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Reciprocal grafting between selenium hyperaccumulator *Neptunia amplexicaulis* and non-accumulator *Neptunia heliophila*

MSc Research Practice Report

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Abstract

Selenium (Se) is an essential micronutrient for humans. Low concentrations of Se can be beneficial for multiple metabolic processes, while being toxic at elevated concentrations. Such biochemical duality of Se also makes it one of the most interesting elements in biology. Selenium hyperaccumulators are a group of plants that can tolerate rather high Se concentrations in contaminated soil without showing any toxic symptoms. *Neptunia amplexicaulis* is one of the strongest know Se hyperaccumulator plants and originates from Australia. Selenium hyperaccumulator synthesize inorganic Se, such as selenate, into organic complexes, especially selenocystathionine in *N. amplexicaulis*. Understanding the mechanism of Se hyperaccumulation is vital for developing phytomining and phytoremediation methods. In order to contribute insights into Se uptake and localization of organo-Se synthesis by roots and shoots, reciprocal grafting attempts between hyperaccumulator *Neptunia amplexicaulis* and non-accumulator *Neptunia heliophila* were conducted utilizing splice and approach grafting methods. The 'splice grafting' method is not ideal for the reciprocal grafting of *N. heliophila* and *N. amplexicaulis*, yet 'approach grafting' might be a more promising alternative. Identification and development of a functional grafting protocol for the target species *N. amplexicaulis* may inform future studies to comprehensively understand the mechanisms of Se hyperaccumulation.

Keywords: Reciprocal grafting, selenium hyperaccumulator, *Neptunia amplexicaulis*

Contents

1. Introduction

1.1. The biological importance of selenium

Selenium (Se), an essential micronutrient for humans, was first discovered in 1817 (Berzelius, 1818) and is listed as an ingredient in multivitamins and dietary supplements (Steinbrenner et al., 2015). For most animals, optimal concentrations of Se can contribute to enzyme activity and support the immunological cycles (Stadtman, 1974, Hoffmann and Berry, 2008), while elevated Se (>400 micrograms/day for adults) can lead to selenosis (Wrobel et al., 2016). Such biochemical duality of Se also makes it one of the most interesting elements in biology.

Selenium is an essential component of various proteins as animals synthetic selenoproteins (25 known in humans), including selenocysteine (SeCys, also known as the 21st protein amino acid) (Kryukov et al., 2003). The presence of SeCys rather than Cys (cysteine) in the active site allows selenoproteins to have better antioxidant functions and plays a vital role in stabilizing and neutralizing the body metabolism (Zoidis et al., 2018, Brown and Arthur, 2007). For example, the involvement of selenoproteins in free radical scavenging may help prevent gastrointestinal and lung cancers (Hatfield et al., 2009). During wound healing process, the combination of specific selenoproteins inhibits inflammatory cytokines and eliminates super radical ions produced during the inflammatory phase (Lei et al., 2009, Cox et al., 2013). Most Se in the body is stored in muscle tissue (van Dronkelaar et al., 2018), although the thyroid gland holds the highest concentration of Se due to various selenoproteins that assist with thyroid function (Ventura et al., 2017). Selenium is involved in the activation and deactivation of various thyroid hormones and their metabolites in every cell that utilizes thyroid hormones (Pakdel et al., 2019). In cases of Hashimoto's disease, in which the body's own thyroid cells are attacked as foreign, a dietary intake of 0.2 mg of sodium selenite per day has been proven to suppress the disease by reducing antibody levels (Silvestrini et al., 2020).

In plants, Se exists more as a bystander micronutrient, but the beneficial effects of small levels of Se on plants cannot be neglected (Ellis and Salt, 2003). It has been demonstrated that low concentrations of Se are capable of improving plant defenses by detoxifying intracellular free radicals and increasing enzymatic and non-enzymatic activities, thereby helping plants to eliminate ROS and prevent oxidative stress (Schiavon et al., 2017, Silva et al., 2020). Non-enzymatic molecules such as glutathione (GSH) and ascorbate act in minimizing ROS are also important for maintaining the cellular redox state (Foyer and Noctor, 2012). Selenium is incorporated into the formation of GSH through the biosynthesis of

selenocysteine (Schiavon et al., 2017), and an increase in glutathione peroxidase effectively sequesters hydrogen peroxide and lipid hydroperoxides, leading to a decrease in the formation of superoxide anions (Hartikainen et al., 2000, Feng et al., 2013).

