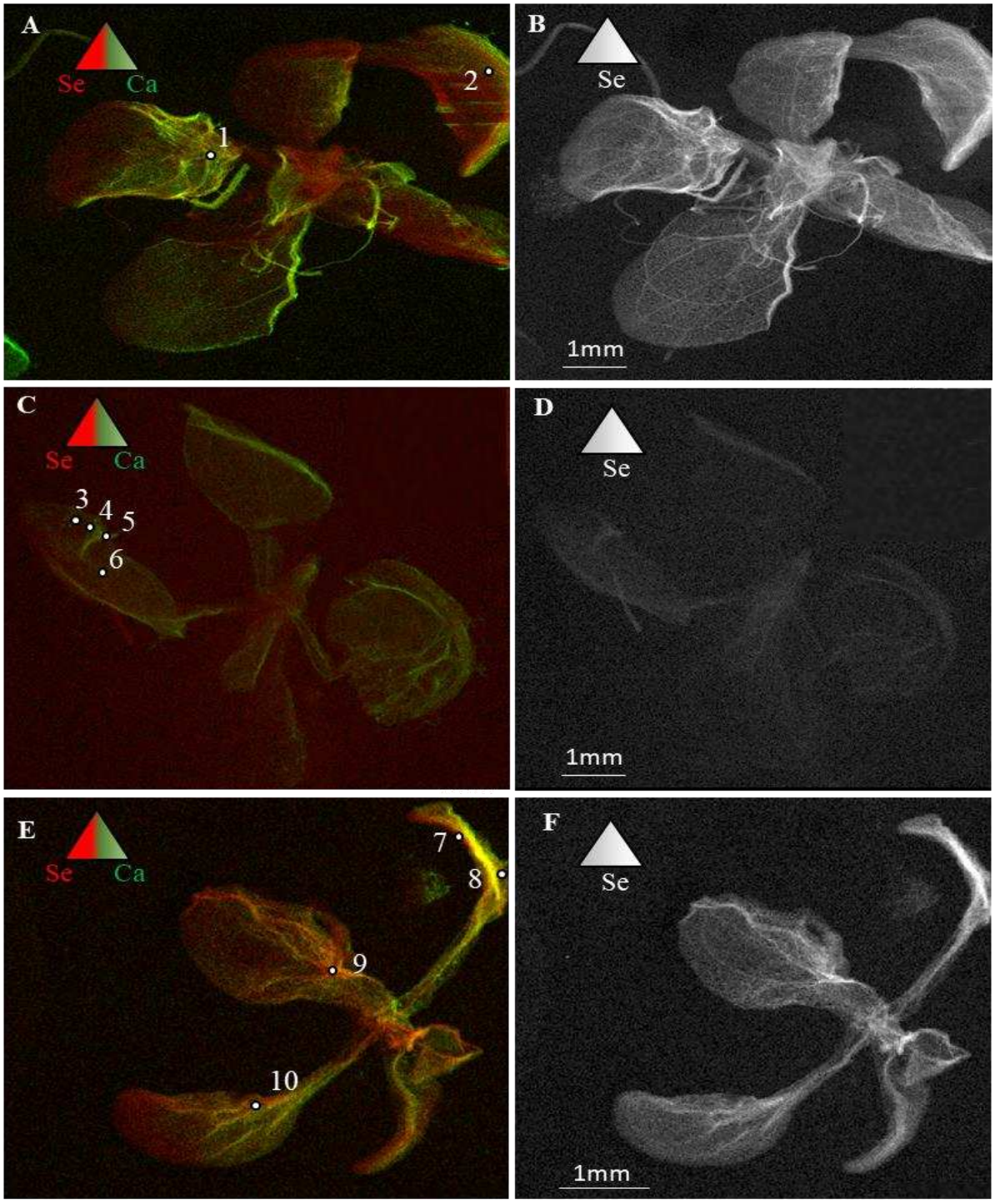

Figure 5-5. Micro X-ray fluorescence elemental distribution maps of *Arabidopsis thaliana* treated with 20 μm selenate for 7 days. Selenium is shown in red or white, and calcium in green. Panels A and B show a seedling of untransformed *A. thaliana* Col-o W.T.; Panels C and D show a seedling of *A. thaliana* Col-o W.T. transformed with the *S. pinnata APS2* gene; Panels E and F show a seedling of *A. thaliana* Col-o W.T. transformed with the *S. elata APS2* gene. Micro X-ray Absorption Near-Edge Structure (XANES) spot locations are shown as numbered white circles; speciation results are shown in Figure 8.

