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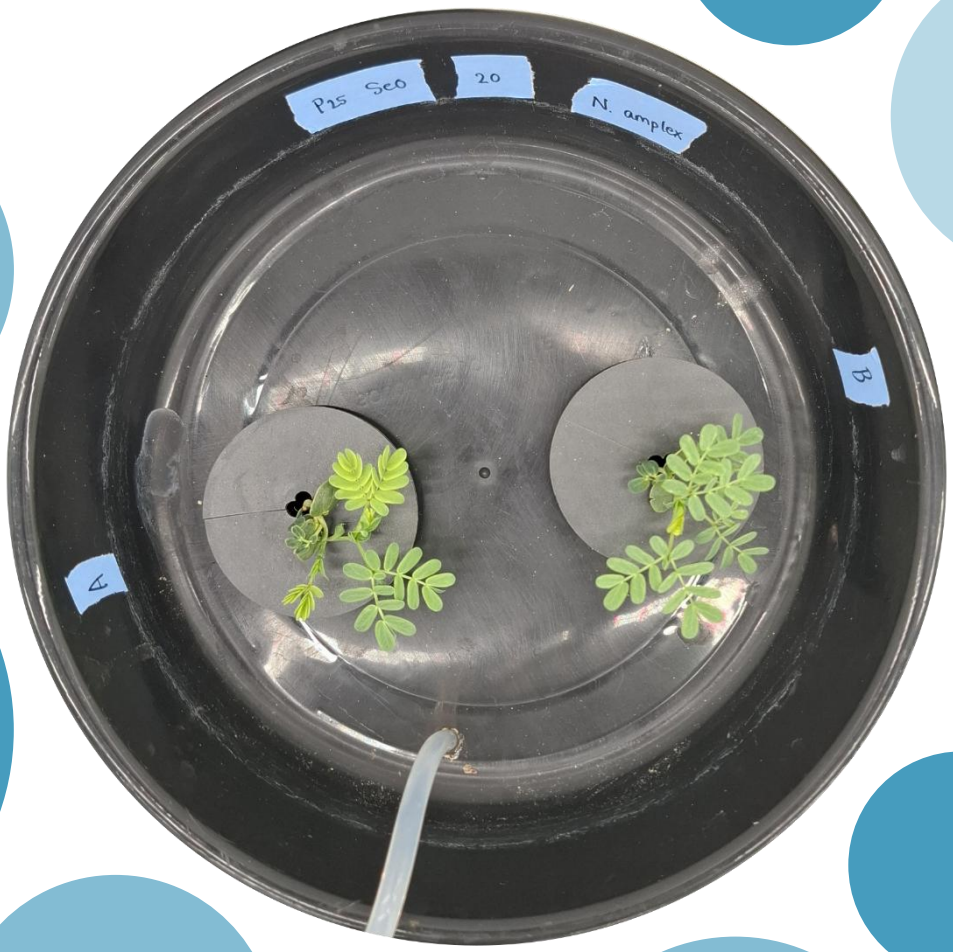
Anniek Blok



Studying the antagonistic relationship between phosphate and selenite uptake in *Neptunia amplexicaulis*

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Abstract

To improve selenium content in crops for human consumption, mechanisms of selenium uptake in selenium hyperaccumulators, such as *Neptunia amplexicaulis*, are studied. *N. amplexicaulis* naturally grows on soil that has both a high abundance of selenium (Se) and very low phosphorus (P) bioavailability. Considering the high concentrations at which *N. amplexicaulis* takes up Se (4,334 µg Se g^{-1} DW in nature), when we better understand the relationship between phosphate and selenite uptake in this plant, that knowledge can be applied to crop management, with the goal of increasing Se content in the plant. Therefore, this study researched the relationship between phosphate and selenite uptake in *N. amplexicaulis*. *N. amplexicaulis* and *N. heliophila*, a non-accumulator and close relative, were treated with different concentrations of phosphate and selenite using hydroponics. Their dry biomass, projected leaf area, photosynthetic efficiency, and P and Se concentrations in the roots and shoots were measured. Increasing phosphate supply decreased selenite uptake and vice versa in *N. amplexicaulis*. However, changing selenite supply concentrations had no effect on phosphate uptake. Moreover, *N. amplexicaulis* had a larger increase in phosphate uptake than *N. heliophila* when P bioavailability increased, suggesting that *N. amplexicaulis* does not sense when enough phosphate has been taken up. *N. amplexicaulis* may have even experienced phosphate toxicity when treated with the highest phosphate concentration (1,000 µM), seeing as growth in projected leaf area and root dry weight was significantly decreased compared to *N. amplexicaulis* treated with 100 µM phosphate. Thus, the previously described antagonistic relationship between phosphate and selenite uptake was partially true in *N. amplexicaulis*, and less phosphate should be applied to increase selenite uptake.

Key words: selenite, phosphate, selenium hyperaccumulator, *Neptunia amplexicaulis*, hydroponics, X-ray fluorescence

Table of Contents

Table of Figures

Table of Tables

1 Introduction

Selenium (Se) is a metalloid, and a vital trace element for many organisms [1]. In mammals, Se is required for the production of 25 selenoproteins, which are involved in processes such as DNA synthesis, ROS scavenging, hormone production, and immunity [2, 3]. Furthermore, Se supplementation helps to alleviate the effects of several viral infections, and is associated with a decreased chance of mutations in RNA viruses [1]. For example, Keshan disease was endemic to a Se-deficient region in China, until the people were provided with Se supplementation, entirely erasing the disease from this region [4]. Thus, it is unfortunate that an estimated 15% of the global population does not meet its required daily intake of 55-200 µg Se, putting their health at risk [3, 5].

The primary source of Se for human consumption is crops, and the main cause for large groups of people being Se-deficient is that these crops are low in Se themselves [5]. For one, this can be the case because the soil they are grown on is naturally low in Se, which is true for many regions in the world [6]. For example, about 50% of China's soil is considered Se-deficient [7] and in the 1980s, Finland had so little bioavailable Se in their soils that they started fertilising with Se, as they were aware of its health benefits [8]. Secondly, crops can be low in Se due to them lacking mechanisms to take up and/or tolerate higher levels of Se [5, 9]. Plants mostly take up Se via the roots in one of its inorganic forms, selenate (SeO_4^{2-}) or selenite (SeO_3^{2-}) [10–12]. Depending on its form, Se enters plants via different pathways: selenate is taken up by sulphur (S) transporters [10] and selenite is taken up, among others, by members of the phosphate transporter (Pht1) family [10, 12]. Although no evidence of Se essentiality in plants is found [13], low doses of Se can improve abiotic and biotic stress resistance [14–21]. However, when concentrations increase, Se quickly becomes toxic, causing oxidative stress when present in excess and protein misfolding and loss-of-function [3, 9, 22, 23].