Although Se-deficient soils are widely distributed globally, making Se deficiency one of the most common micronutrient deficiencies around the world, Se-enriched ('seleniferous') soils have also been observed in some areas (United States, Australia, etc.) (Lyons, 2018). Selenium contamination in soils can be cleaned up by living plants, a process known as phytoremediation (Muthusaravanan et al., 2018). For instance, species within the large genus *Astragalus* (Fabaceae) have great potential as a phytoextractor of Se that can accumulate >10,000 μg Se g^{-1} dry weight in shoots (Rosenfeld and Beath, 2013). Phytoremediation has been proposed as a more cost-effective method of environmental remediation than traditional soil remediation methods. Combining promising and sustainable phytoremediation and biofortification technique (Galić et al., 2021) to increase Se concentrations in edible plant parts, especially widely consumed staple foods such as cereals, through breeding, agronomic and genetic approaches may be an effective component of food system strategies to reduce Se malnutrition.

1.2. Selenium hyperaccumulation in plants

Soil Se exists mainly in the inorganic form of selenate (SeO_4^{2-}) or selenite (SeO_3^{2-}), which is the main form of Se absorbed by roots because of their solubility (White, 2016, Deng et al., 2019). Organic Se compounds such as seleno-amino acids can also be taken up by plants (White, 2016). However, plants do not have significant uptake capacity for the less bioavailable metal selenides or colloidal elemental Se (White and Broadley, 2009). After entering plant cells, selenates will be assimilated into selenocysteine (Se-Cys) and selenomethionine (SeMet) via biochemical pathways involved in sulfate reduction (White, 2016, Gupta and Gupta, 2017, Guignardi and Schiavon, 2017). The substitution of these two seleno-amino acids for the analogues cysteine (Cys) and methionine (Met) disrupts protein folding and leads to protein dysfunction, which is regarded as the main cause of Se toxicity in plants (Brown and Shrift, 1982). To mitigate Se toxicity, Se hyperaccumulators methylate SeCys via enzyme SeCys methyltransferase (SMT) (Sors et al., 2009). The resulting methyl-SeCys can be safely accumulated and constitutes the main (>80%) Se component in hyperaccumulators (Freeman et al., 2006). Methylated SeCys and SeMet can also be further converted to volatile dimethyl(di)selenide (DMDSe) thereby participating in the environmental Se cycle and possibly contributing to the formation of Se-enriched regions (Sors et al., 2005, Winkel et al., 2015).

Due to the chemical similarity between Se and S, plants take up and metabolize Se mainly through sulfate transporters (Sors et al., 2005, Schiavon and Pilon-Smits, 2017). According to the ability to accumulate Se, plants are generally classified into three categories: Se non-accumulators (<100 μg Se g^{-1}), secondary Se accumulators (100–1000 μg Se g^{-1}) and Se hyperaccumulator (>1000 μg Se g^{-1}) (Brown and Shrift, 1982). It was indicated that the major gene involved in root sulfate/selenate uptake (a type 1 high-affinity sulfate transporter or SULTR) was overexpressed in Se hyperaccumulator, which resulted in hyperaccumulators having higher levels of Se and S than comparable non-accumulators (Freeman et al., 2010, Schiavon et al., 2015). Plant tolerance to Se depends in part on their ability to convert inorganic Se to organic Se, as the inorganic form of Se is thought to cause more oxidative stress than the organic form (Van Hoewyk, 2013). After uptake into the roots, selenate/sulfate can be translocated to the plastids for reductive assimilation via group 3 SULTR (Sultr3;1 in non-accumulator Arabidopsis thaliana) (Takahashi et al., 2011, Cao et al., 2013), and the ATP sulfurylases required for assimilation was demonstrated to be highly overexpressed in the hyperaccumulator plant *Stanleya pinnata*, potentially increasing the rate of selenate reduction to organic forms (Schiavon et al., 2015, Freeman et al., 2006).

1.3. *Neptunia amplexicaulis*, a selenium hyperaccumulator

Notably, a new world record holder of Se hyperaccumulation – the Australian legume *Neptunia amplexicaulis*, was recently discovered in central Queensland, which can accumulate up to 13,600 μg Se g^{-1} in the young leaves (i.e. 1.36 wt% of plant dry weight) (Harvey et al., 2020). *Neptunia heliophila* (previous described as *N. gracilis*) which is sympatric with *N. amplexicaulis*, is a Se non-accumulator with tissue concentrations of Se that generally do not exceed 10 μg Se g^{-1}. These two constitute an attractive comparative system to study plant Se metabolism of Se hyperaccumulation. Selenium accumulation in plants has been confirmed to protect the plants from pathogens and herbivores (Quinn et al., 2007). For example, the Se hyperaccumulator *Astragalus spp*. enriched the bioavailable Se in the local soil, thus the enhanced tissue Se level in neighboring plants negatively affected herbivores (through deterrence and toxicity), therefore protecting plants (El Mehdawi and Pilon-Smits, 2012). This also partly provides the basis and ecological drivers for the survival of these plants in seleniferous soils. Selenium hyperaccumulation may have evolved through adaptations to basic nutrient regulation mechanisms shared by all higher plants and is thought to be driven by the induction of Se toxicity in non-vertebrate herbivores (Lima et al., 2018). However, regions of Se-enriched soils are globally scarce and suitable plant materials are rare, thus the evolutionary dynamics and mechanisms of Se hyperaccumulation remain

largely unknown.