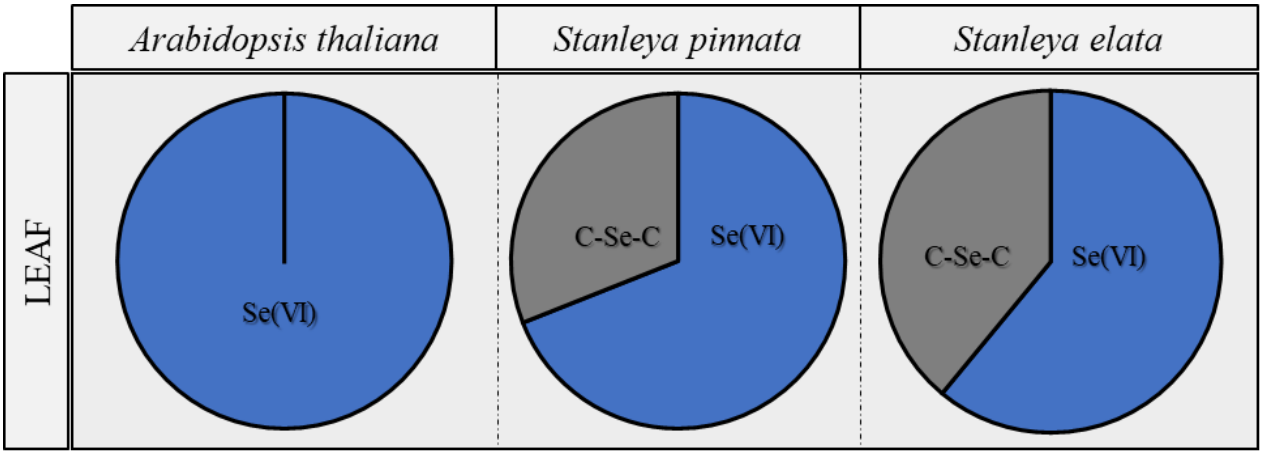

Figure 5-6. Selenium speciation as determined by least-square linear combination fitting of the Micro X-ray absorption near-edge structure (μXANES) spectra collected at the marked spots in the XRF maps (Figure 5). C-Se-C may correspond to the organic forms SeMet, and/or MeSeCys, which are indistinguishable by μXANES. Inorganic Se is represented as blue for Selenate (Se VI). Errors on fits are +/−10%.

