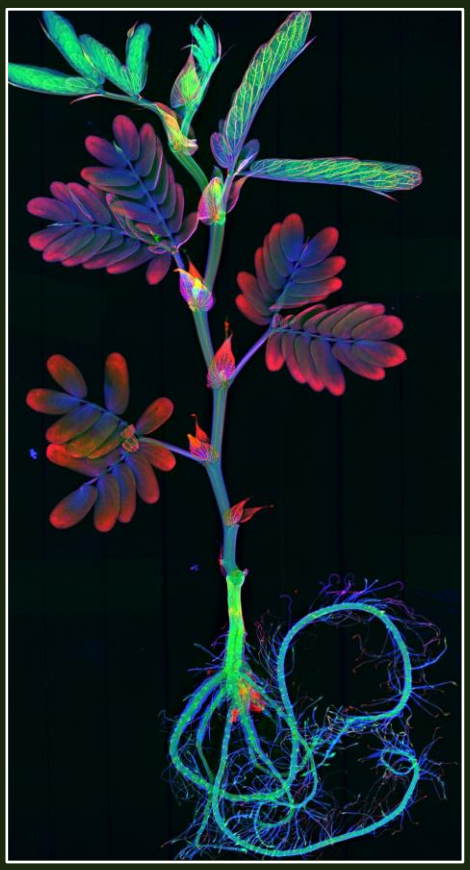
MSc. Research Practice

2023/2024

An essential worker?

Determining the physiological impact of selenium in hyperaccumulator plants

Cyril Stevens



Cover page image references:

Neptunia amplexicaulis (top): (Pinto Irish et al., 2021)

RNA strand (bottom left): Designed using DALL.E 2 generated February 21, 2024 using the prompt "a realistic oil painting of an RNA strand floating in a grey background"

Selenium concentration in *Neptunia amplexicaulis* (bottom centre): Maggie-Anne Harvey, personal communication

Selenium ingot (bottom right): Selenium (Cast ingot). (n.d.). Collect The Periodic Table. Retrieved February 22, 2024, from https://the-collectable-periodic-table2.mybigcommerce.com/selenium-cast-ingot/

Acknowledgements

I would like to express my sincere gratitude to Antony van der Ent, my main supervisor, whose guidance and expertise were instrumental in shaping this thesis. Special thanks to Maggie-Anne for her continuous support as my daily supervisor in the cultivation of plants and experimental design. I am also grateful to Jeroen van der Woude for his invaluable assistance in RNA isolation and experimental execution. Mirko Salinitro played a crucial role in germinating plants and contributing to the design of growing conditions. Louise Logie's expertise in photosynthesis measurements greatly enhanced the quality of this research. Finally, I extend my appreciation to the students and especially Abel Muller and Jacky To which helped me with the analysis of the result and whit whom I had many interesting discussions. Their collective input has enriched my academic experience and contributed to the success of this endeavour.

An essential worker? Determining the physiological impact of selenium in hyperaccumulator plants

Author: Cyril Bertrand Jacques Stevens.

MSc: Plant biotechnology – Specialisation: Molecular plant breeding and Pathology.

GEN79324 - MSc Research Practice Genetics.

Research proposal.

Main supervisor: Dr. Antony van der Ent.

Daily supervisors: Dr. Maggie-Anne Harvey, Jeroen van der Woude.

Examiners: Dr. Francisca Reyes Marquez, Dr. Antony van der Ent.

Laboratory of Genetics, Wageningen University

August 2023 – February 2024

Abstract

Selenium hyperaccumulators such as *Neptunia amplexicaulis* and *Astragalus bisulcatus* might be useful plants to resolve selenium malnutrition or excessive selenium concentration in soil. Their metabolism has been studied over the past years and much has been discovered. However, it is still unknown whether selenium is an essential element to the hyperaccumulator plants and if small selenium concentrations might improve the growth of those plants. To study those subjects, *A. bisulcatus* and *N. amplexicaulis* (selenium hyperaccumulator plants) and *Neptunia heliophila* (selenium tolerant plant) were grown in a controlled environment in which the selenium levels were regulated. The impact of different levels of selenium on the physiology of the plant was assessed. We noted that selenium concentration had a significant impact on the plant size, and not necessarily a negative one on hyperaccumulator plants. The high selenium accumulation negatively impacts the photosystem efficiency of the plant and might also reduce sulphur accumulation. Finally, we presented a novel method to deplete the plants from their physiological selenium by harvesting the very young leaves. Although the essential nature of selenium could not be confirmed or inferred, this study presents promising results and methods to further study this question.

Table of Contents

1. Introduction

1.1 Selenium, an essential element for animals and humans

Selenium (Se) is an essential mineral for many organisms. For instance, humans require Se (~60 µg/day) for the correct function of their metabolism (EFSA Panel on Dietetic Products & Allergies, 2014). Indeed, this element is essential to humans as it is required for selenoproteins to fold correctly (Rayman, 2000). Moreover, some selenoproteins have an antioxidant role as selenocysteine has a lower reduction state than cysteine (Byun & Kang, 2011). However, an opposite effect, a Se excess, can also occur as 400 µg/day can already cause symptoms of Se toxicity, which is known as selenosis (EFSA Panel on Dietetic Products & Allergies, 2014). Since Se is an analogue of sulphur (S), an excess Se will result in a replacement of the S by Se in amino acids (a.a.) which renders many proteins unfunctional (Frenkel et al., 1991). Consequently, many projects have looked into methods to control Se to prevent deficiencies or toxicity.

1.2 Hyperaccumulator plants, a solution for selenium deficiency?

Plants can be used to alleviate Se related problems. One of the most promising approaches to combat Se imbalances consists in using Se hyperaccumulators plants. These plants are capable of concentrating a very high amount of this Se (Beath et al., 1939). It should be noted that Se levels will vary greatly depending on the environment in which the plants are cultivated. Currently, a lot of possible uses for Se hyperaccumulators are being tested. The main applications concern nutrient supplement production, crop biofortification and soil phytoremediation (Zhu et al., 2009). The first two techniques aim at increasing the Se concentrations for human and animal consumption while phytoremediation intends to reduce Se from soils to prevent toxicity. Nutrient supplements (e.g., powder pills of hyperaccumulator plant parts) could be used directly for human consumption if their Se daily intake is too low (Zhu et al., 2009). Similarly, biofortification aims at using the knowledge of how Se hyperaccumulator plants function in order to breed cultivated crops to accumulate more Se and thus enrich the Se in food in general (Nestel et al., 2006). Finally, phytoremediation would be a solution to reduce a soil's Se levels when they are too high and can cause a risk of toxicity for both humans and the environment (Reichenauer & Germida, 2008). This could be done through the cultivation of Se hyperaccumulators on soils with high Se levels and harvesting the plant later, thus reducing Se in the soil. To use those plants efficiently for those different roles, it is important to understand the Se metabolism in hyperaccumulator plants.

Currently, of the hyperaccumulator species examined for their metabolism of Se, *Astragalus bisulcatus* and *Stanleya pinnata* are by far the most studied, while *Neptunia amplexicaulis* is also investigated. Indeed, they were among the first plants to be discovered as accumulating high Se quantities and designated as "indicator plants", i.e., plants indicating the high Se concentrations in the soil. They are found in Midwest region of the United States of America, in the location of a dried-up sea from the Cretacean era (i.e., Western interior seaway) which is the probable reason for high soil Se levels (Beath, 1936; Beath et al., 1939; Stanley, 2005). *Neptunia amplexicaulis* is a plant belongs to the Fabaceae family and can only be found in Central Queensland, Australia. This area is also very rich in Se and is also found in the location of a dried-up sea, the Eromanga sea (Dettmann et al., 1992). The high Se levels have been theorised to be the reason for which *Se* hyperaccumulators have adapted to not only prevent Se toxicity but also to benefit from high Se (Pinto Irish et al., 2021).

1.3 Plant selenium metabolism

Several stages in the Se metabolism have already been discovered. In the soil, plants will assimilate Se mainly in selenate form (SeO_4^{2-}), through sulphate transporters, (SULTR1;2 and SULTR1;2) or in selenite form (SeO_3^{2-}), through phosphate and silicon transporters. It should be noted that other chemical forms such as elemental Se (Se^0) and organic Se can also be assimilated, although to a much lower extent (Chauhan et al., 2019; Dinh et al., 2019). In hyperaccumulator plants, the uptake mechanism is similar, however, their SULTR proteins seem to be able to discriminate between S and Se and have a preference for the latter (Cappa et al., 2015). Furthermore, the SULTR genes are constitutively overexpressed in hyperaccumulators compared to non-hyperaccumulators, resulting in a very high concentration of both Se and S in their tissues (Schiavon et al., 2015). Then, the selenate/selenite will be "assimilated" either in the root cells or the mesophyll cells (van der Ent et al., 2023). There, SULTR1;2 or SULTR1;1 transport the metabolite in the cells which is then taken in the plastids by SULTR1;3 (Schiavon & Pilon-Smits, 2017). In this organelle, SeO_4^{2-} is reduced to APSe, then to selenite and finally Se^{2-} by several proteins (Fig. 1). Afterwards, cysteine synthase produces Selenocysteine (SeCys) by binding Se^{2-} with a serine (Schiavon & Pilon-Smits, 2017). In the Se hyperaccumulator plant species *A. bisulcatus a*, the protein selenocysteine methyltransferase will produce Methyl-SeCys which will then produce dimethyl-diselenide (DMDSe). *Stanleya pinnata* will produce methyl-diselenide (MDSe) through several metabolic steps (Fig. 1). Those molecule are volatile and will be released in the air through the stomata (Schiavon & Pilon-Smits, 2017). In *N. amplexicaulis,* on the other hand, MDSe or DMDSe are not produced (Peterson & Butler, 1967). Consequently, contrary

to the two former plants species, *N. amplexicaulis* releases only very small amount of Se in the atmosphere but holds on to most of the element (van der Ent et al., 2023). However, contrary to the well-established knowledge of cellular mechanisms for Se accumulation, the subsequent metabolic role of the element in plants is still unclear.

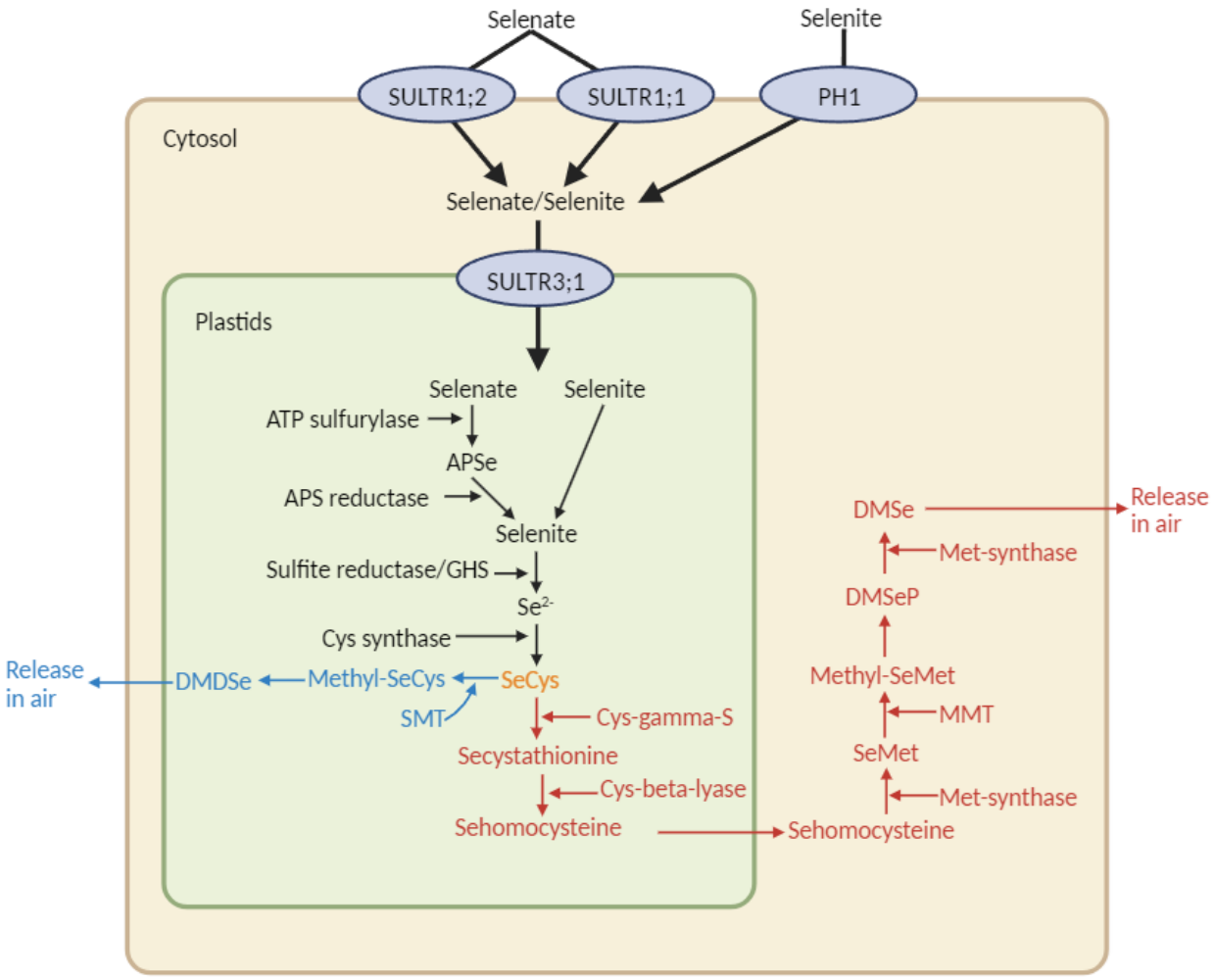


Figure 1: Schematic representation of selenium in plant cells.