1.4. Reciprocal grafting in hyperaccumulators

Grafting is a form of asexual reproduction that theoretically does not alter the genetic characteristics of the progeny (Lee et al., 2010, Harada, 2010). However, growing evidence has shown that various genetic materials are exchanged between rootstocks and scions, demonstrating grafting may alter certain traits and affect inheritance (Duan et al., 2015). For the hyperaccumulators, reciprocal grafting of two ecotypes of *Bidens pilosa* facilitated the growth and Cd extraction capacity of their grafted progeny, suggesting grafting has the potential to change the hyperaccumulation ability in the progenies of grafted plants (Li et al., 2019). In studies of Zn hyperaccumulation, reciprocal grafting between a Zn hyperaccumulator, *Noccaea caerulescens*, and a Zn non-accumulator, *Thlaspi perfoliatum*, determined Zn hyperaccumulation is primarily dictated by root processes and the mechanisms controlling Zn hypertolerance are driven primarily by shoot processes (Guimarães et al., 2009). High expression levels of *HMA4* (Heavy Metal ATPase) and *MTP1* (Metal Tolerance Protein) in *N. caerulescens* appear to provide a feedback loop between the root and shoot (Willems et al., 2007, Hanikenne et al., 2008), which not only increases the uptake and flux of Zn from the soil via the roots to the shoots, but also allows this elevated Zn flux to enter the vacuolar compartment of shoot cells for detoxification and long-term storage (Guimarães et al., 2009). Although the molecular mechanisms of hyperaccumulation and hypertolerance in the Se hyperaccumulator *N. amplexicaulis* are still unclear, we predicted a model similar to that for Zn hyperaccumulation: root is the primary driver of Se hyperaccumulation and shoot Se hypertolerance. Moreover, it is crucial to know exactly where the organo-Se-synthesis of selenocystathionine occurs in *N. amplexicaulis* and hence to disentangle the roles of the root and the shot. To directly test this model, reciprocal grafting between Se hyperaccumulator *N. amplexicaulis* and the non-accumulator *N. heliophila* will be carried out in this study.

1.5. Aim and objectives

Contribute insights into the physiological and genetic mechanisms of Se hyperaccumulation in plants, particularly with regard to Se uptake and distribution by roots and shoots. Determine the relative importance of roots and shoots in Se hyperaccumulation and hypertolerance in relation to organo-Se-synthesis

Objective: 1. Reciprocal grafting between the root of hyperaccumulator (*N. amplexicaulis*) and the shoot of non-accumulator (*N. heliophila*), and *vice versa*.

2. Observation of survival of reciprocal grafted seedlings and testing of selenium accumulation capacity of grafted progenies on the hydroponics system (Se dosing experiment and ionomics analysis)

1.6. Research questions

1. How important are roots and shoots for Se hyperaccumulation? / What is the role of roots and shoots in Se hyperaccumulation and hypertolerance with respect to site of organo-Se-synthesis?

2. Can grafting between Se hyperaccumulator and non-accumulator be successful? What phenotype will the grafted seedlings have? (hyperaccumulating and/or hypertolerant or Se sensitive?)

1.7. Hypotheses

1. Hyperaccumulation of Se in leaves of *N. amplexicaulis* is primarily dictated by root processes, while Se distribution is driven primarily by shoot processes. This can be explained by the localization of organo-Se-synthesis in either the root or the shoot.

2. Reciprocal grafting can be successful
   Phenotype prediction:

	Hyperaccumulate or not	**survival rate after 3 weeks**
Reciprocal graft		
Na shoot scion+ Nh rootstock	×	less than 50%
Nh shoot scion+ Na rootstock	✓ (Not as hyper-accumulative as self-grafted plants, organic selenium can be detected in shoot scion)	less than 50%
Self-graft (control)		
Na shoot scion+ Na rootstock	✓	less than 50%
Nh shoot scion+ Nh rootstock	×	less than 50%

2. Materials and methods

2.1. Plant materials and treatments

Seeds of *N. amplexicaulis* and *N. heliophila* were harvested from plants grown on sandy soil in climate chambers at 23/21°C for day/night, 16/8 hours light/dark and 70% relative humidity (RH). The ancestors of these plants were collected from Richmond, Central Queensland in 2018. Seed surface sterilization was performed prior to puncture: small amounts of seeds were placed in 50 mL capped tubes and sterilization steps were based on Table 1 until the smell of bleach was completely undetectable.