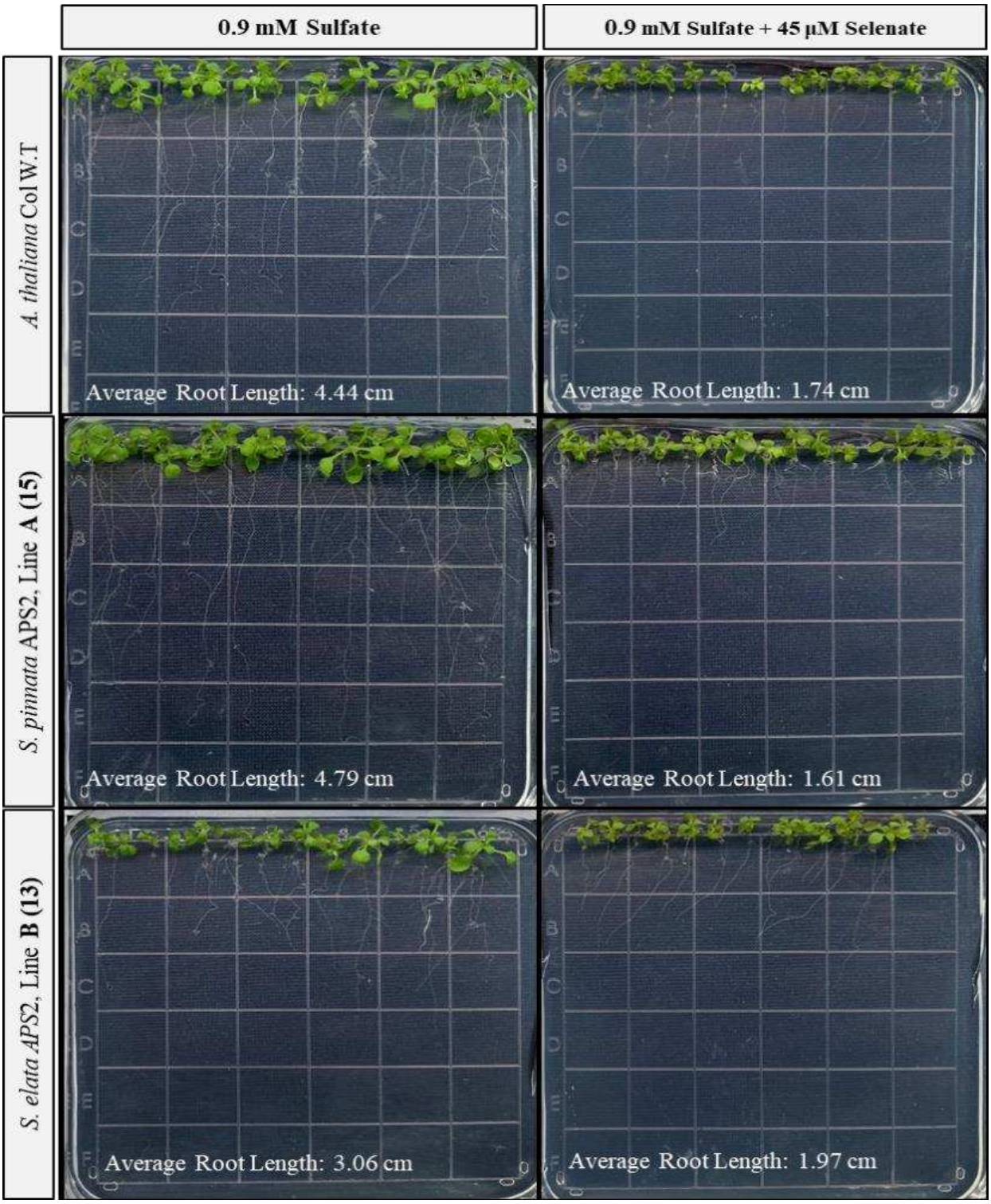

Figure 5-7. Seedling fifteen-days Se tolerance and accumulation experiment using vertical 0.5MS agar plates with or without 45 µM selenate. A visual representation of the data is shown in figure 5-8A (Root length). The values shown in each correspond to the averaged root length (cm) of three different plates per treatment. The transgenics have the *Arabidopsis thaliana* Col-0 WT background.