A small group of plants evolved to take up much higher concentrations of Se than most, called Se hyperaccumulators [24]. These plants can accumulate Se at concentrations of > 1,000 µg g^{-1} DW and are naturally only found on seleniferous soils (> 2 µg Se g^{-1}) [24]. One of these hyperaccumulators is *Neptunia amplexicaulis*, which was found to accumulate up to 4,334 µg Se g^{-1} DW in nature and up to 13,600 µg Se g^{-1} DW in young leaves under experimental conditions [9, 25]. This species originates from a small region in Central Queensland, Australia, where the soil is seleniferous, with concentrations ranging from 10-69 µg Se g^{-1} [25].

Notably, *N. amplexicaulis*' native environment also has very low phosphorus (P) bioavailability [26–29]. P is a vital element for plants, as it is involved in many cellular processes, flower and seed development, and in the formation of energy-rich compounds [26]. The form of P that is easiest to efficiently take up by roots is $H_2PO_4^-$, which is mostly present at pH 5-6, and as soil pH increases, P uptake decreases [26, 27]. P uptake efficiency already decreases to 50% of its maximum when the soil pH is 7 [28]. With the soil pH in the region *N. amplexicaulis* originates from being 7-9.5, adaptions are needed to take up enough P [29].

N. amplexicaulis seems to have adapted to the extremely low P bioavailability that is the case in its natural habitat. Two previous students at the Laboratory of Genetics (WUR) both performed an

experiment with *N. amplexicaulis* and *N. heliophila*, a close relative and non-accumulator, which were exposed to varying concentrations of selenate (Van der Woude et al., unpublished). In the first experiment, the plants were grown on hydroponics, and in the second, they were grown on sand with perlite. At the end of the experiments, all plants were harvested and the P concentrations in the leaves were measured using X-ray fluorescence. When compiling the data of both experiments, a clear difference is visible between how these two species take up P (Figure 1). In hydroponics, P is more bioavailable than in sand with perlite, so more P was taken up by both *N. amplexicaulis* and *N. heliophila* when grown on hydroponics than on sand with perlite. However, for *N. amplexicaulis*, average P concentration in the leaves increased by a factor of 5.9, while for *N. heliophila*, this only increased by a factor of 3.2. Most plants require a P concentration of 2,000 ppm in their tissues [13], which is much lower than the median of ~15,000 ppm in *N. amplexicaulis* when grown on hydroponics. This could suggest that *N. amplexicaulis* has mechanisms in place that result in a constant uptake of P, not sensing whether enough P has already been taken up or not.

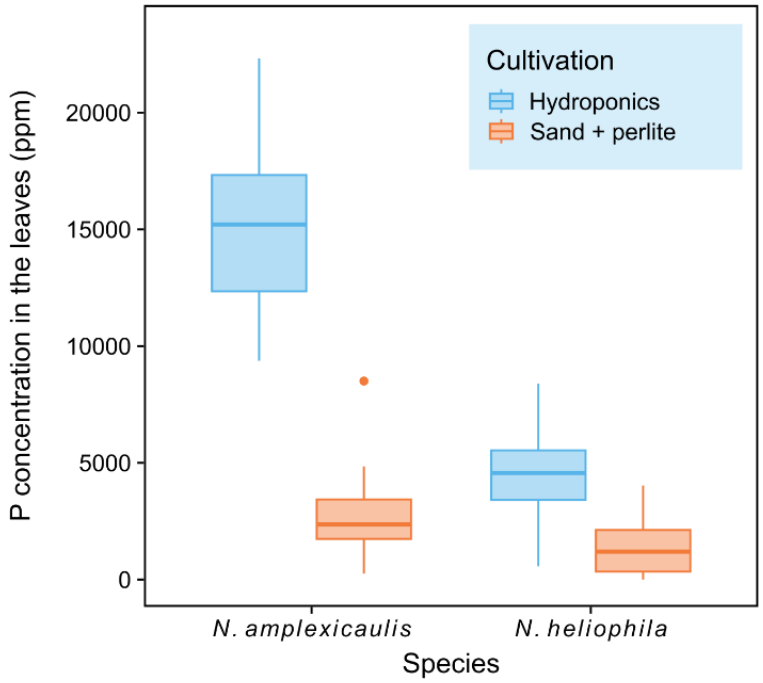

Figure 1. P concentrations in the leaves of *N. amplexicaulis* and *N. heliophila* measured by previous students. Each different method of cultivation was a separate experiment done by a different student (Van der Woude et al., unpublished). The plants in each experiment were treatments with different levels of selenate. P concentration is given in parts per million (ppm). The plants grown on hydroponics received either 0, 10, 15, 20, 40 or 80 µM selenate. The plants grown on sand with perlite received either 0 or 20 ppm selenate.

One of the mechanisms that may facilitate this different pattern of phosphate uptake by *N. amplexicaulis* is the number of phosphate transporter gene copies. The genome of both *N. amplexicaulis* and *N. heliophila* have previously been sequenced and this showed that *N. amplexicaulis* contains eleven more phosphate transporter gene copies than *N. heliophila* (Pinto-Irish et al., unpublished). While more gene copies can lead to one or multiple copies gaining a new function, i.e., neofunctionalisation, the function of the copies can also stay unchanged [30]. This

could result in an increased gene dosage, where the extra gene copies leads to more phosphate transporters, facilitating higher phosphate uptake [30].

Considering *N. amplexicaulis* likely has very active phosphate transporter genes and selenite is taken up via these transporters, it is worth looking further into the relationship between phosphate and selenite uptake. In 1972, Broyer et al. reported an antagonistic relationship between phosphate and selenite uptake for Se hyperaccumulators *Astragalus bisulcatus* and *Astragalus crotolariae*; with increasing selenite concentrations in the growing medium, phosphate uptake decreased, and vice versa [31, 32]. Additionally, they hypothesised that higher selenite application resulted in increased plant growth, not due to Se being important for plant growth, but due to selenite decreasing phosphate uptake and therefore preventing P toxicity [32]. In rice, it was also found that in P-deficient conditions, selenite uptake increased significantly [12]. However, this antagonistic relationship is not found with every plant species. For berseem (*Trifolium alexandrinum*), increased phosphate application resulted in higher selenite uptake and increased selenite application, up to 46 µM, resulted in higher phosphate uptake [33].

Due to these contradicting data, the interaction between selenite and phosphate uptake in Se hyperaccumulators should be validated. Additionally, *N. amplexicaulis* comes from a region where Se concentration is high and P bioavailability is low, making it all the more interesting to study if and how these two elements influence each other. It is possible that by increasing efforts in phosphate uptake in response to low P bioavailability has the side effect of also increasing selenite uptake. Thus, this report tries to answer the question: What is the relationship between phosphate and selenite uptake in *N. amplexicaulis* compared to *N. heliophila*?