Table 1. Seed sterilization

Step	Reagent	Time
1	70% ethanol	3 min
2	bleach	20 min
3	sterile demi water	2 min →5 min →20 min

Due to the hard seed coat of *N. amplexicaulis* and *N. heliophila*, seeds that had been surface-sterilized were stabbed with surgical blades (Swann Morton, England) (only puncturing the seed coat rather than damaging the seed interior or cutting the seed in half) and submerged in demi water for 24 hours. Well-punctured seeds swelled to twice the size of untreated seeds.

2.2. Seed germination tests

2.2.1 Sterile conditions

The plant nutrient medium was made of half-strength Murashige and Skoog medium (including Vitamins, Duchefa Biochemie) and 8 g/L DAISHIN Agar (Duchefa Biochemie) with the final pH adjusted to 5.8 using 1M HCl and/or NaOH. The medium was autoclaved at 121 °C and was respectively decanted into sterile square Petri dishes (120X120X17 mm, Greiner Bio-One) and polypropylene boxes (ECO2BOX (OVAL MODEL 80MM H), Duchefa Biochemie) until solid. To assess optimal seed germination conditions, several treatments were applied: a dark (where the container was covered with aluminium foil for two days) or normal light treatment; water treatment (extra 1 ml demi water) or no water treatment. The two-by-two combinations of different treatment

conditions were dark with water (DW), dark without water (DNW), light with water (LW) and light without water (LNW). 10 seeds were assigned to each of the four treatments and incubated in the tissue culture room at 24°C and 16/8 hours light/dark.

2.2.2 Non-sterile conditions

A layer of sandy soil about 1 cm thick was spread on the bottom of the polypropylene box. Pre-treated seeds were placed on the soil and then covered with sandy soil to approximately 3 cm. A small amount of demi water was added to the box (ensure that the sand is sticky, but there should be no observable flowing water in the box). Seeds were placed in an incubator at 30°C for 48 hours and subsequently placed in the tissue culture room for growth (24°C, 16 /8 hours light/dark).

2.3. Reciprocal grafting experiments

Four grafting types (Table 2) were performed using the splice grafting method, with 10 replicates of each type. There were also attempts utilizing the approach grafting method in the later stage of this project.

Table 2. Grafting type

Reciprocal grafting	**Self grafting**
Na shoot scion+ Nh rootstock	Na shoot scion+ Na rootstock
Nh shoot scion+ Na rootstock	Nh shoot scion+ Nh rootstock

Na indicates the Se hyperaccumulator *N. amplexicaulis*, and Nh indicates the non-accumulator *N. heliophila*.

2.3.1 Splice grafting

Seedlings of *N. amplexicaulis* and *N. heliophila* were used as shoot scion and rootstock respectively. Cotyledons were first removed to reduce transpiration and provide more space for the splicing of the rootstock and shoot scion. To ensure that fragile seedlings did not die due to rapid dehydration, seedlings were kept moist (especially the incision) in Petri dishes containing demi water during all operations. The double-edge blades (AccuTec, USA) were then used to make an oblique cut at an angle of 30 to 45° in the mid-hypocotyl of the seedlings (Figure 1). The scion and rootstock were carefully secured together

with parafilm and remained on the half MS medium. Grafted seedlings were kept in the hydroponics room at 23/21 °C for day/night, 16/8 hours light/dark and 70% RH.