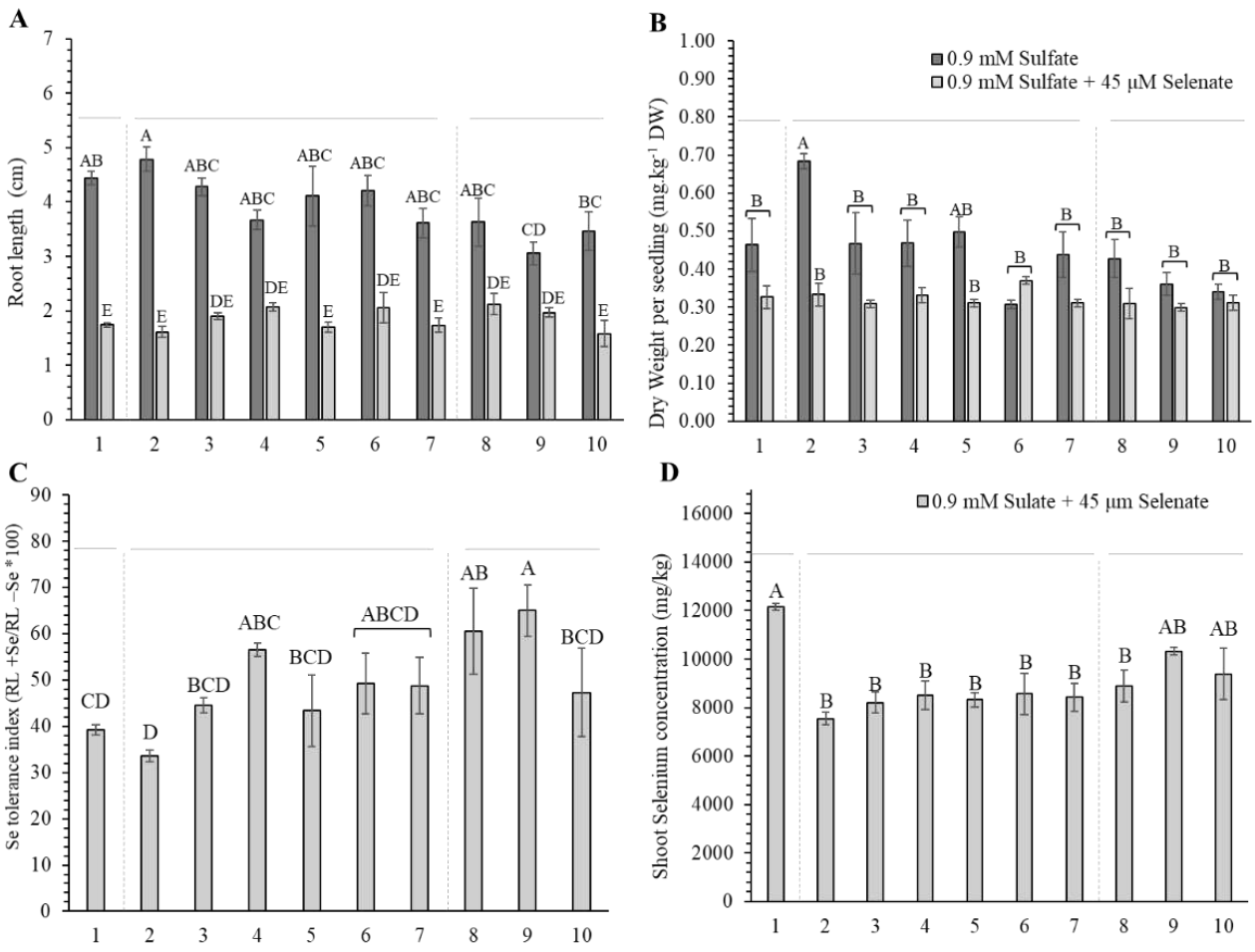

Figure 5-8. Fifteen-days Se tolerance and accumulation experiment using vertical 0.5MS agar plates with or without 45 µM selenate. A: Root length; B: dry weight per seedling; C: Selenium tolerance index (Root length at +Se/ Root length at -Se * 100); RL=Root Length. D: Shoot Se concentration. X-axis numbers represent different plant lines. **1=** Control *A. thaliana* Col-0 WT; **2=** *S. pinnata* APS2 line **A(15)**; **3=** *S. pinnata* APS2 line **B(5)**; **4=** *S. pinnata* APS2 line **B(11)**; **5=** *S. pinnata* APS2 line **C(2)**; **6=** *S. pinnata* APS2 line **C(3)**; **7=** *S. pinnata* APS2 line **C(9)**; **8=** *S. elata* APS2 line **B(3)**; **9=** *S. elata* APS2 line **B(13)**; **10=** *S. elata* APS2 line **B(14)**. The transgenics have the Col-0 WT background. Different letters above bars indicate statistically different means among transgenic lines ($P < 0.05$).