To research this, two hydroponics experiments have been performed with *N. amplexicaulis* and *N. heliophila*, which were treated with different concentrations of phosphate and selenite. These experiments investigated (1) how the level of selenite uptake is influenced by different phosphate supply concentrations, (2) how the level of phosphate uptake is influenced by different selenite supply concentrations, (3) how the level of phosphate uptake is influenced by different phosphate supply concentrations, and (4) whether *N. amplexicaulis* experiences stress due to relatively high phosphate supply concentrations or low selenite supply concentrations. Stress in this scenario was quantified by measuring photosynthetic efficiency (Φ_{PSII}), dry weight (DW), and growth in projected leaf area (PLA), seeing as those parameters are negatively impacted by stress.

Based on literature, it was expected that higher phosphate supply concentrations would decrease selenite uptake for both plant species [31, 34–37]. Additionally, it was expected that higher selenite supply concentration will not decrease phosphate uptake for *N. amplexicaulis*. The plant species originates from a region with extremely low P bioavailability and high selenite soil concentrations [25–29], so a higher selenite supply concentration was not expected to influence the uptake of P, a vital element, that is already naturally in low supply. Furthermore, *N. amplexicaulis* was expected to be more stressed in low selenite/high P conditions, while the opposite was expected for *N. heliophila*.

Lastly, three attempts were made to isolate RNA from *N. amplexicaulis* and *N. heliophila* grown on different concentrations of phosphate and selenite. The goal was to measure the expression levels of the phosphate transporter genes *PHT1-1*, *PHT2-1*, and *PHT4-1*. One or multiple of these phosphate transporter genes were expected to be upregulated under low phosphate conditions [12].

2 Results

2.1 Lower phosphate supply influences Se uptake in *N. amplexicaulis*

To investigate the effects of different concentrations of phosphate supply on selenite uptake in *N. amplexicaulis*, a hydroponics experiment was set up. In this experiment, *N. amplexicaulis* and its close relative *N. heliophila* were exposed to one of three levels of phosphate (50, 1,000, and 5,000 µM) and one of two levels of Se (5 or 25 µM), where Se was supplied as a 1:1 ratio of selenate and selenite. The plants were placed in buckets, with each bucket containing two plants of the same species. The buckets were considered a replicate, and for all measurements, the average phenotypic value per bucket was used during data analysis. With this experimental set-up, there were four replicates for each treatments and plant species combination.

Considering the high phenotypic variation between *N. amplexicaulis* plants (A. van der Ent, personal communication, 18 December 2024), the treatments were changed after six days to have more replicates while maintaining the number of buckets. First, instead of two different concentrations of Se (1:1 ratio selenate and selenite), Se was given to all plants as only selenite at 25 µM. While selenate is the most available form of Se present in the natural habitat of *N. amplexicaulis* [25], selenite is the form of Se that is taken up via phosphate transporters [10, 12]. Therefore, by supplying Se solely as selenite, the focus was now on the relationship between phosphate and selenite uptake by phosphate transporters, and much less on the possible effects of P and Se plant tissue concentrations on each other's uptake. Second, instead of three different concentrations of phosphate, the plants now received either 100 or 1,000 µM phosphate. The plants previously receiving 50 µM phosphate were now given 100 µM phosphate, the plants previously receiving 5,000 µM phosphate were now given 1,000 µM phosphate, and the plants previously receiving 1,000 µM phosphate were now equally divided over the treatments 100 µM P and 1,000 µM phosphate. After changing the treatments, the experiment continued for another 26 days.

Higher phosphate uptake by N. amplexicaulis

First, phosphate uptake by *N. amplexicaulis* and *N. heliophila* in response to different phosphate supply concentrations was investigated. This could give an indication of how different phosphate supply concentrations may affect the activity of phosphate transporters in both species. Additionally, phosphate transporters also take up selenite, and to better understand phosphate and selenite uptake, it is important to investigate what factors affect phosphate transporter activity. The P concentration was measured for both the roots and shoots of all plants using X-ray fluorescence (XRF).

To begin, P concentration in the roots of *N. amplexicaulis* and *N. heliophila* responded differently to the treatments (Figure 2A; Two-way ANOVA; $F(1, 26) = 27.376$, $p < 0.001$). While both *N. amplexicaulis* and *N. heliophila* had higher P concentration in the roots when supplied with a higher phosphate concentration, this effect was much stronger in *N. amplexicaulis*. For *N. heliophila*, a higher phosphate treatment resulted in a 1.7-fold increase in average P concentration in the roots (Tukey

HSD post-hoc: p = 0.025), while this was a 5.2-fold increase for *N. amplexicaulis* (Tukey HSD post-hoc: p < 0.001). Similar results were found for P concentration in the shoots, where *N. amplexicaulis* responded much stronger to the phosphate treatments than *N. heliophila* (Figure 2B; Two-factor ART ANOVA: F(1, 44) = 15.765, p = 0.003). Compared to the low phosphate treatment, the high phosphate treatment resulted in a much larger increase in P concentration for *N. amplexicaulis* (Tukey HSD post-hoc: p < 0.001) than for *N. heliophila* (Tukey HSD post-hoc: p = 0.023), 3.2-fold and 1.7-fold, respectively.