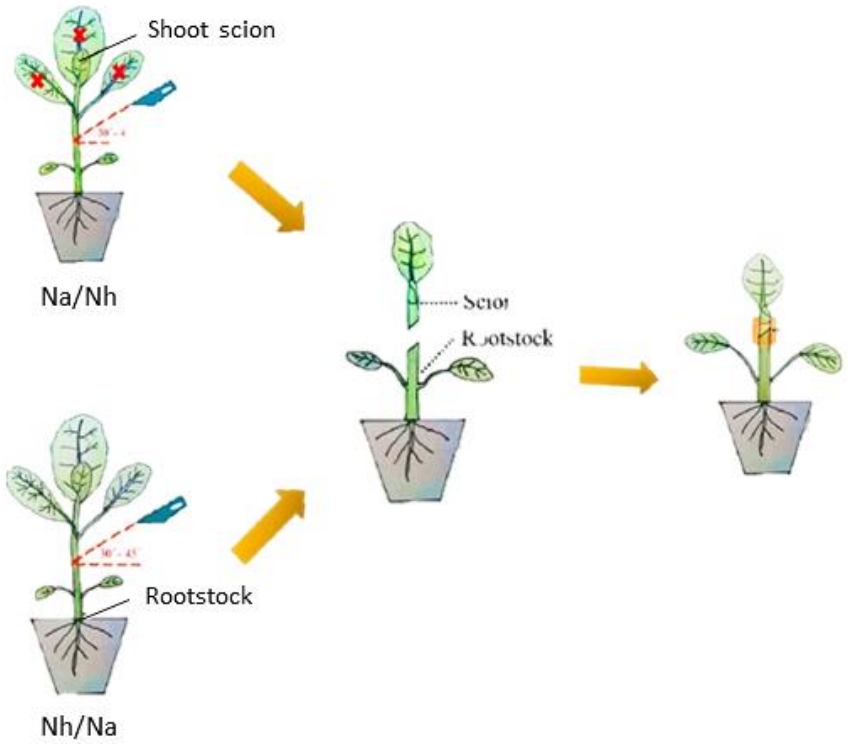

Figure 1. Schematic of splice grafting method. Na indicates the Se hyperaccumulator *N. amplexicaulis*, and Nh indicates the non-accumulator *N. heliophila*. (Image based on Fig. 1 of (Chen et al., 2019)

2.3.2 Approach grafting

Similar to the splice grafting protocol, most of the cotyledons of the seedlings were first removed. A thin, smooth slice was produced 1cm from the root on adjacent surfaces of the scion and rootstock, the two sections were aligned and joined together, secured tightly with grafting clips, and then placed on the hydroponic system (750 mL pots filled with half-strength Hoagland's solution). The pots were covered with clear plastic bags for the first two days to ensure adequate humidity. After the graft union was fully integrated, the top of the rootstock was removed, and the shoot scion was cut from the root below the graft union according to Figure 2. Grafted seedlings were kept in the hydroponics room at 23/21°C for day/night, 16/8 hours light/dark and 70% RH.

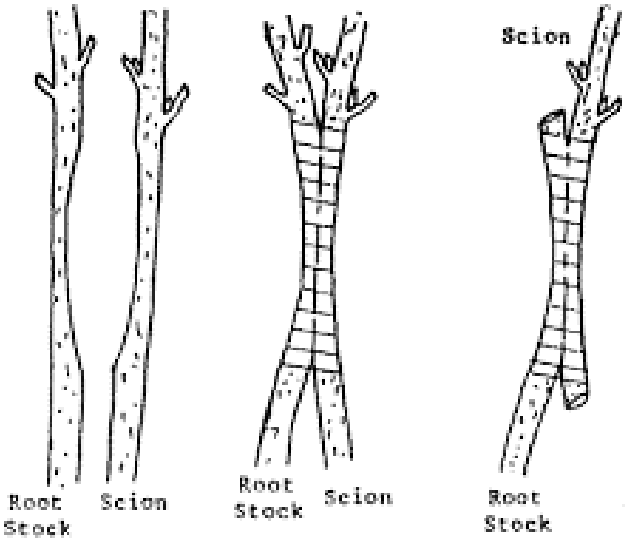

Figure 2. Schematic of approach grafting method. (Image based on https://rfcarchives.org.au/Next/CaringForTrees/Grafting11-92.htm)

3. Results

3.1. Effects of growing conditions on seed germination

Appropriate conditions for seed germination in non-sterile environments have been tested and determined in previous studies (Pinto Irish et al., 2021), but optimal conditions in sterile environments are still equivocal. Hence, experiments were conducted on combinations of different germination containers (Figure 3A), dark or normal light treatments and water treatments. Each treatment contained 10 seeds, the same treatment was not subdivided into different groups, so only size comparisons between germination values were conducted, and no significance testing between data sets could be performed. Under normal circumstances, *N. heliophila* seed germination is expected to reach 90% to 100% in a week. Based on Figure 3B, the mean germination rates of seeds sowed in pots were lower than those in Petri dishes, and DNW-treated seeds showed an unusually low germination rate. There was almost no difference in the average values of germination between dark and normal light treatments for seeds germinated in petri dishes (plates), indicating that the dark treatment had little promoting effect on seed germination.