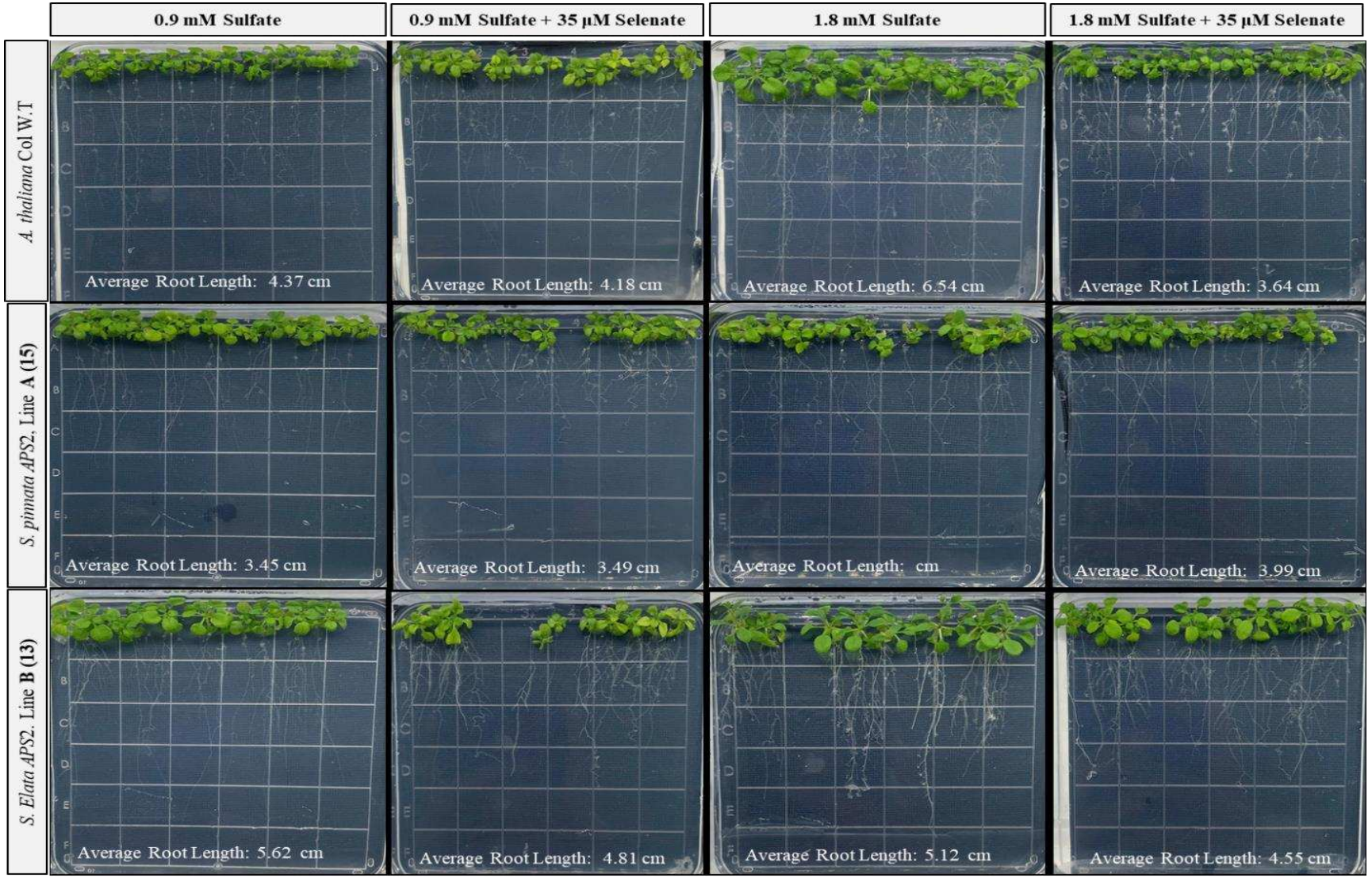

Figure 5-9. Seedling fifteen-days Se tolerance and accumulation experiment using vertical 0.5MS agar plates with or without 45 µM selenate. A visual representation of the data is shown in figure 5-10A (Root length). Values shown in each panel correspond to the averaged root length (cm) of three different plates per treatment. The transgenics have the *Arabidopsis thaliana* Col-0 WT background.