The orange font indicates where the metabolism ends for *N. amplexicaulis*. The bleu font indicates metabolism present in *A. bisulcatus*. The red font indicates metabolism present in most plants, including *S. pinnata*. SULTR, sulphate transporter. PH1, phosphate transporter. ATP, adenosine triphosphate. APSe, adenosine phosphosulphate. APS, adenosine phosphosulphate. GSH, glutathione. (Se)Cys, (seleno)cysteine. SMT, selenocysteine methyltransferase. DMDSe, dimethyldiselenide. (Se)Met, (seleno)methionine. MMT, methyl-methionine methyltransferase. DMSeP, dimethylselenoproprionate. DMSe, dimethylselenide. Adapted from (Schiavon & Pilon-Smits, 2017).

1.4 The inquiry of selenium essentiality

The physiological importance of Se on plant metabolism is still unknown. An important characteristic which is still unclear concerns the essentiality (i.e., an element which is strictly required for an organism to complete its life cycle) and the growth promoting effect of Se in plants. In the mid-20th century, research was conducted on Se hyperaccumulators (mainly *A. bisulcatus*), newly discovered in central and western USA, to determine the properties of Se in the species' metabolism. Beath *et al*. (1936), mapped the presence of hyperaccumulators in relation to the Se concentration in soils and discovered that both distributions correlated quite strongly. Thus, the researchers theorised that the Se might be an essential element for the plants (Beath, 1936). Later, Trelease & Trelease (1938) conducted several experiments on *Astragalus pattersonii* and *Astragalus racemosus* and observed that Se had a beneficial effect on the plant's growth which supported the idea that Se might be an essential element for hyperaccumulators or even plants in general (Trelease & Trelease, 1938). However, Broyer *et al.* (1972) noticed that phosphate toxicity symptoms could be observed on the results from the study of Trelease & Trelease (1938) and conducted a similar experiment in which the phosphate concentrations were decreased. The results of this later study did not show any sign of growth promotion, and the growth promoting effect observed by the former study was theorised to be the result of selenite preventing the plant from assimilating too much phosphate, as selenite is assimilated through phosphate transported proteins (Broyer et al., 1972a, 1972b). Thus, the study of Trelease & Trelease (1938) along with the idea that Se might be an essential element was invalidated. Nevertheless, other experiments were later conducted and did show a correlation between Se concentrations and growth in some plants (Pilon-Smits et al., 2009). Since then, research aiming to identify if Se is essentiality in plants was left abandoned and has thus not been answered properly. Consequently, it is still unclear whether Se could be indeed essential or not to (some) plants.

1.5 Knowledge gap

For the purposes of this study, it is important to understand what the differences between an essential element and a beneficial (growth promoting) element is. Arnon & Stout (1939) (Arnon & Stout, 1939) defined the criteria which should be met for an element to be considered essential: (I) A given plant must be unable to complete its lifecycle in the absence of the element; (II) The function of the element must not be replaceable by another element, and (III) The element must be directly involved in plant metabolism (e.g., as a component of an essential plant constituent such as an enzyme) or it must be required for a distinct

metabolic step (*e.g.,* an enzyme reaction). On the other hand, an element is considered beneficial element if it stimulates growth but either: (I) is not essential according to the three characteristics mentioned above; (II) it is essential but only for certain plant species, or (III) only essential in specific conditions (Broadley et al., 2012). Consequently, as of this date and according to those definitions, Se is considered a beneficial element as it improves growth but does not seem to be required for the completion of a plant's life cycle and no direct action in the metabolism has been found (Brown et al., 2022).

In summary, it is currently not clear whether Se is an essential element for hyperaccumulator plants. Furthermore, even though it is widely accepted that Se is beneficial to plant growth, it is not clear yet what the optimum Se concentrations are for efficient plant growth for specific plant species and how they react to varying Se levels.

1.6 Objectives & research questions

Therefore, in this project, I undertook the following:

(I) investigating whether Se is an essential mineral for Se hyperaccumulators and, if so, what the minimum Se requirement is for the plants and what are the symptoms of Se deficiency.

(II) the potential growth promoting effect of the Se on plants will also be studied and an optimum Se concentration will be investigated.

(III) I will study the impact of Se concentration on the genes involved in the Se metabolism (e.g., SULTR1;1, SULTR1;2, Cysteine synthase).

2. Materials & Methods

2.1 Seed germination

2.1.1 Germinating *Astragalus bisulcatus* seeds:

In total, 75 seeds were used for germination (52 + ~50% to account for non-germinating seeds). The seeds were punctured with a razor blade. They were immersed in tap water and allowed to soak for approximately 16 hours until they absorbed water and increased in size.

Plastic boxes (emptied 1000 µL pipette tips) were filled to half with dampened sand. One box was used for 12 seeds. The seeds were placed on the sand. The seeds were covered by 2mm of sand. The sand was dampened with 2mm of water dropped with a 1000 µL pipette. These boxes were transferred to a fridge set at 4°C for 3 days for stratification. The boxes were transferred to a germination room set at 25°C, 50% relative humidity, 16/8 hrs of light and a photosynthetic photon flux density 200–250 µmol m^2 s^{-1} light. After this period, the seeds were ready for use in experiments.

2.1.2 Germinating *Neptunia amplexicaulis* seeds:

60 seeds were used for germination (52 + ~33% to account for non-germinating seeds). The seeds were punctured with a razor blade. They were immersed in tap water and allowed to soak for approximately 16 hours until they absorbed water and increased in size.

Plastic boxes (emptied 1000 mL pipette tips) were filled to half with dampened sand. One box was used for 12 seeds. The seeds were placed on the sand. The seeds were covered by 2mm of sand. The sand was dampened with 2mm of water dropped with a 1000 µL pipette. The boxes were then transferred to a germination room set at 25°C, 50% relative humidity, 16/8 hrs of light and a photosynthetic photon flux density 200–250 µmol m^2 s^{-1} light. After this period, the seeds were ready for use in experiments.

2.1.3 Germinating *Neptunia heliophila* seeds:

60 seeds were used for germination (52 + ~33% to account for non-germinating seeds). The seeds were punctured with a razor blade. They were immersed in tap water and allowed to soak for approximately 16 hours until they absorbed water and increased in size.

Plastic boxes (emptied 1000 µL pipette tips) were filled to half with dampened sand. One box was used for 12 seeds. The seeds were placed on the sand. The seeds were covered by 2mm of sand. The sand was dampened with 2 mm of water dropped with a 1000 µL pipette. The boxes were then transferred to a germination room set at 25°C, 50% relative humidity, 16/8

hrs of light and a photosynthetic photon flux density 200–250 µmol m^2 s^{-1} light. After this period, the seeds were ready for use in experiments.

2.2 Hoagland's medium preparation

An adapted ½ Hoagland's medium was prepared according to the recipe (Tables 2–3). 0.39 g/L (2 mM) MES pH buffer was added to the preparation and the pH was set to 5.8 by addition of KOH. The medium was kept in a cold and dark place until use. Batches of 60 L of ½ Hoagland's solution were prepared each week. Once the medium was poured in the pots, sodium selenate (0.1 M) was added to the ½ Hoagland's medium according to the experiment treatment (Table 4).

Table 2: Molar concentration of the stock solution used for the Hoagland's medium. A single stock solution was prepared for the combined micronutrients.

	Stock (g/L)	Stock (M)	Element
Macronutrients			
KNO_3	101.10	1.00	N/K
$Ca(NO_3)_2 \bullet 4\ H_2O$	236.15	1.00	Ca/N
$NH_4H_2PO_4$	115.03	1.00	N/P
$MgSO_4 \bullet 7\ H_2O$	246.47	1.00	Mg/S
Micronutrients (1 X)			
KCl	0.075	1.00	Cl
H_3BO_3	1.546	25.00	B
$MnSO_4 \bullet 4\ H_2O$	0.446	2.00	Mn
$ZnSO_4 \bullet 7\ H_2O$	0.575	2.00	Zn
Other			
Fe(Na)EDDHA	8.704	20	Fe/Na

Table 3: Volume of stock solution required to prepare 1 l of ½ Hoagland's medium.

Stock solutions:	ml
KNO_3	3.0
$Ca(NO_3)_2 \bullet 4\ H_2O$	2.0
$NH_4H_2PO_4$	1.0
$MgSO_4 \bullet 7\ H_2O$	0.5
Fe(Na)EDDHA	0.5
1 X Micronutrients	0.5

Table 4: Volume of Sodium selenate (0.1M) added to the hydroponics medium according to the selenium (Se) treatment.

Selenium concentration (µM)	Volume of Sodium selenate added (ml)	
	0.75 l pots	1 l pots
0	0.000	0.000
10	0.075	0.100
15	0.100	0.150
20	0.150	0.200
40	0.300	0.400
80	0.600	0.800

2.3 Hydroponics setup

The room used for the hydroponics experiment had the following conditions: 16/8 hours of light and dark cycle, 200-250 µmol of light intensity, 25/20 °C, ~50% relative humidity.

The setup for the hydroponics experiment involved the following steps:

To create an even surface for the experiment, a polystyrene patch was laid and secured with tape on the bench. In cases where several patches were used, they were fixed together using tape. A plastic sheet was then placed over the polystyrene and fixed to it with tape.

The plastic pots for the hydroponics experiment were arranged and evenly spaced on top of the plastic sheet.

One 1-liter (13 cm high by 12.2 cm wide) plastic pot with a fixable lid (Sirclecup) was used for three plants of the same species. The lid had four evenly spaced holes, three 12 mm holes for the plants and one 8 mm hole for the air tube. Five pots were aligned in each column.

A plastic tube, for air circulation, extended from the bottom of the pot, where a water stone was inserted into the tube, then passed through the lid hole and connected to a faucet.

A five-faucet complex was present for each column and was fixed to the wall. Each faucet complex was linked by a plastic tube to the faucet complex of the adjacent column. The faucets on the sides were connected to an air 400 pump (EHEIM GmbH & Co. KG.) via another set of plastic tubes.

The pots were subsequently filled with a 1/2 Hoagland's medium + selenate stock solution. The air pump was activated, and the air flow was adjusted using the faucets to create a gentle current, ensuring it did not harm the plant roots.

The plantlets inserted through a rock-wool patch of a similar size to the plant's lid holes, the plant was then placed in one of the 12 mm holes. Care was taken to ensure that the roots were in contact with the medium.

The hydroponics medium and the selenium (Se) was fully replaced weekly. The plants were monitored daily for any signs of disturbances and the experiment set up was cleaned from dead leaf tissue.