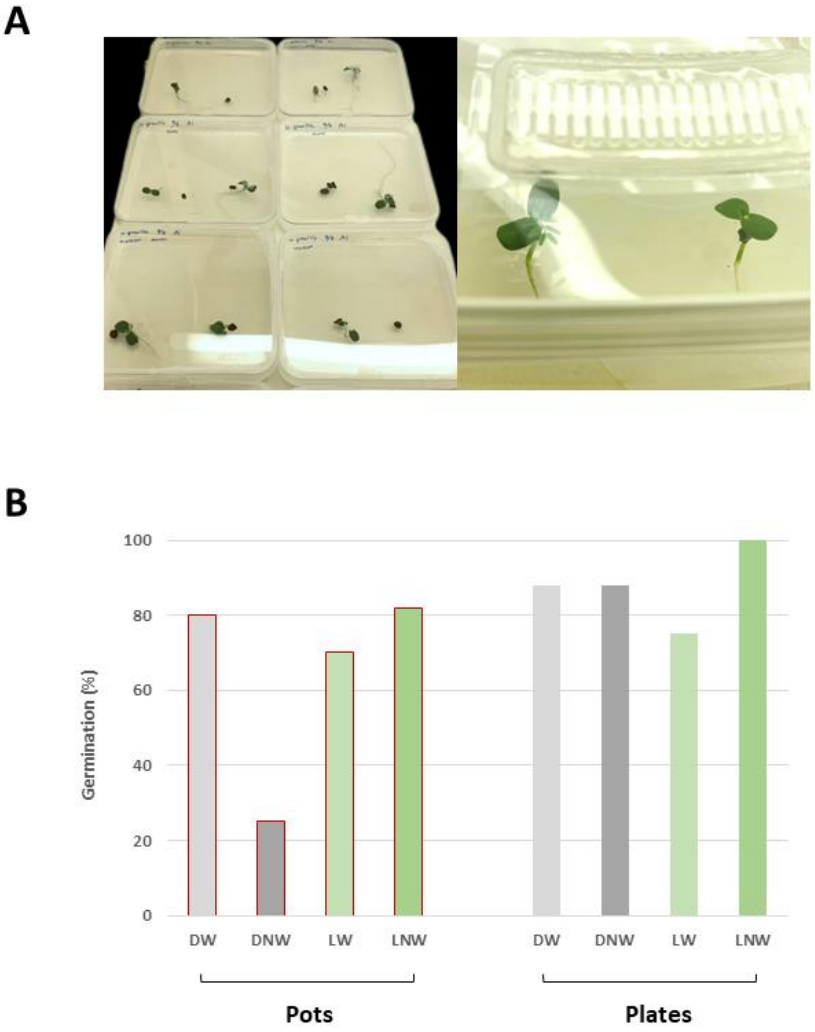

Figure 3. Images of germination tests. (**A**) Seedlings grown on plates (left) and pots (right). (**B**) Germination rate after two weeks under different treatment conditions. The combinations of different treatments were dark with water (DW), dark without water (DNW), light with water (LW) and light without water (LNW). Seeds were sown in pots and petri dishes (plates) respectively.

3.2. Effects of different grafting methods

Most grafting was attempted using the splice grafting method (Figure 4A), and the survival rates of grafted seedlings after three weeks were indicated in Table 3. Overall, splice grafting is not an ideal method: the mortality of self-grafted and reciprocal-grafted seedlings was above 60% and 50%, respectively. The remaining seedlings were not grafted successfully in any case, but all showed developed adventitious roots from the shoot scion (Figure 4C). Morphological differences can be observed between *N. amplexicaulis* and *N. heliophila* seedlings, even at the same growth period, seedlings of *N. amplexicaulis* are generally larger than those of *N. heliophila.* This led to difficulties in maintaining

consistency in the joined area between shoot scion and rootstock when oblique cuts had been made in the hypocotyl. Additionally, the use of parafilm for scion and rootstock fixation was not an ideal method to achieve the desired results. Although the parafilm was clamped as tightly as possible with forceps during the operation to avoid possible gaps that would prevent the scion and rootstock from joining together, there were still a few seedlings showed separation of scion and rootstock during the growing phase.

Since the splice grafting method did not achieve the expected results, attempts using the approach grafting method were made later in this project (Figure 4B). The first attempt contained six seedlings, five of which all showed the death of either the shoot scion or the rootstock after two weeks, and only one (Nh/Na) formed a clear fusion of the hypocotyls (Figure 4D). For this "survivor", the shoot scion was cut from the root below the graft union and the top of the rootstock was removed. However, three days after cutting, this sole candidate also showed mortality (Figure 4E).

Table 3. Survival rate of grafted seedlings

	Nh/Nh	**Na/Na**	**Na/Nh**	**Nh/Na**
Adventitious roots from scion	5	0	6	6
Dead	9	9	5	5
Total	14	9	11	11