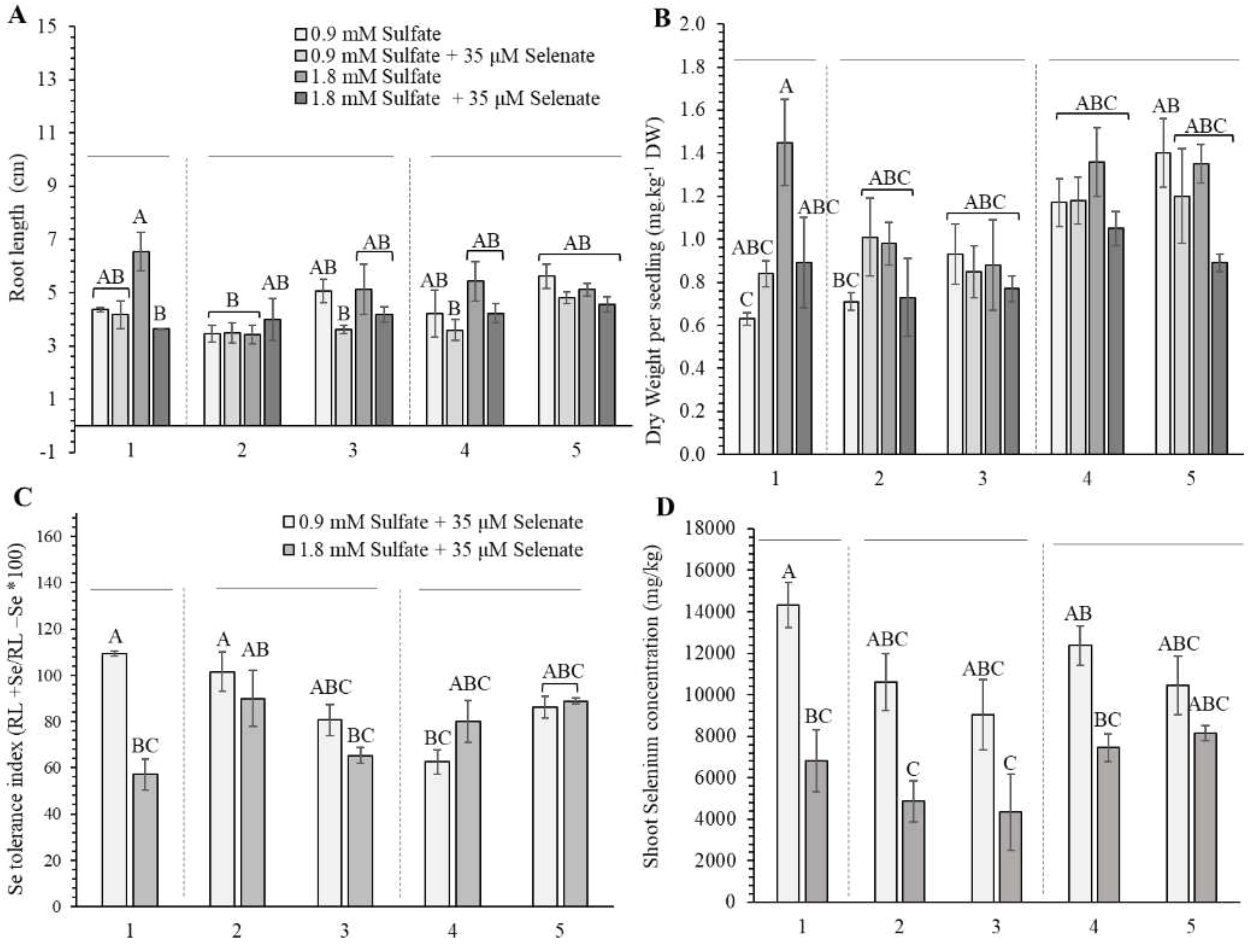

Figure 5-10. Fifteen-days Se tolerance and accumulation experiment using vertical 0.5MS agar plates with adequate or high Sulfur, in combination with or without 35 µM selenate. A: Root length; B: dry weight per seedling; C: Selenium tolerance index (Root length at +Se/ Root length at -Se * 100); RL=Root Length. D: Shoot Se concentration. X-axis numbers represent different plant lines. **1=** *A. thaliana* Col-o WT; **2=** *S. pinnata* APS2 line **A(15)**; **3=** *S. pinnata* APS2 line **C(9)**; **4=** *S. elata* APS2 line **B(3)**; **5=** *S. elata* APS2 line **B(13)**. Different letters above Standard Error bars indicate statistically different means among transgenic lines ($P < 0.05$).