2.4 Work package 1 – Selenium growth promotion

36 pots were used in total for this experiment. 12 pots were assigned to each species which were divided into six levels of Se (table 3). Two pots were assigned for each Se treatment. The position of the pots was randomly assigned using the "RAND()" formula in Microsoft Excel® in a complete randomised design. The plants were transferred from their germination box to their pots after 7-14 days. The plants were then left to grow for 6 weeks until the measurements were made.

2.5 Work package 2 – Selenium essentiality

18 pots were used for this experiment. 6 pots were assigned to each species which further divided into two levels of treatment (Control, Se deficient plants). The control plants were provided with a bit of Se, the Se deficient plants were grown in a Se depleted medium. After

four weeks of growth, the very young leaves of the plants were picked so as to reduce the Se in the plants. The plants were monitored for any signs of deficiency. After two additional weeks of picking their young leaves, the measurement phase began.

2.6 Measurements:

2.6.1 Measurement of the photosynthetic capacity:

This measurement was conducted at the end of the growing period before the other measurements. The multispec (PhotosynQ Inc.) was used. The "Photosynthesis RIDES 2.0" protocol was used. The measurements were conducted on leaves from a fully mature composed leaf.

2.6.2 Measuring plant fresh weight:

The roots of the plants which had to be measured were untangled carefully using tweezers. The plant was transferred to a paper towel to take the water. The plant was then sectioned at the root crown using scissors. Each part (root and shoot) was weight using a precision scale which had been balanced prior.

2.6.3 Harvesting material for the qRT-PCR experiment:

2ml screw cap Eppendorf tubes (Qaigen Inc.) were labelled and filled with two 3mm or five 2mm zirconia Yttria beads (LabTIE International). Shoot and root samples were harvested and placed in separate tubes for each plant. The tissue material corresponded to 15-50 mg of fresh plant material. The samples were then flash frozen in liquid nitrogen and placed in a freezer (-80°C) until further use.

2.6.4 Measuring the Se concentrations:

The root and shoot tissues were placed in a different labelled 95 by 145 mm carton wage envelope with a gummed flap (Quantore B.V.). The envelopes were then placed in a drying oven at 60°C for 4 to 6 days. Once the samples had been completely dried, they were transferred to a 2ml labelled 2ml Eppendorf tube with a screw cap (Qaigen Inc.). Two 3mm zirconia yttria beads (LabTIE International) had previously been added to the tubes. The samples were then crushed using a Retsch MM 400 mixer mill set at 30 fzs for 90 sec. The samples were then transferred on a 6 µm polypropylene thin film (Chemplex Industries Inc.) which had been fixed with a 0.5 x 5 mm plastic ring (Chemplex Industries Inc.) on a custom XRF sample holder. The ground tissue was then covered by a second plastic film, fixed to the sample holder by a second plastic ring, to squeeze and evenly spread the powder over the measuring area and between the two plastic films. The XRF analysis of the plant and soil

powdered material and the soil extracts was performed using a Z-Spec JP500 and E-lite instruments (Z-Spec Inc.). These instruments use monochromatic X-ray fluorescence excitation at 17.48 keV and 4.5 keV to analyse elements Z = 11 (Na) to Z= 21 (Sc) and Z = 15 (P) to Z= 39 (Y) on the K-lines and up to Z= 92 (U) on the L-lines with optimum sensitivity for elements Cu–Se and Hg-Tl-Pb with LODs ranging from 0.009–0.025 mg kg^{-1}. Samples were analysed for 30 sec. in leaf mode.

2.7 Work package 3 – RNA isolation methods

Several RNA isolation methods were performed to test for best yield. Initially, the CiAR isolation method was used (Oñate-Sánchez & Verdonk, 2021). The RNA easy plant isolation kit was also performed. Finally, a CTAB based method (combining the Acosta-Maspons et al., (2019) and the TRIzol (Invitorgen) method), developed by Dr. Subash Rai, was performed (Acosta-Maspons et al., 2019). The samples were either from flowers, leaf or root tissue. The methods were tested on samples which had either been ground directly in a 1.5 ml safe lock Eppendorf tube (Qaigen Inc.) using a Pellet pestle Eppendorf (Dulis Belgium), ground using the mixer mill MM 400 (Retsch Inc.) set at 30 fzs for 90 sec., or hand ground using a mortar and pestle. After each RNA isolation methods, the yield and quality of the isolation was tested using the DS-11 Series Spectrophotometer/Fluorometer (DeNovix Inc.). If a sufficient yield was obtained (>25 ng), the RNA was dyed using a gel loading dye purple 6X and was the migrated on an 1% agarose gel + 15µg Ethidium bromide. The procedure was set at 120 volts for 60 min using an electrophoresis machine (Biorad). Then, a picture of the gel was taken using the Geldoc programme.

2.8 Statistical analysis

The phenotypic data and all statistical data were analysed and interpreted using the R® software with the packages "ggplot2" to generate graphs, "agricolae" and "PMCMRplus" for the statistical analysis and "tidyverse" and "data.table" for data visualisation. The plant weight from the same pot was summed together to calculate the plant weight of a single pot and reduce the impact of competition between plant of the same pot (i.e., 5 in this experiment). Those calculations were done using Microsoft Office Excel® software. To determine if the plant line differed significantly for the relevant traits, a Kruskal-Wallis followed by a Conover's test was performed. To determine the relation between the measured metabolites and the average larva weight a Spearman's rank correlation coefficient test was conducted.

3 Results

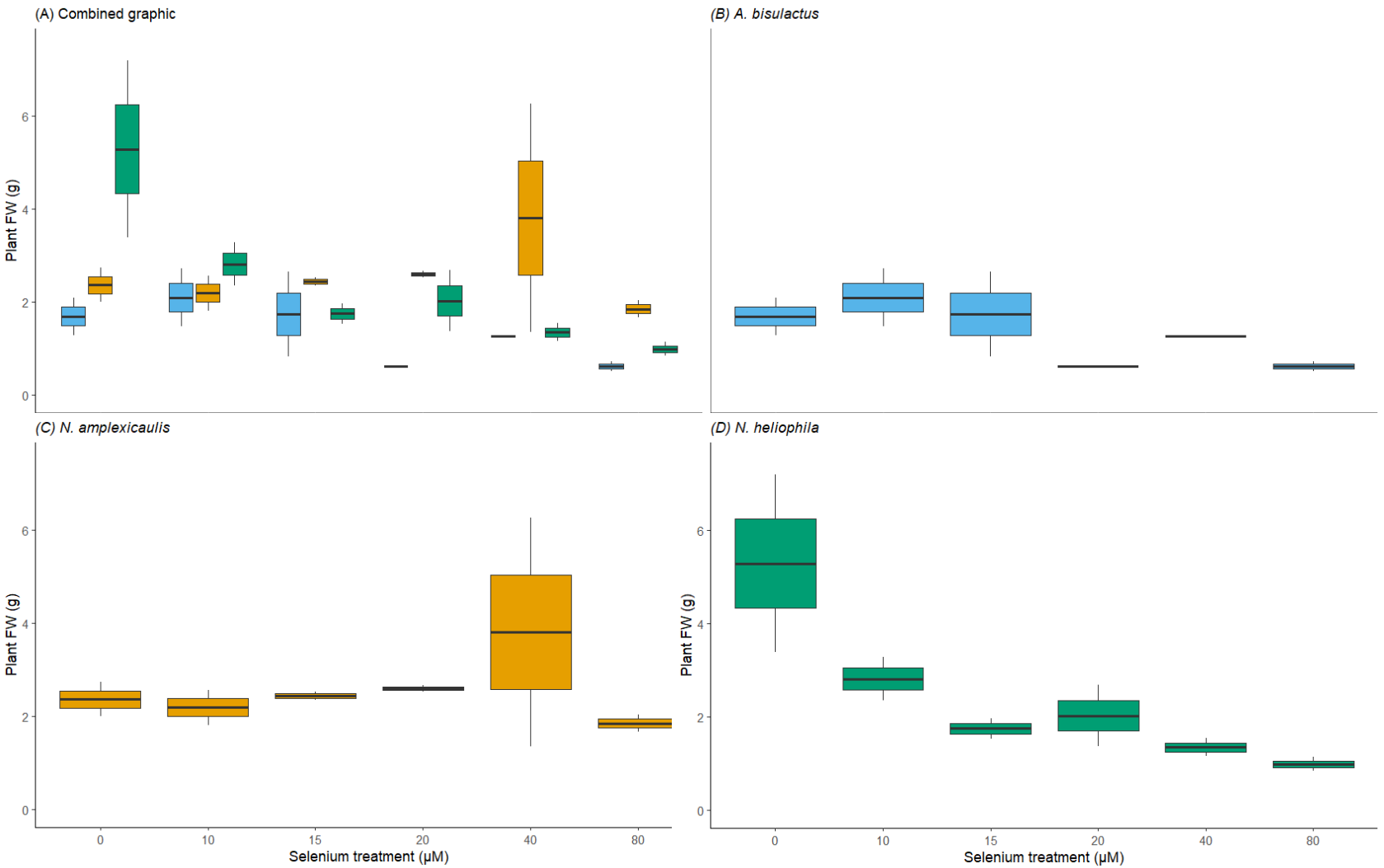
3.1 Impact of selenium on growth

To determine the impact of selenium (Se) concentration in the environment on the growth and the physiology of Se hyperaccumulator plants, three plant species, two hyperaccumulators and one tolerant plant (*Astragalus bisulcatus*, *Neptunia amplexicaulis* and *Neptunia heliophila* respectively), were grown for six weeks in hydroponics solutions with varying concentration levels of Se. The plants were grown in a controlled environment. Once the period had ended, the photosynthetic capacity of the plant, their weight and their physiological Se concentrations was measured. Root and shoot samples have also been harvested for qRT-PCR experiments. To reduce the impact of competition between plants growing in a same pot, the weight of the plants growing in a single pot was summed, which reduced the variability of the trait. However, it also reduced the number of replicated for this measurement to two.

3.1.1 The concentration of Se has a significant impact on the plant weight:

Se has a more negative impact on plant size on the tolerant plants than on the hyperaccumulator plants. The impact of the Se treatment is presented in figure 2. At low Se, the weight of the hyperaccumulators is lower than in *N. heliophila*. However, once Se increases, the weight of *N. heliophila* drops while the weight of the hyperaccumulators stays relatively stable. Thus, at ≥15µM of Se the weight of *N. amplexicaulis* is higher than that of *N. heliophila*. The same observation cannot be made for *A. bisulcatus*, even though the size of the plant is relatively stable, it reduces after 15µM.

Small concentrations of Se could have a positive impact on the hyperaccumulators weight. The optimum Se level for *A. bisulcatus* is the 10 µM, after that, the size of the plant decreases as Se increases. It should be noted however that, due to low germination rate, only one pot was used for Se concentrations 20 µM and 40 µM for *A. bisulcatus*. This might skew the results in relation to the optimum Se level for this species*. Neptunia* amplexicaulis seemed to have an optimum Se level at 40 µM. However, this level presents some variability due to a pot having only a single plant left alive (the other two had died soon after the transplant) at the moment of the measurement. Consequently, this level of Se still seems to yield the heaviest *N. amplexicaulis* plants. A Kruskal-Wallis' test did not reveal any significant differences between the treatments. This was probably caused by the low number of replicates (two per species per Se level).



<u>Figure 2</u>: The concentration of selenium in the environment increases the selenium concentration within the plant. The plants were grown in different levels of selenium for 42 days (6 weeks), after which, the plant weight was measured on the plants. A Kruskal-Wallis test did not reveal a significant impact of the selenium treatment on this measurement. The colours panel indicate specific plant species, bleu represents *Astragalus bisulcatus*, orange represents *Neptunia amplexicaulis*, green represents *Neptunia heliophila*. (A) Combined graph. (B) *Neptunia amplexicaulis* only. (C) *Neptunia amplexicaulis* only. (D) *Neptunia heliophila* only.