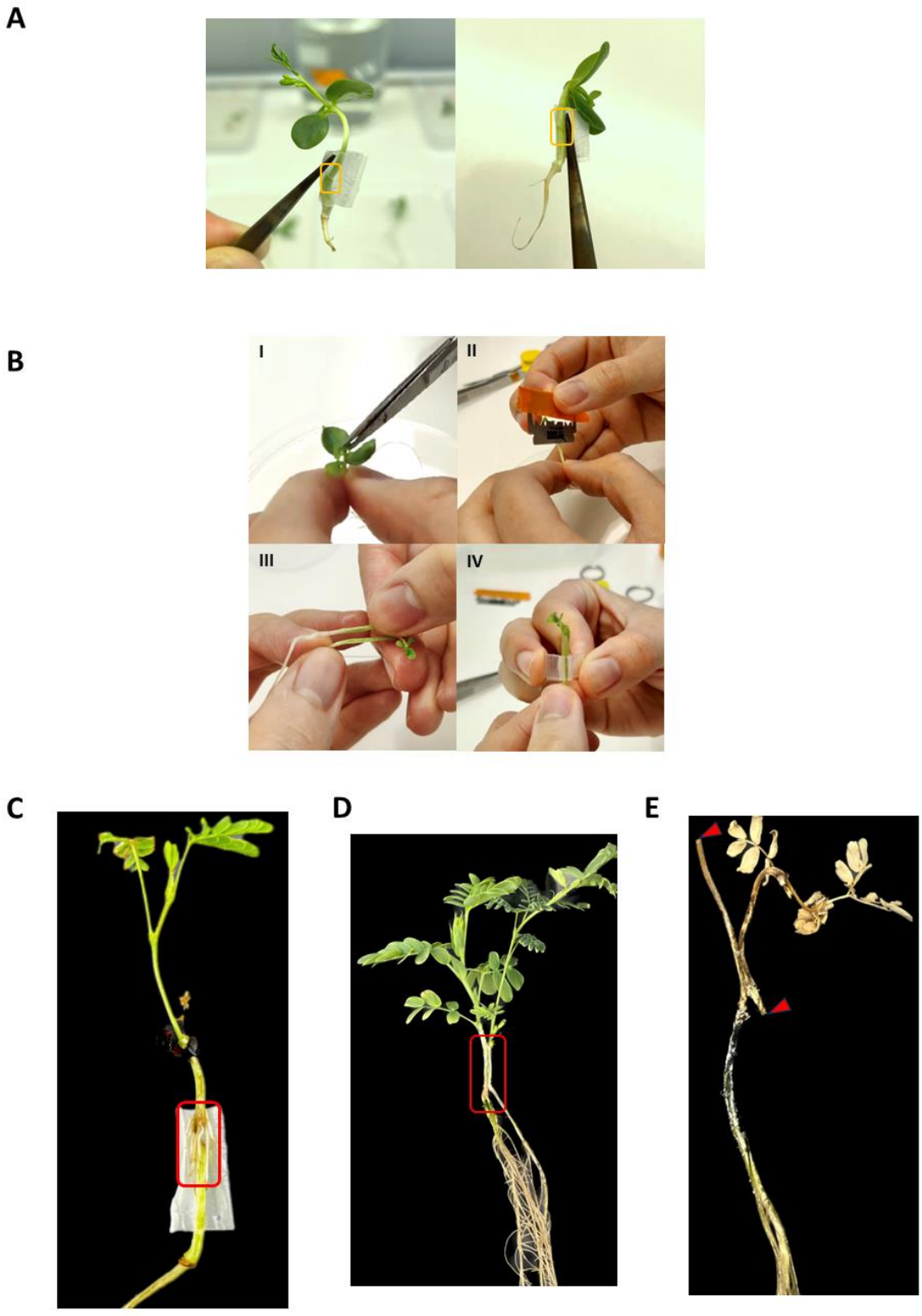

Figure 4. Images of attempts using two grafting methods. (**A**) *N. heliophila* shoot scion + *N. amplexicaulis* rootstock (left), *N. amplexicaulis* self-grafted (right) samples. The area in the yellow box represents the graft union section. (**B**) Procedures for approach grafting: I. Cotyledon removal, II. Production of slices on scion and rootstock, III. Alignment of adjacent surfaces, IV. Securing with grafting clips. (**C**) Self-grafted *N. heliophila* seedlings developed adventitious roots from the shoot scion, area in the red box indicates the adventitious roots. (**D**) Fusion of adjacent surfaces (red box) of grafted seedlings using approach grafting

method. (**E**) Grafted seedling using approach grafting method appears dead after cutting, red arrowhead indicates the cutting sites of shoot scion and rootstock.

4. Discussion

The objective of this project was to determine the relative importance of roots and shoots in Se hyperaccumulation and hypertolerance by attempting to make the reciprocal grafts between the Se hyperaccumulator and non-accumulator species *N. amplexicaulis* and *N. heliophila*. The unviability of the splice grafting method resulted from this study, and we suggested a suitable treatment for seed germination in sterile conditions.

4.1. Abnormalities in seed germination rates

In order to investigate the appropriate growth conditions for seed germination under sterile conditions, several treatments were conducted. Under normal growing conditions, seed germination is estimated to be close to 100% after one week. Yet not all treatments achieved the desired germination rates in the performed tests, with the main problem centered on seeds sown in pots: 1. Seed germination from all four treatments could not reach 90% after two weeks; 2. Germination of DNW-treated seeds was unusually below 30%. Possible explanations for the unexpected result are as follows: *N. amplexicaulis* and *N. heliophila* are tropical plants that grow in seleniferous arid areas and their seeds have a preference for germinating in high temperatures and high humidity conditions. The 24°C germination temperature used for the beginning of this project was not sufficient to meet the heat demands. Besides, pots are larger than plates, thus the RH is conversely lower. Low germination rates are understandable when neither temperature nor humidity can attain the ideal conditions for seed germination. Therefore, in further studies, we expect a faster and higher germination rate under our proposed sterile germination conditions: seeds are sown in plates with 1 mL demi water, and the germination temperature is adjusted to 30°C with a 12-hour photoperiod.