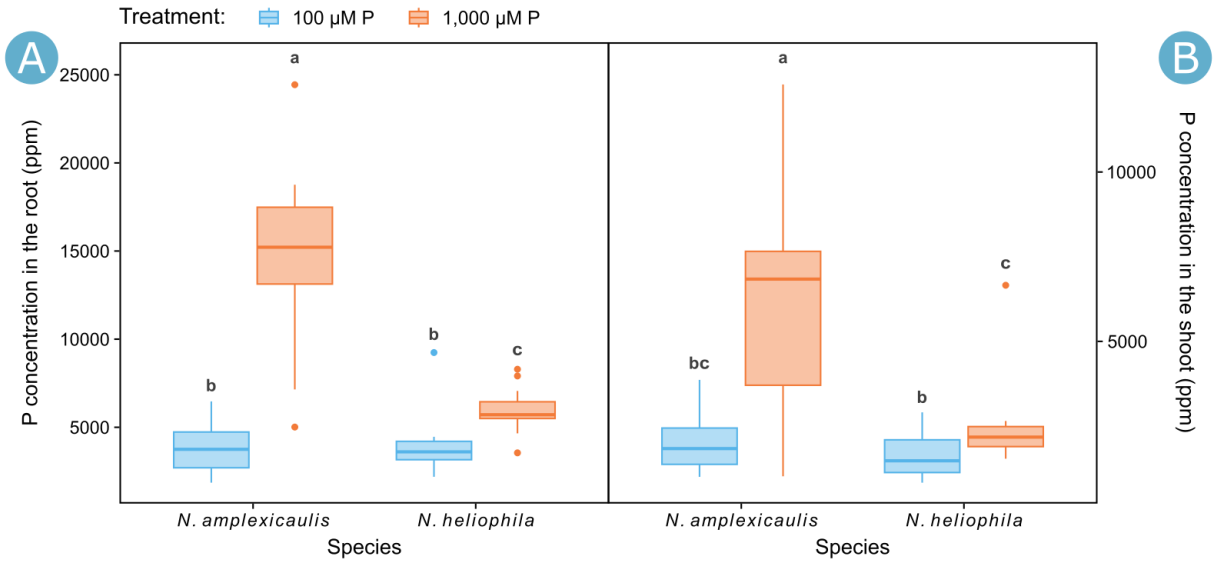

Figure 2. P concentration in the (A) root and (B) shoot of *N. amplexicaulis* and *N. heliophila*. (A, B) The treatments were either 100 or 1,000 µM phosphate. All plants received 25 µM selenite. Each replicate is the average of two plants within a single bucket, resulting in twelve replicates. Significant differences between different treatments were calculated with Tukey HSD test (α = 0.05).

Antagonistic relationship between phosphate and selenite

Next, the aim was to investigate whether different phosphate supply concentrations influenced selenite uptake in *N. amplexicaulis* and *N. heliophila*. To do so, Se concentrations were measured for both root and shoot tissues of *N. amplexicaulis* and *N. heliophila* using XRF.

First, the phosphate treatments significantly affected Se concentration in the root for both species (Figure 3A; Two-way ANOVA: F(1, 25) = 12.232, p = 0.002). With the high phosphate treatment, Se concentration in the root of *N. amplexicaulis* was significantly lower compared to that with a low phosphate treatment (Tukey HSD post-hoc: p = 0.028). For *N. heliophila*, Se concentration in the root was not significantly affected by the treatments. Additionally, *N. amplexicaulis* had higher Se concentration in the root compared to *N. heliophila*, independent of the treatment (Two-way ANOVA: F(1, 25) = 10.706, p = 0.003). Specifically for the low phosphate treatment, *N. amplexicaulis* had significantly higher Se concentration in the root than *N. heliophila* (Tukey HSD post-hoc: p = 0.044).

In contrast, the different phosphate supply concentrations did not significantly affect Se concentration in the shoot for both *N. amplexicaulis* and *N. heliophila* (Figure 3B). While a two-factor ART ANOVA showed the species to have a significant effect on Se concentration in the shoot (Two-

factor ART ANOVA: F(1, 44) = 5.197, p = 0.028), a Tukey HSD post-hoc did not reveal any groups to be significantly different. Notably, there is a relatively large variation in Se concentration in the shoot for *N. amplexicaulis* treated with high phosphate.

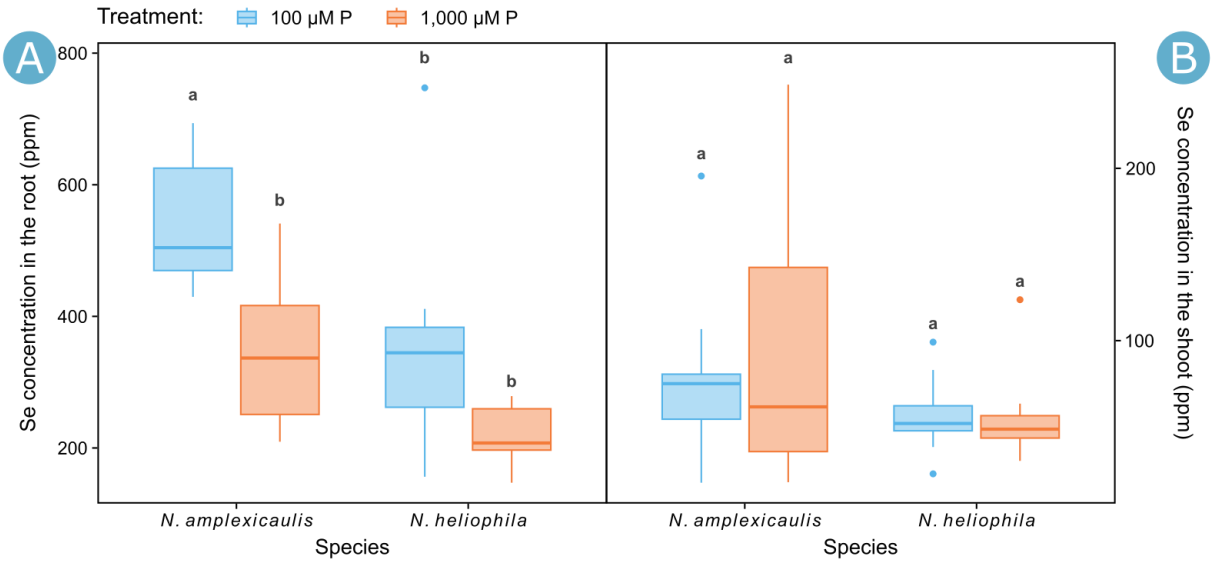

Figure 3. Se concentration in the (A) root and (B) shoot of *N. amplexicaulis* and *N. heliophila*. (A, B) The treatments were either 100 or 1,000 µM phosphate. All plants received 25 µM selenite. Each replicate is the average of two plants within a single bucket, resulting in twelve replicates. Significant differences between different treatments were calculated with Tukey HSD test (α = 0.05).

In addition to the ionomics data, a possible indication of the strong uptake of selenite by *N. amplexicaulis* under low phosphate conditions was visible for one of the plants by eye (Figure 4). This plant had strong red colouring on the roots, which was likely caused by a high accumulation of elemental red Se (Se^0) on the surface (Harvey, unpublished). Red roots such as these were only found with a few *N. amplexicaulis* plants grown under low phosphate conditions.

Figure 4. Red roots of *N. amplexicaulis* as indication of accumulated Se^0. The red colouring on the surface of the roots were only found with a few *N. amplexicaulis* plants grown on 100 µM phosphate.

Effect of increasing phosphate supply on plant growth and photosynthetic efficiency

When determining the optimal growing conditions for *N. amplexicaulis* to accumulate high concentrations of Se, environmental factors should not only be scored on whether they increase Se accumulation, but also on whether they cause any stress to the plants. One possible cause of stress for *N. amplexicaulis* could have been phosphate toxicity due to excessively high phosphate uptake, relative to *N. heliophila* [32]. A good indicator of stress is the photosynthetic efficiency (Φ_{PSII}) [38], which was measured during this experiment one day after the treatments were last refreshed. From this data, the phosphate treatments were not found to be significantly affected by Φ_{PSII} (Figure 5A). However, the two species did have significantly different Φ_{PSII}, independent of the treatments (Two-factor ART ANOVA: $F(1, 44) = 63.003$, $p < 0.001$). *N. amplexicaulis* had lower Φ_{PSII} than *N. heliophila* under both low and high phosphate conditions (for both, Tukey HSD post-hoc: $p < 0.001$).