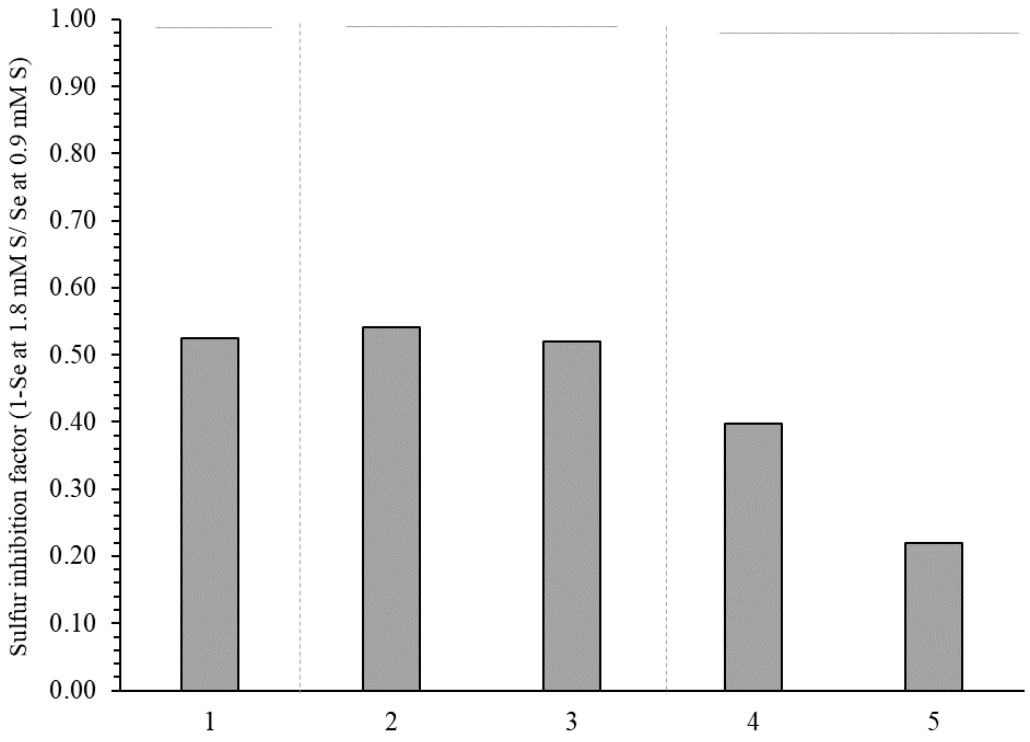

Figure 5-11. Selenium accumulation inhibition by sulfate (sulfate inhibition factor). The sulfate inhibition factor was calculated based on the data shown in figure 5-10D. The shoot Se concentration at 1.8 mM sulfate was divided by the shoot Se concentration at 0.9 Mm S. **1=** *A. thaliana* Col-o WT; **2=** *S. pinnata* APS2 line **A(15)**; **3=** *S. pinnata* APS2 line **C(9)**; **4=** *S. elata* APS2 line **B(3)**; **5=** *S. elata* APS2 line **B(13)**.

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