3.1.2 The Se treatment worked:

The physiological concentration of Se in the plants increases as the Se increases in the environment (Fig. 3). A Kruskal-Wallis' test revealed a significant relationship between the treatment and the Se concentration of plants (p-value = 2.575e-10). This observation can be made in all the three species but, the relationship is not linear. The physiological concentration of Se does not increase by the same amount as the environment Se does. Furthermore, there are some differences between each of the species. Indeed, the two hyperaccumulators plants seem to accumulate Se more than the Se tolerant plant. *Neptunia heliophila* has constitutively less Se than the hyperaccumulators. It should also be noted that *N. amplexicaulis* has a high constitutive level of Se compared to the other species. Indeed, at 0 µM of Se, *N. amplexicaulis* plants have a mean physiological Se concentration of 249 mg/kg while *A. bisulcatus* and *N. heliophila* have 24.33 and 0.68 mg/kg of Se respectively. I should also be noted that more variability can be observed for this trait in the hyperaccumulator plants than in *N. heliophila*. This is especially evident in *A. bisulcatus*, where the significance groups overlap over more treatments than in the two *Neptunia* species.

3.1.3 The physiological sulphur (S) concentration:

The physiological S concentrations are not significantly impacted by the Se treatment (Fig. 4). Indeed, A Kruskal-Wallis' test did not reveal any significant impact of the Se treatment on the plant's S concentrations. Nevertheless, it should be noted that a dip in S can be observed in the three species at the 80µM of Se. The S levels of *A. bisulcatus* are higher than for the two *Neptunia* species. *Neptunia heliophila* and *Neptunia amplexicaulis* had similar levels of S.

3.1.4 The Se treatment impacted the efficiency of the plant's photosystem:

The increase of Se seemed to have a negative impact on the efficiency of photosynthesis in the plant species (Fig. 5). This can be observed for *A. bisulcatus* for which the mean value decreases continuously as Se increases (except for concentration 40µM). Indeed, the photosynthetic capacity of *A. bisulcatus* is lower than the two other species. This might be constitutive of that species, or this might be caused by the growth conditions which had a negative impact of the plant. *Neptunia amplexicaulis* on the other hand has a high ΦpsII compared to the other plants. It is also relatively stable as the Se increases, although a downward trend can be observed until the level of Se reaches 80 µM, where the photosynthetic capacity decreases significantly compared to the previous conditions. The Dunn's test revealed that the plants treated with 80 µM had a significantly lower photosynthe-

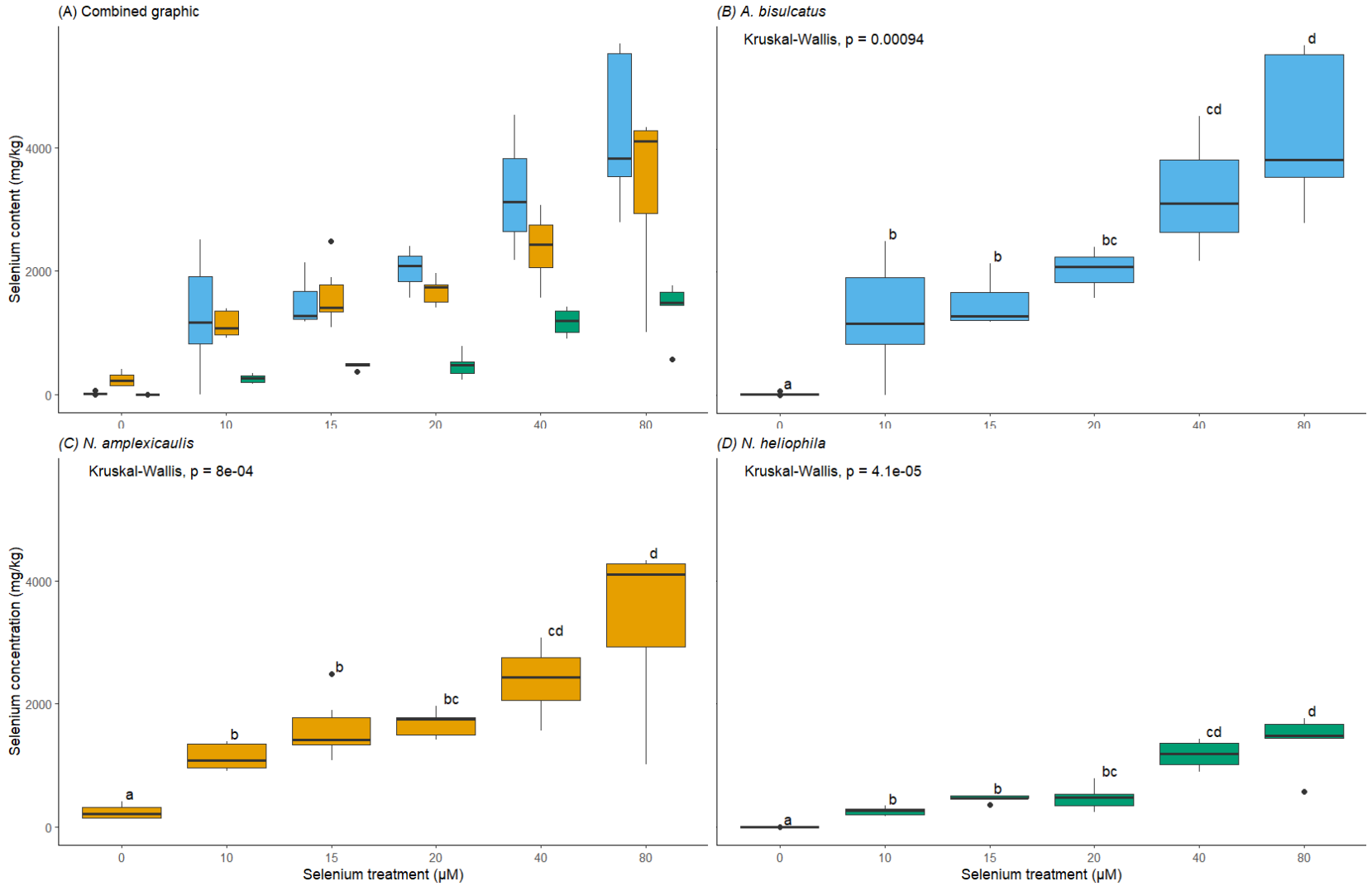
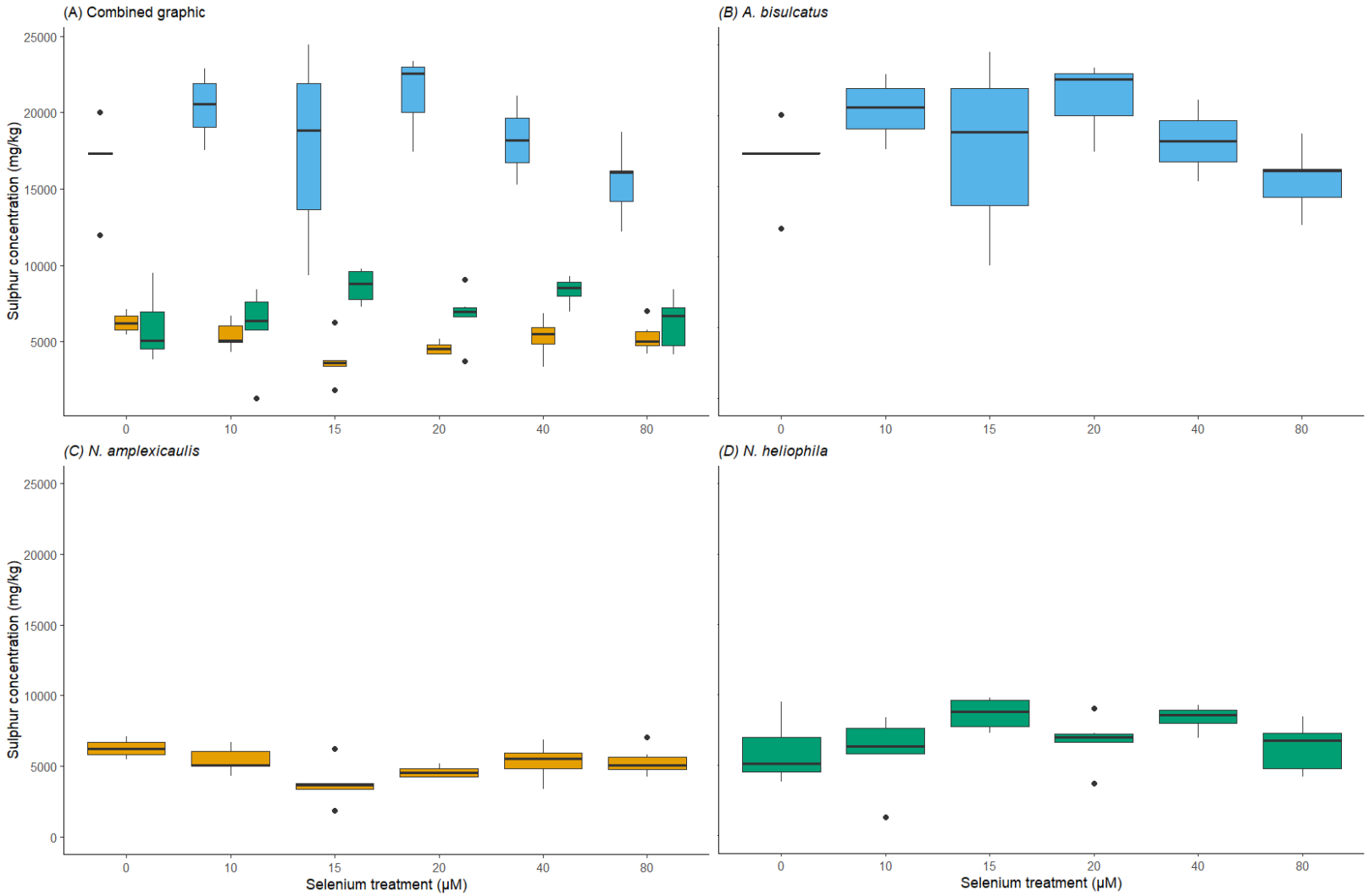


Figure 3: The concentration of selenium in the environment increases the selenium concentration within the plant. The plants were grown in different levels of selenium for 42 days (6 weeks), after which, the selenium concentration was measured on the plants. A Kruskal-Wallis test revealed a significant impact of the treatment ($p < 0.05$), a subsequent Dunn test revealed which comparisons were different ($p < 0.05$) indicated on the figure by the lower case letters. The colours panel indicate specific plant species, bleu represents *Astragalus bisulcatus*, orange represents *Neptunia amplexicaulis*, green represents *Neptunia heliophila*. (A) Combined graph. (B) *Neptunia amplexicaulis* only. (C) *Neptunia amplexicaulis* only. (D) *Neptunia heliophila* only.



<u>Figure 4</u>: The concentration of selenium in the environment did not impact the sulphur concentration within the plant. The plants were grown in different levels of selenium for 42 days (6 weeks), after which, the photosynthetic capacity was measured on the plants. A Kruskal-Wallis test did not reveal a significant impact of the selenium treatment on this measurement. The colours panel indicate specific plant species, bleu represents *Astragalus bisulcatus*, orange represents *Neptunia amplexicaulis*, green represents *Neptunia heliophila*. (A) Combined graph. (B) *Neptunia amplexicaulis* only. (C) *Neptunia amplexicaulis* only. (D) *Neptunia heliophila* only.