Another effect of phosphate toxicity is a decrease in plant growth [39]. Therefore, the relative growth in projected leaf area (PLA) was calculated for each plant by dividing the PLA on the last day of the experiment by the PLA on the first day treatments were given. Relative growth in PLA for *N. amplexicaulis* and *N. heliophila* was differently affected by the treatments (Figure 5B; Two-way ANOVA: $F(1, 44) = 7.120$, $p = 0.011$). While there was no significant difference in relative growth in PLA for *N. heliophila* treated with low and high phosphate, the high phosphate treatment did result in a significantly lower relative growth in PLA for *N. amplexicaulis* compared to the low phosphate treatment (Tukey HSD post-hoc: $p = 0.0495$).

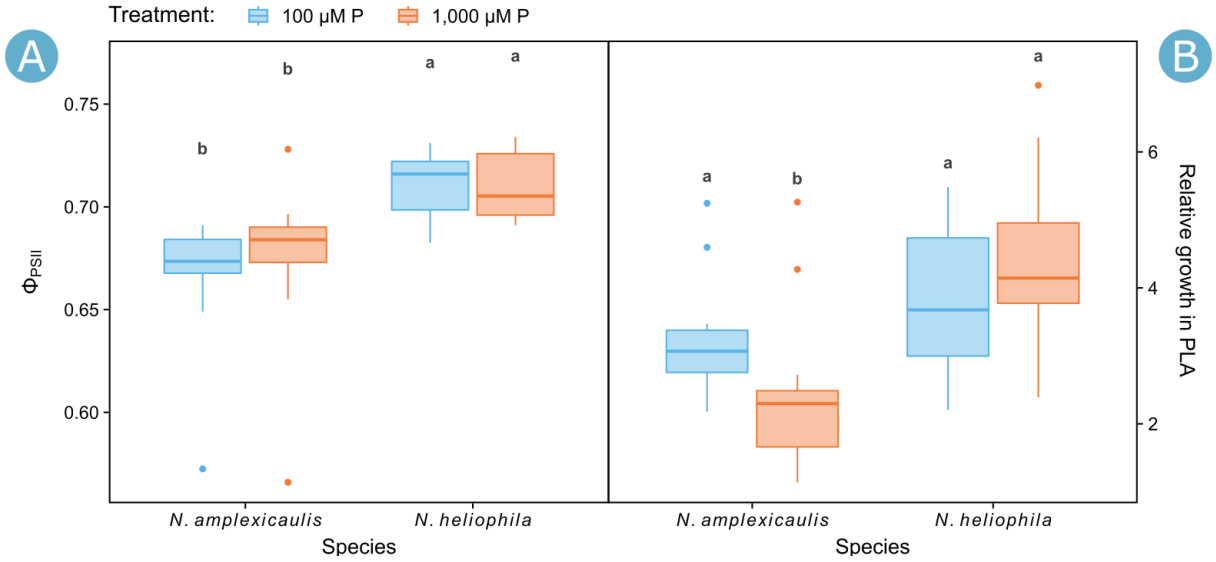

Figure 5. (A) Φ_{PSII} and (B) relative PLA growth of *N. amplexicaulis* and *N. heliophila*. (A, B) The treatments were either 100 or 1,000 µM phosphate. All plants received 25 µM selenite. Each replicate is the average of two plants within a single bucket, resulting in twelve replicates. Significant differences between different treatments were calculated with Tukey HSD test ($\alpha = 0.05$).

Lastly, the final dry weight (DW) of the plants were measured, which could give an indication of whether plant growth may have been affected during the experiment. For this, the roots and shoots of each plant were cut away from each other and separately dried and weighed. First, root DW of *N. amplexicaulis* and *N. heliophila* was differently affected by the treatments (Figure 6A; Two-way

ANOVA: F(1, 42) = 16.006, p < 0.001). While *N. heliophila* did not have significantly different root DW due to changing phosphate supply concentrations, *N. amplexicaulis* had significantly lower root DW when treated with high phosphate compared to low phosphate (Tukey HSD post-hoc: p < 0.001). Similarly, the treatments had different effects on shoot DW for both species (Figure 6B; Two-way ANOVA: F(1, 42) = 15.286, p < 0.001). Only this time, *N. amplexicaulis* was not affected, while *N. heliophila* had significantly higher shoot DW when treated with high phosphate compared to low phosphate (Tukey HSD post-hoc: p = 0.019).

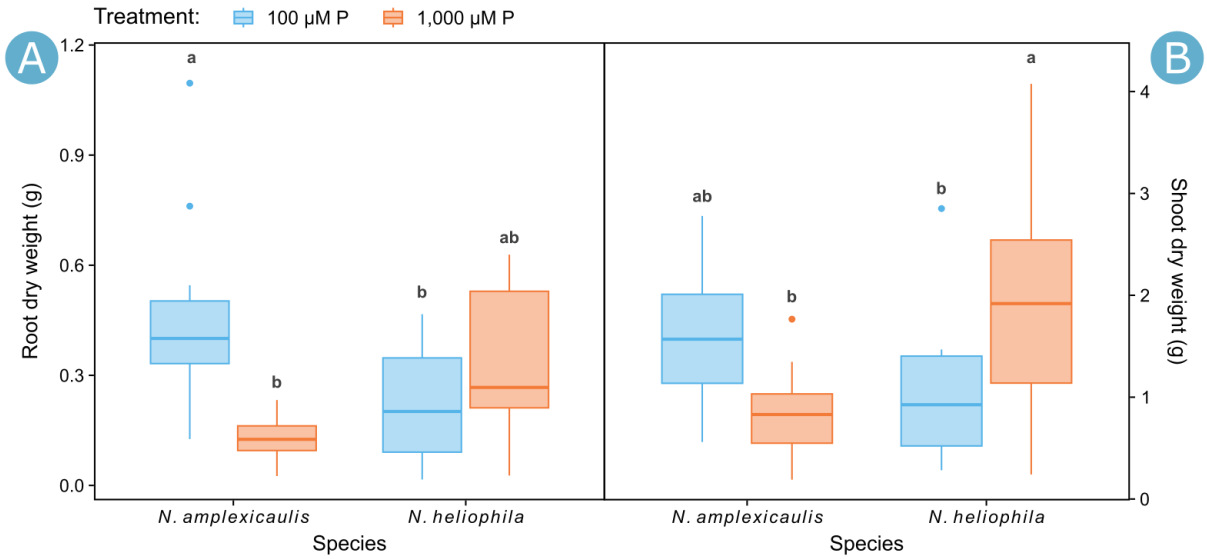

Figure 6. (A) Root and (B) shoot dry weight (DW) of *N. amplexicaulis* and *N. heliophila*. (A, B) The treatments were either 100 or 1,000 µM phosphate. All plants received 25 µM selenite. Each replicate is the average of two plants within a single bucket, resulting in twelve replicates. Significant differences between different treatments were calculated with Tukey HSD test (α = 0.05).