Although this project did not systematically test the effect of temperature on seed germination, when *N. amplexicaulis* seeds were incubated for two days at 30°C on non-sterile sandy soil, 90% germination was achieved in the first week. It is reasonably anticipated that temperature has a dominant influence on the seed germination process, compared to light and additional water treatments. Previous studies have basically incubated non-sterile seeds at around 25°C (Pinto Irish et al., 2021), however, not much has been reported for the optimum

temperature or temperature ceiling for germination of sterile seeds. Future research could also be centered around temperature effects: investigating the effect of different temperatures on seed germination, or even the effect of different combinations of temperature, humidity, and light conditions on seed germination.

4.2. Limitations of the grafting methods

The two grafting methods tried in this project have a common flaw: Grafted seedlings are not produced and grown under completely sterile conditions. The experimental subjects are seedlings with an average age of 14 days (days after germination), which are relatively vulnerable and unstable. These seedlings have to face not only possible mortality due to rapid dehydration during the grafting process, but also stress caused by potential fungal or bacterial infections. Therefore, in order to improve the survival rate of the grafts, future research should ensure that micrografting is carried out under sterile conditions, and grafted seedlings should wait until they are older and fully stabilized under aseptic conditions before transferring to non-sterile conditions.

4.2.1 Splice grafting

In grafted seedlings conducted using the splice grafting method, it was detected that scion formation of adventitious roots was present in more than 50% of the samples. This may be due to the fact that the incision produced by splice grafting leads to stress responses in the plant and the scion produces its own roots as a survival mechanism, which helps the scion to access water and nutrients independently of the rootstock system (Steffens and Rasmussen, 2016). For reciprocal grafting, there may be compatibility problems between *N. heliophila* and *N. amplexicaulis*. In the case of incompatibility, shoot scions and rootstocks may differ in the structure and function of the vascular tissues (xylem and phloem), which are responsible for the distribution of water and minerals absorbed by the roots and sugars and nutrients produced in the shoots, respectively (Jung and Park, 2007). Whether it is the separation of the scion from the rootstock due to inappropriate securing tools, or the improper alignment between the vascular tissue of the scion and rootstock due to incompatibility, the efficient movement of water and nutrients between the two parts will be impeded. This can negatively affect the normal growth of the plant and also weaken the natural defenses of the plant, ultimately leading to plant death and grafting failure.

4.2.2 Approach grafting

With the unsatisfactory splice grafting method, we made new attempts utilizing approach grafting in the later stage of this project. Based on the results, only one of the six plants is currently being fully completed with all steps of approach grafting (including the final cutting). However, the death of this plant does not prove the unviability of the approach grafting method, as it is possible that we conducted the cutting too early (there are no photomicrographs to prove whether the vascular cells of the scion and rootstock are actually fused or not, and the fusion that we observed does not necessarily represent a true "fusion", but probably only partial). Therefore, observation of cell fusion under the microscope may be necessary in subsequent studies.

4.3. Future perspectives

Due to time constraints, we were unable to obtain the answer on whether approach grafting was successful or not. From observations of the seedling grafted using this method, approach grafting seems to be milder and more feasible. Future experiments could continue developing a successful grafting model for *N. heliophila* and *N. amplexicaulis* based on the approach grafting method. Once the reciprocal grafting methods are successfully determined, phenotype analysis can be performed. For instance, a Se dosing test can be conducted to test whether the reciprocal-grafted seedlings still have the ability to hyperaccumulate (can grafted seedlings survive healthily in nutrient solutions containing selenium at 0, 25, 50, and 75 μM, respectively?); Whether organic Se (inorganic to organic selenium transformation is considered an important selenium detoxification process) can be detected in shoot or leaf tissues of Nh/Na grafted seedlings, thus demonstrating that hyperaccumulation of Se in leaves of *N. amplexicaulis* is primarily dictated by root processes.

5. Conclusions

This project suggested a potentially successful protocol for germination of *N. heliophila* and *N. amplexicaulis* seeds in sterile environments: seeds are sown in plates with ½ MS medium and 1 mL demi water, the germination temperature is adjusted to 30°C with a 12-hour photoperiod. Furthermore, the splice grafting method is not ideal for the reciprocal grafting of *N. heliophila* and *N. amplexicaulis*, yet approach grafting might be a more promising alternative. The formulation of ideal grafting protocols between these two species will be a powerful tool for experimentation and analysis of Se accumulation and tolerance.

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