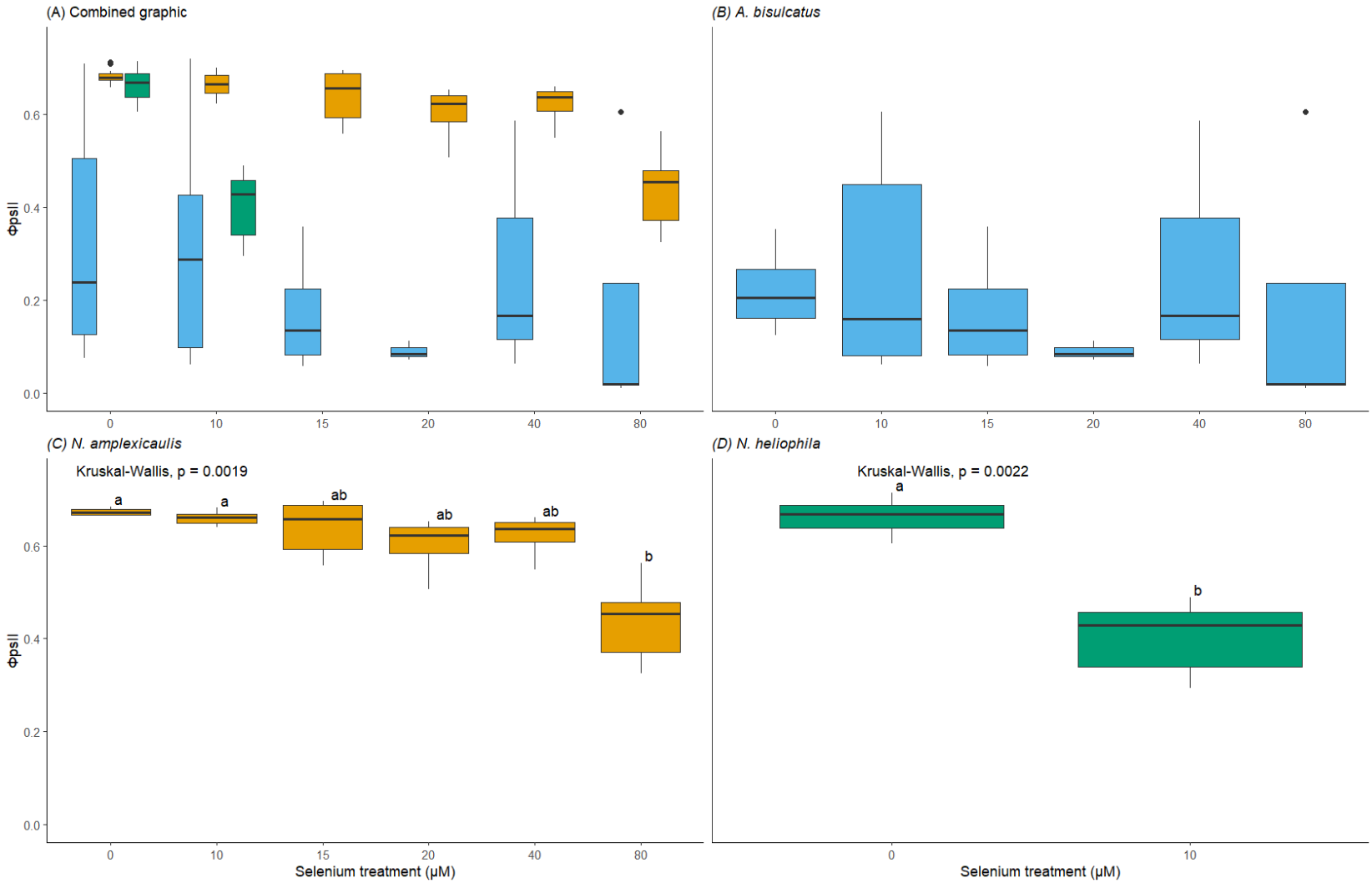
-tic capacity than the plants treated with 0 and 10 µM. The measurements of ΦpsII for *N. heliophila* lacks data points as measurements for Se levels 15 onwards were overlooked on the day of the measurement. Nevertheless, data was collected for conditions 0 and 10 µM of Se. At 0 µM the mean ΦpsII is 0.662 and decreases to 0.402 at the 10 µM of Se treatment. It should be noted that this measurement seems to vary similarly in the *Neptunia* plants but varies more in *A. bisulcatus*. This is probably why the statistic test did not reveal any significant differences between the different conditions for *A. bisulcatus*.

3.1.5 Relationship between physiological Se concentration and photosystem efficiency:

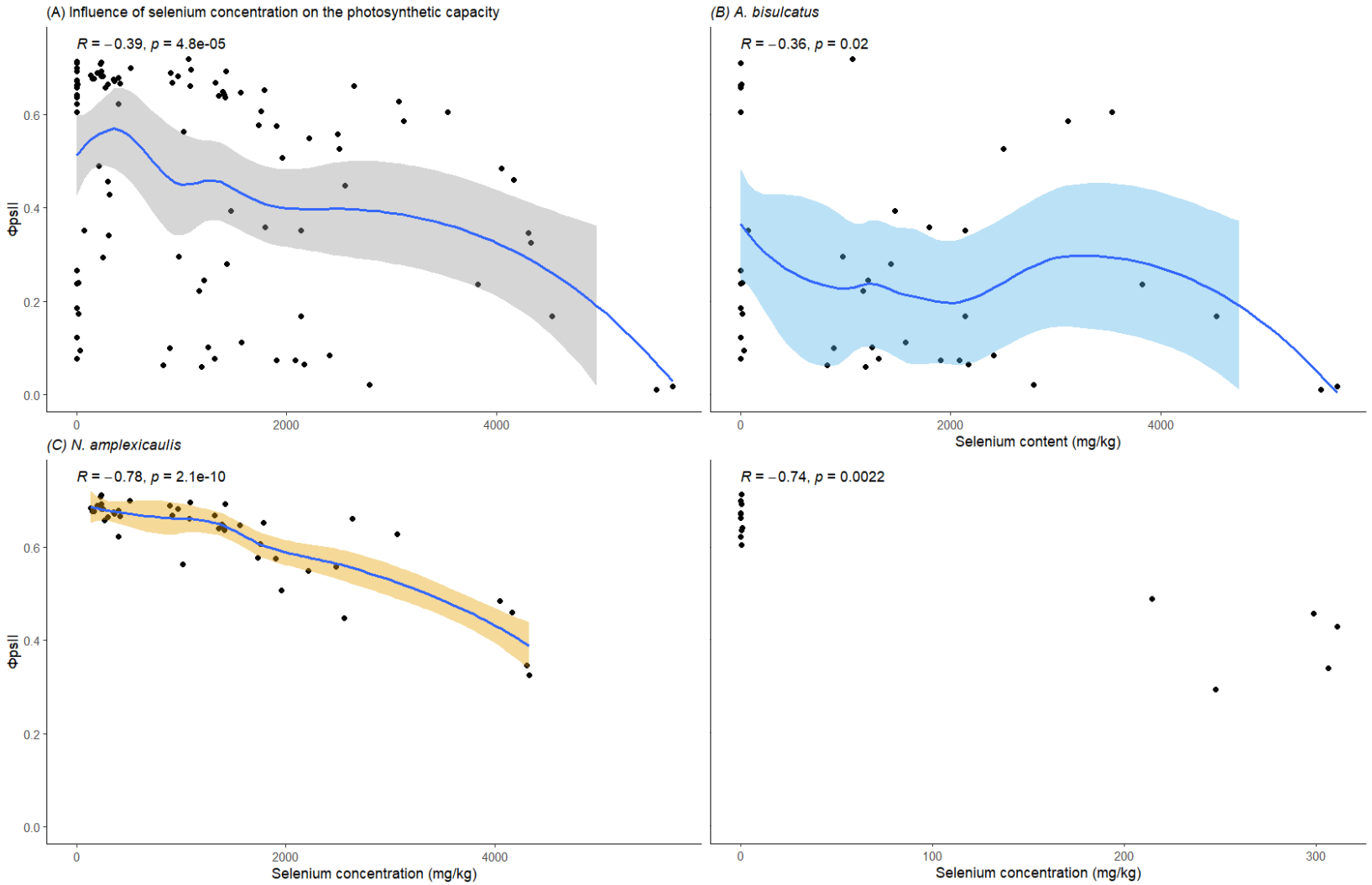
The increase in physiological Se concentration negatively impacts the photosynthetic efficiency (Fig. 6). This is also observed when looking at individual species. A local regression (LOESS) test revealed a significant relationship in all species. For *A. bisulcatus*, high variation can be observed, but is overall lower than for *N. amplexicaulis* and *N. heliophila*. *Neptunia amplexicaules'* and *N. heliophila'* ΦpsII efficiency is high at low Se and decreases to ~0.4 when Se concentration reaches ~4000 or 250 mg/kg. This shows the low tolerance of *N. heliophila* compared to *N. amplexicaulis* when it comes to Se. For *N. heliophila*, the Se concentration is very low compared to the hyperaccumulators plants. It should be noted that, due to the lack of measurements made on *N. heliophila* plants, the photosystem efficiency has only been measured on plants treated with 0 and 10 µM of Se. On another point, it seems some data points of *A. bisulcatus* and *N. heliophila* do not have any physiological Se, but those measurements had very low but not null Se concentrations.

3.1.6 Relationship between the plant weight and photosystem efficiency:

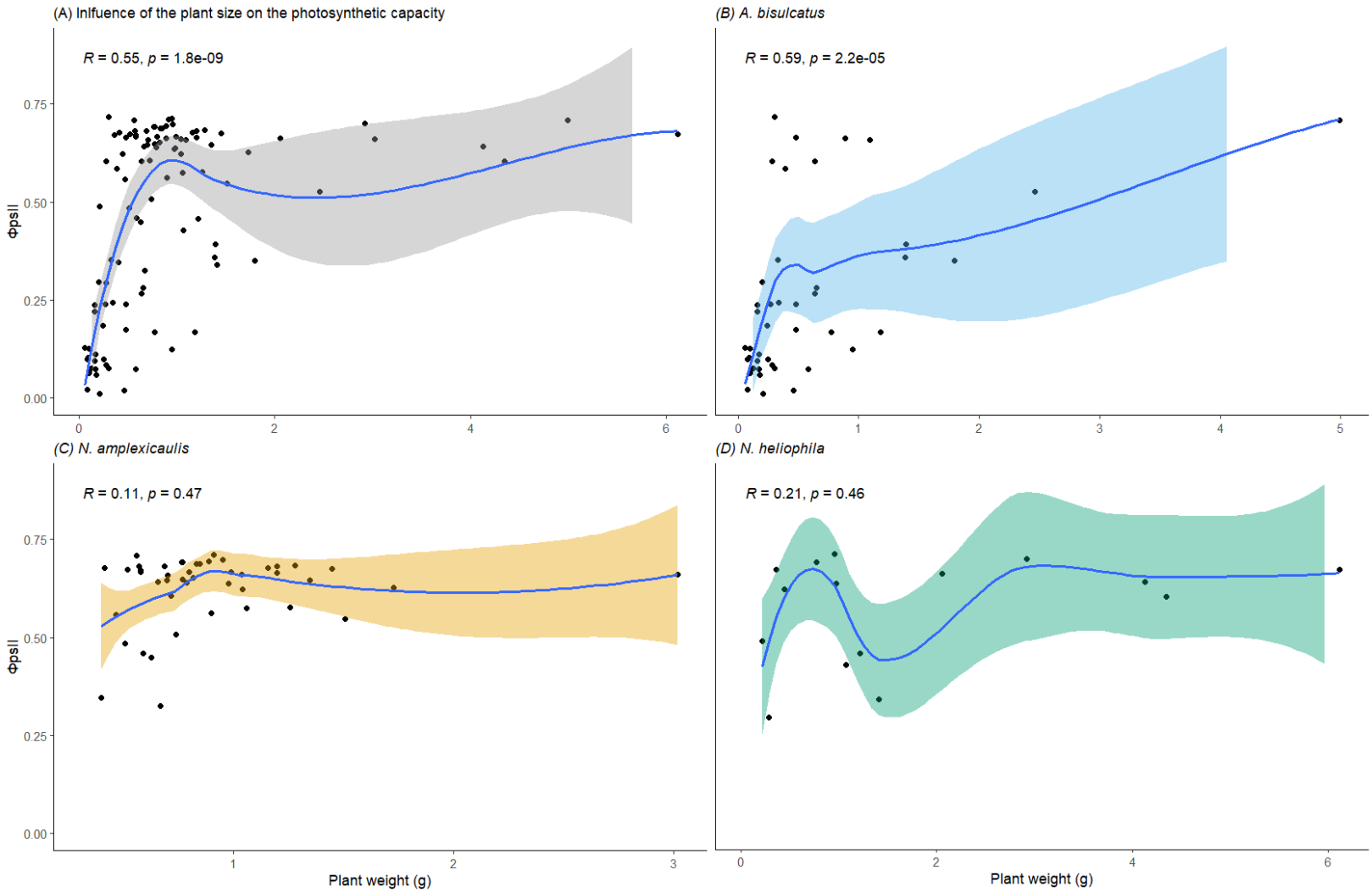
The plant weight is positively correlated with the photosystem efficiency (Fig. 7). The combined graph shows that the photosystem efficiency increases as the plant weight does, until it reaches 0.50–0.75 photosystem efficiency. Above that point, the photosystem efficiency is relatively high and stable. However, although the statistical analysis revealed a significant relationship between plant size and photosynthetic capacity when grouping the different species together, the relationship is only significant for *A. bisulcatus* and not *N. heliophila* and *N. amplexicaulis*. The lack of significance is probably due to a lack of small plants in both *Neptunia* species and a lack of data points for *N. heliophila*.