2.2 Relationship between phosphate and selenite uptake

In the first hydroponics experiment, only the phosphate supply differed between treatments while selenite concentration was constant. However, it is not only valuable to research which concentrations of phosphate supply possibly increase selenite uptake, but also whether the concentration of selenite supply influences phosphate uptake. P is a vital element for plants, required for many cellular processes and the production of energy-rich compounds [26]. When plants are grown with the purpose of harvesting high levels of Se, they also need to take up enough P to grow well. With that in mind, a second hydroponics experiment was conducted in which both phosphate and selenite supply concentrations were altered. This allowed the investigation of not only the effects of phosphate supply concentrations on selenite uptake, but also the effects of selenite supply concentrations on phosphate uptake in *N. amplexicaulis* and *N. heliophila*. In this experiment, the plants received one of three treatments: 25 µM phosphate and no selenite (+P/-Se), 25 µM phosphate and 25 µM selenite (+P/+Se), and 5 µM phosphate and 25 µM selenite (-P/+Se). The phosphate concentrations in this experiment was much lower than in the first hydroponics experiment in order to trigger a stronger P deficiency response in the plants [34, 40]. Additionally, in the treatment where phosphate and selenite were supplied at the highest concentration, they were

both supplied at 25 µM. This made them equal competition in being taken up by phosphate transporters.

Effect of different treatments on growth of N. amplexicaulis

To investigate under which conditions *N. amplexicaulis* and *N. heliophila* grew best, at the end of the second hydroponics experiment, all plants were harvested, dried, and their dry weight (DW) was measured, root and shoot separately. The results showed that the treatments had no different effects on root DW of the two species (Figure 7A). Nevertheless, the treatments did have an effect on root DW (Two-way ANOVA: $F(2, 42) = 19.034$, $p < 0.001$). More specifically, *N. amplexicaulis* grown on -P/+Se had a significantly lower root DW than when grown on +P/+Se (Tukey HSD post-hoc: $p = 0.008$). Additionally, species was found to significantly influence root DW (Two-way ANOVA: $F(1, 42) = 31.113$, $p < 0.001$). Namely, when treated with +P/-Se, *N. amplexicaulis* had significantly lower root DW than *N. heliophila* grown under the same conditions (Tukey HSD post-hoc: $p < 0.001$).

Next, while +P/-Se and +P/+Se did not cause differences in shoot DW for *N. amplexicaulis*, it did for *N. heliophila* (Figure 7B; Two-way ANOVA: $F(2, 42) = 7.348$, $p = 0.002$). N. heliophila treated with +P/+Se had significantly lower shoot DW than when treated with +P/-Se (Tukey HSD post-hoc: $p = 0.009$). Treatment also had a strong effect on shoot DW for both species (Two-way ANOVA: $F(2, 42) = 38.484$, $p < 0.001$). The treatment -P/+Se caused a significantly lower shoot DW compared to +P/+Se in *N. amplexicaulis* (Tukey HSD post-hoc: $p < 0.001$), as well as in *N. heliophila* (Tukey HSD post-hoc: $p = 0.001$). Furthermore, as was the case for root DW, the species had a significant effect on shoot DW (Two-way ANOVA: $F(1, 42) = 44.596$, $p < 0.001$). This was visible for the plants grown on +P/-Se, where *N. amplexicaulis* again had significantly lower DW than *N. heliophila* (Tukey HSD post-hoc: $p < 0.001$).

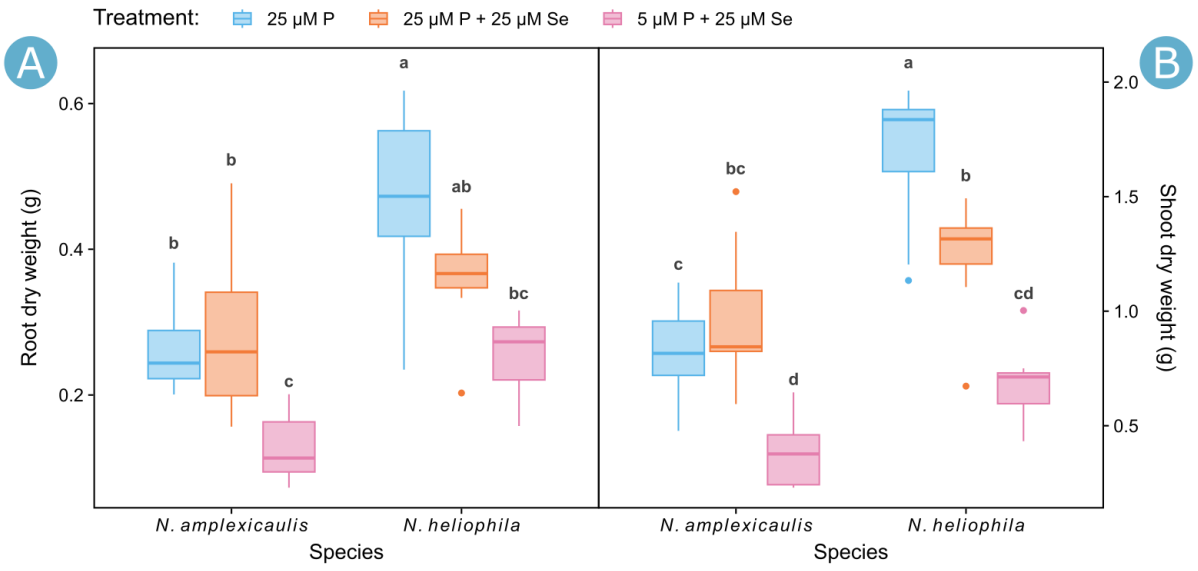

Figure 7. (A) Root and (B) shoot dry weight (DW) of *N. amplexicaulis* and *N. heliophila*. (A, B) The plants received one of three treatments: 25 µM phosphate and no selenite, 25 µM phosphate and 25 µM selenite, or 5 µM phosphate and 25 µM Se. Each replicate is the average of two plants within a single bucket, resulting in eight replicates. Significant differences between different treatments were calculated with Tukey HSD test ($\alpha = 0.05$).

Phosphate uptake by N. heliophila was influenced by changing Se supply concentrations

To research whether phosphate uptake by *N. amplexicaulis* and *N. heliophila* is influenced by selenite supply concentrations, P concentrations in root and shoot tissues of both species were measured using XRF. The two species responded differently to the treatments in terms of P concentration in the roots (Figure 8A; Two-way ANOVA: $F(2, 42) = 6.515$, $p = 0.003$). While there were no differences between P concentrations in the root for *N. amplexicaulis*, *N. heliophila* grown on +P/-Se had lower root P concentration than those grown on +P/+Se (Tukey HSD post-hoc: $p = 0.009$). Species also had a significant effect on root P concentration (Two-way ANOVA: $F(1, 42) = 35.858$, $p < 0.001$), though this was only visible with the treatment +P/-Se. With that treatment, *N. heliophila* had significantly lower P concentration in the root than *N. amplexicaulis* (Tukey HSD post-hoc: $p < 0.001$). In the shoot, P concentration was only significantly influenced by the species (Figure 8B; Two-way ANOVA: $F(1, 42) = 38.677$, $p < 0.001$). *N. heliophila* had lower P concentration in the shoot compared to *N. amplexicaulis* both when treated with +P/-Se (Tukey HSD post-hoc: $p = 0.001$) and -P/+Se (Tukey HSD post-hoc: $p = 0.005$).

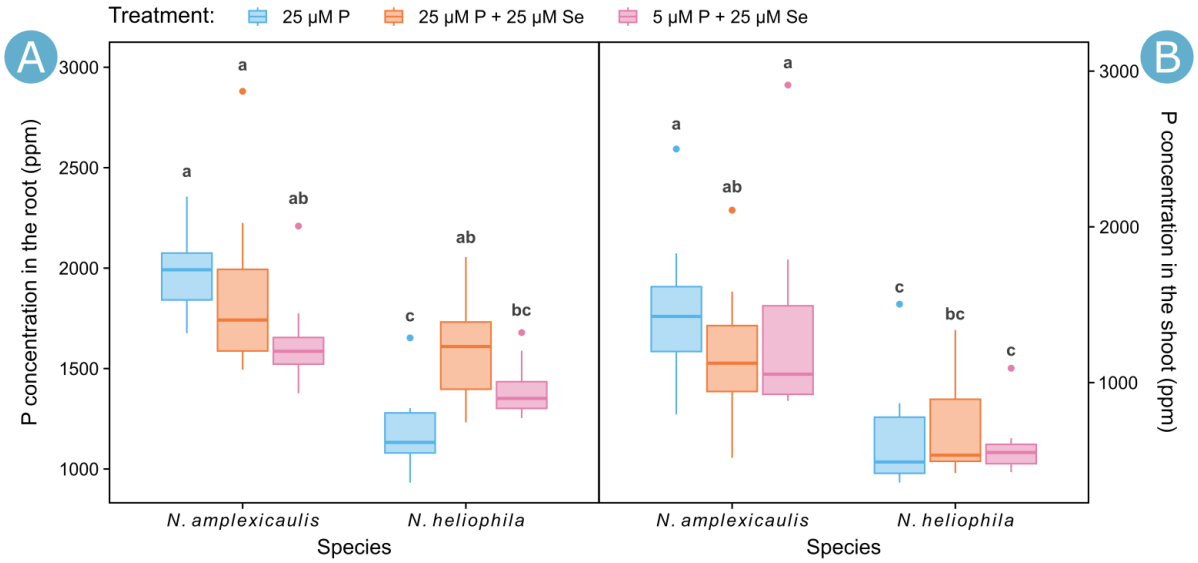

Figure 8. P concentration in the (A) root and (B) shoot of *N. amplexicaulis* and *N. heliophila*. (A, B) The plants received one of three treatments: 25 µM phosphate and no selenite, 25 µM phosphate and 25 µM selenite, or 5 µM phosphate and 25 µM Se. Each replicate is the average of two plants within a single bucket, resulting in eight replicates. Significant differences between different treatments were calculated with Tukey HSD test ($\alpha = 0.05$).

Selenite uptake was influenced by changing P supply concentration

In the first hydroponics experiment, phosphate was given at either 100 or 1,000 µM, a ten-fold difference. There is more phosphate transporter activity when plants are P-deficient, so, to get an even higher activity of phosphate transporters, phosphate was given at either 5 or 25 µM in this second hydroponics experiment. To research whether selenite uptake is also influenced by these low phosphate supply concentrations, Se concentrations in the root and shoot of *N. amplexicaulis* and *N. heliophila* was measured with XRF.

Root Se concentration of the two species was differently affected by the treatments (Figure 9A; Two-factor ART ANOVA: $F(2, 42) = 11.889$, $p < 0.001$). However, the only treatment in which *N. amplexicaulis*

had different response in root Se concentration than *N. heliophila* was +P/-Se, where *N. amplexicaulis* had an average of 75.70 ppm Se and *N. heliophila* on average 4.11 ppm Se (Tukey HSD post-hoc: $p = 0.009$). Next, the plant species had a significant effect on root Se concentration (Two-factor ART ANOVA: $F(1, 42) = 77.248$, $p < 0.001$), with *N. amplexicaulis* having higher root Se concentrations than *N. heliophila* in the case of all three treatments (for both +P/+Se and -P/-Se, Tukey HSD post-hoc: $p < 0.001$).

In the case of Se concentration in the shoot, the treatments had different effects on the *N. amplexicaulis* and *N. heliophila* (Figure 9B; Two-way ANOVA: $F(2, 42) = 188.58$, $p < 0.001$). While, *N. heliophila* had no different shoot Se concentration when treated with +P/+Se or -P/-Se, *N. amplexicaulis* had higher shoot Se concentration when treated with -P/-Se compared to when treated with +P/+Se (Tukey HSD post-hoc: $p = 0.002$). Additionally, species had a clear effect on Se concentration in the shoot (Two-way ANOVA: $F(1, 42) = 503.78$, $p < 0.001$), seeing as *N. amplexicaulis* had higher Se concentrations than *N. heliophila* with all treatments (for +P/-Se, Tukey HSD post-hoc: $p < 0.001$; for +P/+Se, Tukey HSD post-hoc: $p = 0.006$; for -P/+Se, Tukey HSD post-hoc: $p < 0.001$).