<u>Figure 5</u>: The concentration of selenium in the environment decreases the photosystem efficiency of the plant.
The plants were grown in different levels of selenium for 42 days (6 weeks), after which, the photosynthetic capacity was measured on the plants. A Kruskal-Wallis test revealed a significant impact of the treatment ($p < 0.05$), a subsequent Dunn test revealed which comparisons were different ($p < 0.05$) indicated on the figure by the lower case letters. The colours panel indicate specific plant species, bleu represents *Astragalus bisulcatus*, orange represents *Neptunia amplexicaulis*, green represents *Neptunia heliophila*. (A) Combined graph. (B) *Neptunia amplexicaulis* only. (C) *Neptunia amplexicaulis* only. (D) *Neptunia heliophila* only.



<u>Figure 6</u>: The photosystem efficiency of the plant negatively correlates with the physiological selenium concentration. The plants were grown in different levels of selenium for 42 days (6 weeks), after which, the selenium concentration and the photosynthetic capacity was measured on the plants. A spearman correlation test revealed a significant influence between the two measurements. The p-value and the slope of the relationship are written on the graphs. The colours panel indicate specific plant species, bleu represents *Astragalus bisulcatus*, orange represents *Neptunia amplexicaulis*, green represents *Neptunia heliophila*. (A) Combined graph. (B) *Neptunia amplexicaulis* only. (C) *Neptunia amplexicaulis* only. (D) *Neptunia heliophila* only.



<u>Figure 7</u>: The concentration of selenium in the environment increases the selenium concentration within the plant. The plants were grown in different levels of selenium for 42 days (6 weeks), after which, the plant weight and the photosynthetic capacity was measured on the plants. A spearman correlation test revealed a significant influence between the two measurements. The p-value and the slope of the relationship are written on the graphs. The colours panel indicate specific plant species, bleu represents *Astragalus bisulcatus*, orange represents *Neptunia amplexicaulis*, green represents *Neptunia heliophila*. (A) Combined graph. (B) *Neptunia amplexicaulis* only. (C) *Neptunia amplexicaulis* only. (D) *Neptunia heliophila* only.

3.1.7 Comparison between shoot and root tissue Se concentration:

The root tissue contains less Se than the shoot tissue. This can be observed in figure 8. Furthermore, this can also be observed in all three plant species (data not shown). On average, from the slope of the Pearson correlation, root tissues are 20% less concentrated than shoot tissues. However, this is not the case for every data point. Finally, it should be noted that several root tissues could not be measured because of small size of the plants and thus cannot be compared to their shoot tissue counterpart.

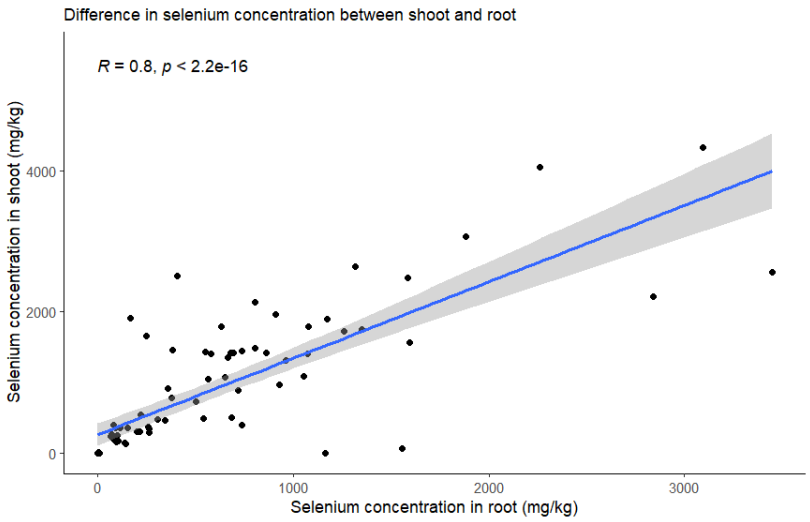


Figure 8: The selenium concentration is lower in roots than shoot. The plants were grown in different levels of selenium for 42 days (6 weeks), after which, the selenium concentration of the root and shoot tissue were measured. A linear relationship between shoot and root tissue. A spearman correlation test revealed a significant influence between the two measurements. The p-value and the slope of the relationship are written on the graphs.

3.2 Depleting physiological Se by picking young leaves:

Picking young leaves reduces physiological Se concentration in plants growing in Se rich environments. Figure 9 presents the Se concentration measured in the leaves of the plants. At 0 µM of Se in the environment, there seems to be no significant difference between the plants from which the young leaves have been picked and the non-treated plants. On the other hand, a significant difference can be observed in the plants growing in the 10 µM selenate environment. On average, the mean was reduced from >1000 mg/kg to <500 mg/kg of Se. Thus, the plants of which leaves have been picked dropped below the Se threshold required to reach to be considered a hyperaccumulator plant.

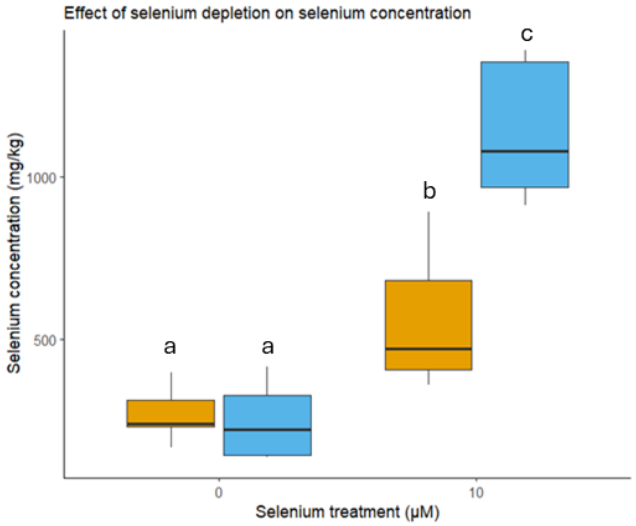


Figure 9: The picking the young leaves reduced the plant's selenium concentration.
The plants were grown in different levels of selenium for 28 days (4 weeks), after which, the young leaves of the plants were harvested for two weeks (one composite leaf per week). Afterwards, the physiological selenium concentration was measured. A Kruskal-Wallis test revealed a significant impact of the treatment ($p < 0.05$), a subsequent Dunn test revealed which comparisons were different ($p < 0.05$) indicated on the figure by the lower case letters. The colours panel indicate the different treatment the plants were subjected to, orange: plants which had their young leaves picked, bleu: plants for which no leaves have been picked.

3.3 Efficiency of the RNA isolation methods

3.3.1 RNA isolation yield

Table 5 presents the yield of RNA obtained during the first attempt, using the RNA isolation method developed by Oñate-Sánchez & Verdonk (2021). The yield obtained during this experiment was low for the *N. amplexicaulis* samples, not exceeding 50 ng/µL for the shoot samples and less than 5 ng/µL for the root samples. On the other hand, *Hirschfeldia incana* samples yielded high (>200 ng/µL) results except when glass beads were used to crush the samples. It can be observed that, in the shoot but not in the root tissues, the samples using high amount of material have a lower yield than the ones using low amount of material. It should be noted that the precision measurements of the nanodrop is of ±5 ng/µL. Thus, measurements below that threshold should not be considered.

Additionally, the table presents the RNA yield obtained during the attempt using the RNeasy plant kit by Qaigen Inc. The *N. amplexicaulis* samples, yielded between 0 and 4.093 ng/µL RNA (i.e., below the measurement threshold). The samples of *Arabidopsis thaliana*, used as a control in here, yielded high results from 198.260 to 467.900. The quality was also high.

The yield of RNA obtained during the third attempt, using the method developed by Acosta-Maspons *et al.*, (2019) is also shown. The root and shoot tissue have a low (<10 ng/µL) yield except for one root and one shoot tissue (26.84 and 15.94 ng/µL respectively). In contrast, the flower tissues have presented a high yield in two samples (119.48 and 83.59 ng/µL). The quality of the 119.48 ng/µL flower sample has a quality level of 1.16 and 1.55 for the 260/230 and the 260/280 ratio respectively. The quality of the other flower sample has a quality level of 2.179 and 2.030 for the 260/230 and the 260/280 ratio respectively.

Table 5: RNA yield and quality obtained through the different RNA isolation methods. The *Hirschfeldia incana* and the *Arabidopsis thaliana* plants have been used as positive controls in those experiments. Low medium and high amounts of material refer to ~10 mg ~20mg and >100mg of fresh tissue used in the experiment.

Method	Species	Tissue	Amount of material	Grinding method	Yield	Quality
CiAR	*N. amplexicaulis*	Shoot	High	Mixer mill (glass bead)	33.000	N/A
					-0.210	N/A
				Mixer mill (zirconia bead)	0.000	N/A
					27.320	N/A
			Low	Mixer mill (glass bead)	44.270	N/A
					19.340	N/A
				Mixer mill (zirconia bead)	29.730	N/A
					43.150	N/A
		Root	High	Mixer mill (glass bead)	-0.290	N/A
					2.084	N/A
				Mixer mill (zirconia bead)	2.719	N/A
					1.802	N/A
			Low	Mixer mill (glass bead)	4.067	N/A
					N/A	N/A
				Mixer mill (zirconia bead)	0.899	N/A
					0.590	N/A
	H. incana	Shoot	High	Mixer mill (glass bead)	9.660	N/A
					267.464	N/A
		Root	High	Mixer mill (glass bead)	36.880	N/A
					361.527	N/A
Rneasy plant kit	*N. amplexicaulis*	Shoot	Low	Mixer mill (zirconia bead)	3.183	N/A
					-1.042	N/A
					4.093	N/A
					-1.851	N/A
		Root	Low	Mixer mill (zirconia bead)	1.619	N/A
					3.600	N/A
					3.296	N/A
					1.970	N/A
	A. thaliana	Shoot	Low	Mixer mill (zirconia bead)	257.237	1.964; 2.155

					459.436	2.277; 2.163
					198.260	2.487; 2.155
					467.900	2.300; 2.100
CTAB based	*N. amplexicaulis*	Flower	High	Mortal & Pestle	-1.013	N/A
					26.838	1.199; 2.006
					3.341	N/A
		Shoot	High	Mortal & Pestle	1.973	N/A
					7.612	N/A
					15.935	0.824; 1.824
		Root	High	Mortal & Pestle	0.623	N/A
					119.483	1.157; 1.554
					83.587	2.179; 2.030

3.3.2 Quality check of the flower samples

The *A. thaliana* samples and the *N. amplexicaulis* samples of the Acosta-Maspons et al. (2019) experiment were migrated on a gel to test their quality (Fig 10). It can be observed that the samples of *A. thaliana* have a good quality and a high yield. Similarly, the flower samples of *N. amplexicaulis* revealed intense and clear bands. However, this is not the case for the root and shoot samples of N. amplexicaulis, their bands are faded although the quality is still good. The faded bands are probably due to the low yield of the plants. As expected, the samples treated with the DNase are more intense than the others, however, the bands are less separated.

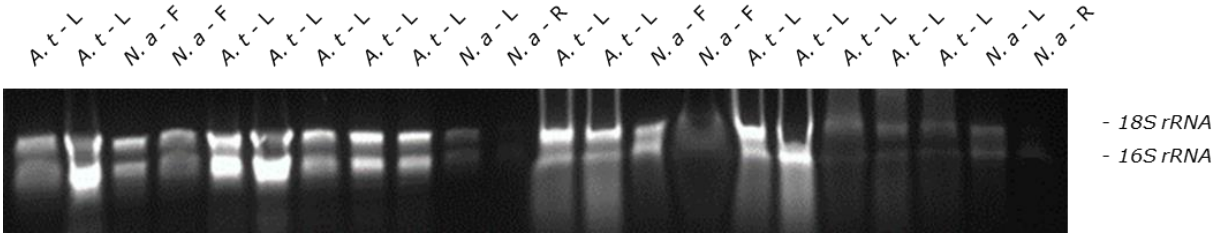


figure 10: RNA samples migrated on an agarose gel.
The samples were migrated on an 1% agarose gel with 15µg of ethidium bromide. The first eleven samples were treated with DNase while the last eleven were. The Acronyms are as follows. First part refers to the species: A. t = Arabidopsis thaliana; N. a = Neptunia amplexicaulis. The second part refers to the tissue of the plant: R = root; L = leaf; F = flower.

4 Discussion

4.1 Result summary

The main purpose of this research practice consisted in finding out if Se concentrations could improve the growth of hyperaccumulator plants and if Se depletion in hyperaccumulator plants results in deficiency symptoms. To this end, several experiments have been conducted. Firstly, two hyperaccumulator plants (*Neptunia amplexicaulis* and *Astragalus bisulcatus*) and one non-hyperaccumulator plant (*Neptunia heliophila*) were grown in hydroponics solutions with varying levels of Se. Afterwards, the plant's weight, photosynthetic capacity and Se concentration was measured. Secondly, another experiment in which the three aforementioned species were used was done. The plants were grown in Se deficient or Se poor hydroponics solutions. After some time, their young leaves were picked to harvest their physiological Se and then the same measurements as the ones done in the first experiment were done. Finally, young leaflets and root tips have been harvested from the plants from both experiments, and RNA isolation methods were tried to test their efficiency on *Neptunia amplexicaulis*.

4.2 Plant growth

4.2.1 Response to Se increase:

It can be observed in figure 2 that the hyperaccumulator plants are less susceptible to the increase of Se in the environment compared to the non-hyperaccumulator plant. Indeed, their weight is relatively constant or even increases. However, at a certain level of selenate, the toxicity of the element is overwhelming and the plant weight drops. On the other hand, *N. heliophila*' (the non-accumulating, Se tolerant plant) weight drops immediately at 10 µM of Se as selenate in the environment. Consequently, even though the *N. heliophila* has a higher weight than the hyperaccumulator plants at 0 µM of Se, this changes as Se increases, the hyperaccumulators start having a similar or even higher weight than non-hyperaccumulator plant. This should be expected as plants which have developed an adaptation to a niche environment (e.g., hyperaccumulator plants) usually do not grow as good as generalist species (e.g., *N. heliophila*) in beneficial environment but out-perform the generalist plants when grown in their niche environments as they have developed adaptation to better support the stress (Kirsch & Kaproth, 2022). A recent study by Harvey *et al.*, (unpublished) noted that *N. amplexicaulis* and *N. heliophila* plants had a near 10-fold tolerance difference with regards to Se dosing when growing in hydroponics.

4.2.2 Optimal Se concentration:

One of the main goals of this experiments was to identify an optimum Se concentration in which the hyperaccumulator plants would grow best. Regarding *A. bisulcatus*, the optimum Se concentration seems to be situated around 15 µM of Se as selenate. Broyer *et al.*, noted that a dosing of 0.25 µg of Se (in form of selenite) atoms per litre was beneficial for the growth of *A. bisulcatus*. For N. amplexicaulis, the optimum level of Se seems to be situated around the 40 µM Se as selenate in hydroponics solution. Harvey *et al.*, (Unpublished) found that an optimal concentration of Se as selenite at 100 µM or 25-50 µM of Se as selenate. This difference might be explained by the high variability of plant weight and the lack of replicates used for this experiment. It is also important to note that, this study focuses on selenate, and that there are differences between selenate and selenite in the accumulation and metabolization of the molecule, as shown above by the different optimal concentrations found by Harvey et al. (Unpublished). Thus, those concentrations do not apply to selenite.

4.2.3 Impact of the light intensity:

It should be noted that differences might also be caused by the sub optimal growing conditions in which the plants were cultivated. The light conditions used in this experiment (200-250 µmol m^2 s^{-1} light) were lower than the conditions commonly used to grow *N. amplexicaulis* and *A. bisulcatus* (*i.e.*, 550 µmol m^2 s^{-1} light) (personal communication). Those sub-optimal conditions and the half Hoagland solution (a poorer nutrient medium) concentration used in the experiment (chosen to prevent any phosphate toxicity) have probably hampered the growth of the plants. Consequently, the most beneficial concentration of Se might be harder to observe as variations between the different Se concentrations are reduced due to the less beneficial environment (low light and less rich environment).

4.3 Selenium and sulphur accumulation

The Se treatment to the environment increased the physiological concentration of Se in all the plant species, although the hyperaccumulator plants acquired more Se than *N. heliophila*. On the other hand, sulphur (S) accumulation did not seem to be significantly affected by the increase of selenate in the environment although a small dip can be observed at 80 µM of selenate. Furthermore, while *N. heliophila* and *N. amplexicaulis* had similar levels of S, *A. bisulcatus* had systematically higher levels of that element.

While the increase of physiological Se correlating with the increase of Se in the environment was expected, it is interesting to note that the plant's S concentration is not significantly

impacted by the increased presence of Se in the environment. Indeed, the metabolic pathway of selenate shares most of its transport proteins with that of S (Schiavon & Pilon-Smits, 2017). Consequently, it could be expected that the increase in selenate would reduce the accumulation of sulphate. This would be similar to what was observed by Trelease & Trelease, (1938), when an increase in selenite reduced the uptake of phosphate and prevented the appearance of symptoms due to phosphate toxicity. When providing the plants with selenite, the accumulation of phosphate was reduced (because selenite enters the plants and its cells through the phosphate transporters). However, this is not what is observed. Several explanations might justify this observation. First, the sulphur transporters (SULTR) genes were not saturated by the increase of selenate in the environment. This is likely as the constitutive levels of physiological S are ten-fold higher than that of Se. This might explain why, only when providing very high amount of selenate in the environment (80 µM) does the physiological S concentration start to decrease. A second explanation might be the stimulation to increase the transport capacity of the plant. It has been observed that exposure to Se in the environment increase the transcription of SULTR genes in hyperaccumulator plants such as *A. bisulcatus* (El Mehdawi & Pilon-Smits, 2012). Thus, the increased transcription of SULTR genes would then increase the plant's ability to accumulate Se but prevent it to become deficient in S. This latter argument might explain why, when combining the physiological concentrations of selenate and sulphur, an increase can be observed in the combined concentrations (data not shown) while the S levels stay constant (Fig. 4). Finally, it could be that some transporters have a specialty or a preference to transport selenate over sulphate. This might also justify why the levels of S are constant and similar between *N. amplexicaulis* and *N. heliophila* but differ in regards Se. This would have to be investigated further in a later project.

4.4 Photosynthetic capacity and selenium concentrations

As shown in figures 6 and 7, ΦpsII is correlated with the weight of the plant and the physiological Se concentration. Similar experiments conducted on sweet potato revealed that very small doses of Se could increase the photosynthetic capacity of the plants, however, over that dose it would decrease (Chen et al., 2022). The latter observation was also noted in the experiments but not the increase in ΦpsII, probably because the levels of Se dosed were already too high. This positive effect of Se on the photosystem of the plant requires further investigation. The negative impact of Se on ΦpsII might be explained by the localisation in which the Se is transported for its assimilation. Indeed, the metabolization of Se requires

several steps of reduction which requires antioxidants (e.g., glutathione). Consequently, high physiological Se levels might deplete the antioxidant pools in the chloroplasts (Noctor & Foyer, 1998). This latter observation was made in a study which measured the glutathione concentration in *Arabidopsis* plants which had been supplied with Se (Hugouvieux et al., 2009). As a result, this metabolite pool cannot be used to eliminate the reactive oxygen species (ROS) produced during the normal functioning of the photosynthetic apparatus. To preserve its chloroplasts from the damage of ROS, the plant might reduce the activity of its photosynthetic machinery and thus reducing ΦpsII. Another theory might be that the production of ROS cannot be efficiently controlled as Se increases which starts damaging the plant's chloroplast. In both cases however the consequences are similar, the reduced photosynthesis and a stunted growth. Indeed, the reduced production of sugars thanks to photosynthesis limitation results in lack of resources for the plant to grow efficiently and, in the worst cases, to sustain itself. This is what was observed in some plants which died overtime when in the high Se concentrated environments.

Those results show that measurement of photosynthesis can be a useful tool to measure how the Se impact the health of the plant. The measurements of photosynthetic capacity are non-destructive and, depending on the techniques used, can be easy to conduct and in a timely manner. It would be interesting to see how the photosynthetic capacity of the plant is affected over time, as the plant grows, from an initial exposure onwards. Harvey (personal communication) theorized that the loss of old leaves at the beginning of the growth of Neptunia amplexicaulis plants (when exposed to Se) was a response to the high physiological Se concentration and a way to reduce its Se levels. The measurements of photosynthetic capacity might be a way to determine if over time, as the plant grows in a certain concentration of Se, the overall health of the plant improves as the plant's capacity to handle the Se improves.

4.5 Root and shoot selenium concentrations

Figure 8 shows that, on average, there is a lower concentration of Se in the roots than the shoot. This can be expected because, although the Se is taken up by the roots, it is sent to the shoot tissue through the tap root. It then accumulates in the young leaf tissues of the plant where it is metabolised (Fig 11). This might explain why increased Se was measured in the shoot than the roots. It should be noted however that, another section which concentrated a lot of Se is the root crown section of the plant as shown by Harvey et al., (2022). However, it probably did not have an impact on the results as the root crown was sectioned in two in

our experiments to separate the two tissues, thus distributing the Se of the root crown equally between shoot and root tissue.

4.6 Efficiency of selenium depletion treatment

The results presented how picking the young leaves of *N. amplexicaulis* managed to decrease the concentration of Se in the plant when Se was added to the environment. However, when the physiological Se concentration is low, this process did not seem to significantly impact the concentration in the plant. A probable reason is that, as the Se concentration drops, the element becomes more defuse and less Se is present in the young leaves. Consequently, conducting this treatment for a longer amount of time could, in time, manage to reduce the physiological Se concentration in those plants. According to that, it should be noted that, as Se decreases it will become more and more difficult to harvest more Se in the plant. Those results do prove that the Se is indeed present in the highest concentrations in the plant's young leaves. It would be interesting to measure the Se concentration of the harvested young leaves and compare it to the rest of the plant tissue to determine how much of the Se is directed to the young leaves and how much each harvesting is depleting the plants from the Se. Hence, this experiment showed that this method of Se harvesting is a viable option to study the subject.

4.7 RNA isolation methods

RNA isolation depends on two main factors, the grinding method and the protocol used to lyse the cells and isolate the RNA molecules. Three methods of grinding were tested (i.e., the mixer mill, a plastic pestle used directly in the tubes, mortar and pestle combination) and three RNA isolation protocols were performed (i.e., CiAR, RNeasy plant kit and the CTAB based method). Initially, the mixer mill method and, to a lesser extent, the plastic pestle used directly in the tubes were initially preferred and tried on the CiAR and RNeasy kit. The high through put and reproducibility of those method were their main benefits. However, neither outcome of the CiAR or the RNeasy method were satisfactory, and it was decided to try another RNA isolation method (i.e., the CTAB based method). It should be noted that, from the control plants that were harvested, using zirconia beads instead of glass beads revealed to be more efficient. Unfortunately, the CTAB based method required high levels of material and, the high throughput methods were consequently not available. The results obtained from the CTAB method seemed promising as RNA with acceptable quality was harvested from the

flower samples. However, the leaf and root samples did not yield any good results. The success of the CTAB method with the mortar and pestle grinding and the fact that the flower samples but not the root and leaf samples (sturdier than flowers) yielded results, shows that the grinding method might have been (and possible still for root and shoot samples) is the main problem. It should be noted that, although it does not explain a yield difference that wide, root and leaf tissue concentrate less RNA than flower samples. Once reliable RNA yield will be obtained from root and leaves, the grinding method might be optimised to enable the use of high through put grinding methods such as the mix mill.

4.8 Critical assessment of the study

4.8.1 Limitations

This study presents several limitations which negatively impacted the study. The main factor was the low light intensity in which the plants were grown. Indeed, this lack of photons reduced the potential growth of the plants and caused problems when measuring the Se concentration of the plant as the minimum required amount of the plant was not attained and an alternative method had to be used. Furthermore, if the RNA isolation uses the amount of sample used in the CTAB method (the only one which worked), there would not be enough material. Finally, the lack of growth reduced the negative and positive impact of the Se concentration in the environment and rendered it difficult to spot a beneficial or negative impact of Se. It might have also been interesting to observe how the Se in the plant and the environment affect the flowering of the plant. Unfortunately, this was not possible to be measured as no plants measured due to the low light intensity. The impact of bad growing conditions might have been reduced by extending the experiment, however, due to time restrictions (*i.e.*, deadline of the MSc. Research practice) this was not possible. In this study, only selenate was used (as it is the main form of Se in the soil) thus, some observation, especially those relating to sulphur accumulation, might have been different for selenite. The impact of Se on the growth of the plant lacks precision due to the use of two replicates for this measurement. A final limitation is the overlooking of some measurements of the photosynthetic capacity of *N. heliophila* which prevents the precise assessment of Se treatment on ΦpsII in that plant.

4.8.2 Strengths

On the other hand, this study has several strengths. The large panel of Se concentration used in the first work package enable a precise observation into which treatment was optimal. Multiple types of measurements could confirm each other and thus allowed us to draw more

robust conclusions. Furthermore, the hydroponics set up ensured that the Se concentration in the environment was controlled. Finally, the use of several species, of which a non-Se hyperaccumulator, yielded interesting results in regard to how hyperaccumulators and non-hyperaccumulators reacted differently to high Se concentrations.

4.8.3 Future recommendations

Although the final conclusions of this study did not manage to determine whether a lack of Se results in deficiency symptoms in the plant, the method used in this study seem to be promising. Thus, I recommend to reconduct the study but by changing the characteristics in which the plants were grown. Increasing the light intensity to 800–1000 μmol m^2 s^{-1} light will probably be better for both *N. amplexicaulis* and *A. bisulcatus*. Increasing the richness of the nutrient solutions might also improve the plant's growth, although not as much as the light intensity. Furthermore, this latter factor might have an impact on the Se accumulation of the plants so it would one be implemented if the increased light intensity is not improving the growth enough. Selenium accumulation and plant size is a variable trait, so to improve the accuracy of determining which Se concentration in the plant might be optimum, it would be best to increase the number of replicates used in the study. Furthermore, to prevent any competition between plants it would also be best to grow plants in either individual pots or very large ones. This latter recommendation might increase the space required for each experiment. Consequently, it might be recommended to conduct the experiments over several staggered periods to prevent being overwhelmed by the activities. Finally, the multispec tool is easy to use and non-destructive when it measures the plant's photosynthetic capacity. Thus, I would recommend using it constantly while the plant is growing to monitor how the Se impacts the plant across time.

4.9 Conclusion

Altogether, this study arrived at several conclusions based on the results obtained during the experiments. First, Se has a significant impact on the plant size, but not a necessarily negative impact on hyperaccumulator plants when Se concentrations are low. Second, high Se accumulation negatively impacts the photosystem efficiency of the plant and might reduce sulphur accumulation. Finally, we presented a novel method to impoverish the plants in Se by harvesting the very young leaves. Although the essential nature of Se could not be confirmed or inferred, this study presents promising results and methods to further study this question. Therefore, we recommend optimising the method used in this study to improve plant growth and then reconduct those experiments to assess if the impact of Se on the plant's growth.

References

Acosta-Maspons, A., González-Lemes, I., & Covarrubias, A. A. (2019). Improved protocol for isolation of high-quality total RNA from different organs of Phaseolus vulgaris L. *BioTechniques*, *66*(2), 96-98. https://doi.org/10.2144/btn-2018-0129

Arnon, D. I., & Stout, P. R. (1939). The Essentiality of Certain Elements in Minute Quantity For Plants With Special Reference to Copper. *Plant Physiology*, *14*(2), 371-375. https://doi.org/10.1104/pp.14.2.371

Beath, O. A. (1936). Selenium in Native Range Plants Occurring on Soils Derived from Permian or Triassic (?) Sediments. *Science*, *83*(2144), 104-104. https://doi.org/doi:10.1126/science.83.2144.104.a

Beath, O. A., Gilbert, C. S., & Eppson, H. F. (1939). The Use of Indicator Plants in Locating Seleniferous Areas in Western United States. I. General. *American Journal of Botany*, *26*(4), 257-269. https://doi.org/10.2307/2436499

Broadley, M., Brown, P., Cakmak, I., Ma, J. F., Rengel, Z., & Zhao, F. (2012). Chapter 8 - Beneficial Elements. In P. Marschner (Ed.), *Marschner's Mineral Nutrition of Higher Plants (Third Edition)* (pp. 249-269). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-384905-2.00008-X

Brown, P. H., Zhao, F.-J., & Dobermann, A. (2022). What is a plant nutrient? Changing definitions to advance science and innovation in plant nutrition. *Plant and Soil*, *476*(1), 11-23. https://doi.org/10.1007/s11104-021-05171-w

Broyer, T., Johnson, C., & Huston, R. (1972a). Selenium and nutrition of Astragalus: I. Effects of selenite or selenate supply on growth and selenium content. *Plant and Soil*, *36*, 635-649.

Broyer, T., Johnson, C., & Huston, R. (1972b). Selenium and nutrition of Astragalus: II. Ionic sorption interactions among selenium, phosphate, and the macro-and micronutrient cations. *Plant and Soil*, *36*, 651-669.

Byun, B. J., & Kang, Y. K. (2011). Conformational preferences and pKa value of selenocysteine residue. *Biopolymers*, *95*(5), 345-353. https://doi.org/https://doi.org/10.1002/bip.21581

Cappa, J. J., Yetter, C., Fakra, S., Cappa, P. J., DeTar, R., Landes, C., Pilon-Smits, E. A. H., & Simmons, M. P. (2015). Evolution of selenium hyperaccumulation in Stanleya (Brassicaceae) as inferred from phylogeny, physiology and X-ray microprobe analysis. *New Phytologist*, *205*(2), 583-595. https://doi.org/https://doi.org/10.1111/nph.13071

Chauhan, R., Awasthi, S., Srivastava, S., Dwivedi, S., Pilon-Smits, E. A., Dhankher, O. P., & Tripathi, R. D. (2019). Understanding selenium metabolism in plants and its role as a beneficial element. *Critical Reviews in Environmental Science and Technology*, *49*(21), 1937-1958.

Chen, H., Cheng, Q., Chen, Q., Ye, X., Qu, Y., Song, W., Fahad, S., Gao, J., Saud, S., Xu, Y., & Shen, Y. (2022). Effects of Selenium on Growth and Selenium Content Distribution of Virus-Free Sweet Potato Seedlings in Water Culture [Original Research]. *Frontiers in Plant Science*, *13*. https://doi.org/10.3389/fpls.2022.965649

Dettmann, M. E., Molnar, R. E., Douglas, J. G., Burger, D., Fielding, C., Clifford, H. T., Francis, J., Jell, P., Rich, T., Wade, M., Rich, P. V., Pledge, N., Kemp, A., & Rozefelds, A. (1992). Australian cretaceous terrestrial faunas and floras: biostratigraphic and biogeographic implications. *Cretaceous Research*, *13*(3), 207-262. https://doi.org/https://doi.org/10.1016/0195-6671(92)90001-7

Dinh, Q. T., Wang, M., Tran, T. A. T., Zhou, F., Wang, D., Zhai, H., Peng, Q., Xue, M., Du, Z., Bañuelos, G. S., Lin, Z.-Q., & Liang, D. (2019). Bioavailability of selenium in soil-plant

system and a regulatory approach. *Critical Reviews in Environmental Science and Technology*, *49*(6), 443-517. https://doi.org/10.1080/10643389.2018.1550987
EFSA Panel on Dietetic Products, N., & Allergies. (2014). Scientific opinion on dietary reference values for selenium. *EFSA Journal*, *12*(10), 3846.
El Mehdawi, A. F., & Pilon-Smits, E. A. H. (2012). Ecological aspects of plant selenium hyperaccumulation. *Plant Biology*, *14*(1), 1-10. https://doi.org/https://doi.org/10.1111/j.1438-8677.2011.00535.x
Frenkel, G. D., Falvey, D., & MacVicar, C. (1991). Products of the reaction of selenite with intracellular sulfhydryl compounds. *Biol Trace Elem Res*, *30*(1), 9-18. https://doi.org/10.1007/bf02990338
Hugouvieux, V., Dutilleul, C., Jourdain, A., Reynaud, F., Lopez, V., & Bourguignon, J. (2009). Arabidopsis putative selenium-binding protein1 expression is tightly linked to cellular sulfur demand and can reduce sensitivity to stresses requiring glutathione for tolerance. *Plant Physiol*, *151*(2), 768-781. https://doi.org/10.1104/pp.109.144808
Kirsch, A., & Kaproth, M. A. (2022). Defining plant ecological specialists and generalists: Building a framework for identification and classification. *Ecology and Evolution*, *12*(11), e9527. https://doi.org/https://doi.org/10.1002/ece3.9527
Nestel, P., Bouis, H. E., Meenakshi, J. V., & Pfeiffer, W. (2006). Biofortification of staple food crops. *J Nutr*, *136*(4), 1064-1067. https://doi.org/10.1093/jn/136.4.1064
Noctor, G., & Foyer, C. H. (1998). ASCORBATE AND GLUTATHIONE: Keeping Active Oxygen Under Control. *Annu Rev Plant Physiol Plant Mol Biol*, *49*, 249-279. https://doi.org/10.1146/annurev.arplant.49.1.249
Oñate-Sánchez, L., & Verdonk, J. C. (2021). Citrate-Citric Acid RNA Isolation (CiAR) for Fast, Low-Cost, and Reliable RNA Extraction from Multiple Plant Species and Tissues. *Curr Protoc*, *1*(12), e298. https://doi.org/10.1002/cpz1.298
Peterson, P. J., & Butler, G. W. (1967). Significance of Selenocystathionine in an Australian Selenium-accumulating Plant, Neptunia amplexicaulis. *Nature*, *213*(5076), 599-600. https://doi.org/10.1038/213599a0
Pilon-Smits, E. A., Quinn, C. F., Tapken, W., Malagoli, M., & Schiavon, M. (2009). Physiological functions of beneficial elements. *Curr Opin Plant Biol*, *12*(3), 267-274. https://doi.org/10.1016/j.pbi.2009.04.009
Pinto Irish, K., Harvey, M.-A., Erskine, P., & Ent, A. (2021). Root foraging and selenium uptake in the Australian hyperaccumulator Neptunia amplexicaulis and non-accumulator Neptunia gracilis. *Plant and Soil*, *462*, 1-15. https://doi.org/10.1007/s11104-021-04843-x
Rayman, M. P. (2000). The importance of selenium to human health. *Lancet*, *356*(9225), 233-241. https://doi.org/10.1016/s0140-6736(00)02490-9
Reichenauer, T. G., & Germida, J. J. (2008). Phytoremediation of organic contaminants in soil and groundwater. *ChemSusChem*, *1*(8-9), 708-717. https://doi.org/10.1002/cssc.200800125
Schiavon, M., & Pilon-Smits, E. A. (2017). The fascinating facets of plant selenium accumulation–biochemistry, physiology, evolution and ecology. *New Phytologist*, *213*(4), 1582-1596.
Schiavon, M., Pilon, M., Malagoli, M., & Pilon-Smits, E. A. H. (2015). Exploring the importance of sulfate transporters and ATP sulphurylases for selenium hyperaccumulation—a comparison of Stanleya pinnata and Brassica juncea (Brassicaceae) [Original Research]. *Frontiers in Plant Science*, *6*. https://doi.org/10.3389/fpls.2015.00002
Stanley, S. M. (2005). *Earth system history*. Macmillan.
Trelease, S. F., & Trelease, H. M. (1938). Selenium as a stimulating and possibly essential element for indicator plants. *American Journal of Botany*, 372-380.
van der Ent, A., Salinitro, M., Brueckner, D., Spiers, K. M., Montanari, S., Tassoni, A., & Schiavon, M. (2023). Differences and similarities in selenium biopathways in

Astragalus, Neptunia (Fabaceae) and Stanleya (Brassicaceae) hyperaccumulators. *Annals of Botany*, mcad110.

Zhu, Y.-G., Pilon-Smits, E. A., Zhao, F.-J., Williams, P. N., & Meharg, A. A. (2009). Selenium in higher plants: understanding mechanisms for biofortification and phytoremediation. *Trends in plant science*, *14*(8), 436-442.