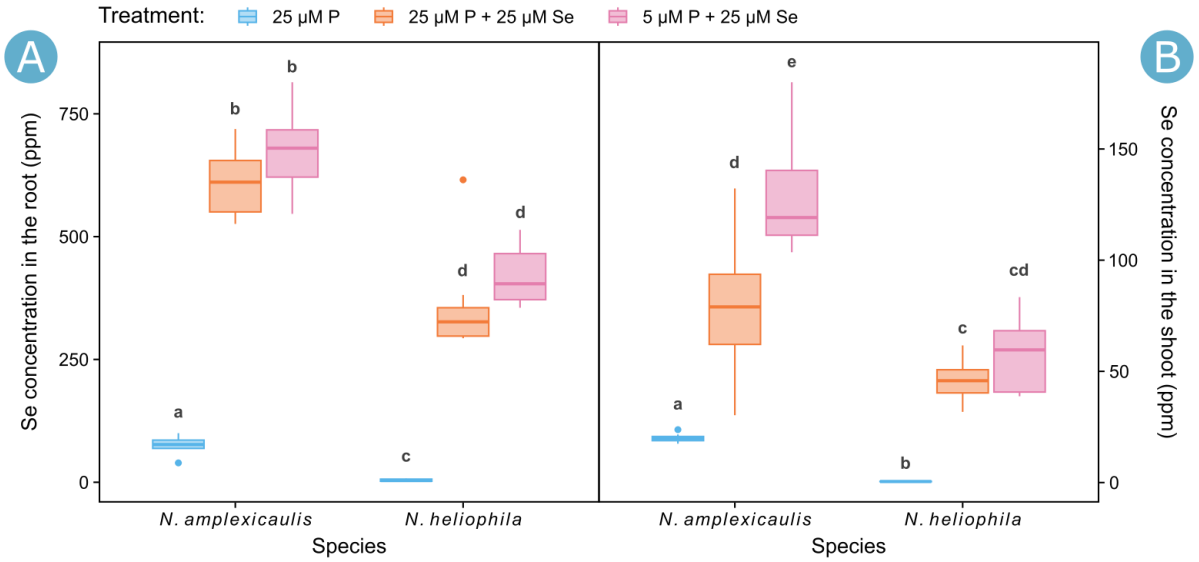

Figure 9. Se concentration in the (A) root and (B) shoot of *N. amplexicaulis* and *N. heliophila*. (A, B) The plants received one of three treatments: 25 µM phosphate and no selenite, 25 µM phosphate and 25 µM selenite, or 5 µM phosphate and 25 µM Se. Each replicate is the average of two plants within a single bucket, resulting in eight replicates. Significant differences between different treatments were calculated with Tukey HSD test ($\alpha = 0.05$).

2.3 Expression levels of phosphate transporter genes

RNA isolation

The goal was to measure the expression levels of several phosphate transporter genes in *N. amplexicaulis* and *N. heliophila* in response to the different treatments in the second hydroponics experiment using RT-qPCR. This data, combined with the previously measured phenotypic data, could give an idea of how phosphate transporter genes respond to different phosphate and selenite supply concentrations and which phosphate transporters are more involved in the observed responses to the treatments than others. To do so, the first step was to isolate RNA from *N.*

amplexicaulis and *N. heliophila* root and shoot tissues that grew in the second hydroponics experiment. These samples were taken 37 days after the treatments started, one day after the nutrient solution was last refreshed.

To isolate RNA, a protocol specifically for RNA isolation from *N. amplexicaulis* plant tissues was followed, which was provided by Subash Rai from the University of Queensland (Appendix B). This protocol takes the extraction method from Acosta-Maspons et al. [41] and uses a TRIzol method in the final steps. According to the protocol by Subash Rai, final RNA concentrations of 146-164 ng/µL were to be expected.

In total, three trials were run in an attempt to isolate enough RNA, however, none of the trials were considered to be successful (Appendix C). First, while a minimum RNA concentration of 146 ng/µL was to be expected according to the protocol, the highest RNA concentration obtained during the three trials was approximately 40 ng/µL. That is less than a third of the expected concentration. Additionally, the results were very inconsistent. While from some samples an RNA concentration of around 30 ng/µL was obtained, close to 0 ng/µL was obtained from most other samples according to the NanoDrop™. This indicates that the protocol was not properly performed.

The first trial was set up as a test run, which used samples from both the first and second hydroponics experiment. Due to too little plant tissue being sampled from plants in the first hydroponics experiment, all samples from the same treatments (species, treatment, and plant material sampled) were combined into one sample for the RNA extraction. Next, from the second hydroponics experiment, two root and two shoot samples were taken from a *N. amplexicaulis* plant that received +P/+Se treatment and from a *N. heliophila* plant that received +P/-Se treatment. These test samples were taken 26 days after treatments started.

During the first trial, the protocol was followed exactly as it was first provided. In this first edition from Subash Rai, one of the steps (step 6) was accidentally not included. In this step, 1 mL should have been taken from the ground up sample that was previously homogenised with 3 mL preheated RNA extraction buffer and dispensed into a new 2 mL tube. The following step was then to add 1 mL chloroform:isoamyl alcohol. However, the chloroform:isoamyl alcohol should have been added in equal volume as the RNA extraction buffer, and by skipping step 6, the chloroform:isoamyl alcohol was now added at one-third volume.

Furthermore, by not transferring 1 mL of homogenised sample suspension to a 2 mL tube, the suspension was kept in a 15 mL tube, requiring a larger centrifuge that was only able to go up to 3,220*g* instead of 10,000*g* in step 7. Finally, there was no spermidine trihydrochloride present in the lab when running this trial, so spermidine was used instead. Out of the sixteen samples, RNA was only successfully isolated from two leaf samples that both were taken from *N. heliophila* in the first hydroponics, one grown on low P and the other on high P. The isolated RNA had a concentration of 30-40 ng/µL.

The second trial of RNA isolation was done with only samples taken from plants in the second hydroponics experiment. A single root and shoot sample was taken for every treatment (species and treatment). The first change in the protocol for this trial was that spermidine trihydrochloride was now available and used instead of spermidine. This was also the case for the third trial. Additionally, after communication with Subash Rai, it was found out that the 6th step was missing from the protocol. However, due to the viscosity, it was incredibly difficult to pipet exactly 1 mL of homogenised sample suspension. Therefore, this step was skipped again. Finally, the drying during step 18 took much longer than according to the protocol, roughly 25 minutes.

In the second trial, only three out of twelve samples had an isolated RNA concentration of more than 20 ng/µL: root sample of *N. amplexicaulis* with treatment +P/-Se (22.762 ng/µL), root sample of *N. heliophila* with treatment +P/+Se (26.862 ng/µL), and root sample of *N. heliophila* with treatment -P/+Se (21.757 ng/µL) (Appendix C). These concentrations are much lower than the expected 146-164 ng/µL.

In the third trial, only root samples were used from plants in the second hydroponics experiment, mainly due to a lack of time and the focus of the project being more on phosphate and selenite uptake by the roots than other genetic mechanisms happening further downstream.

Before running this trial, Francisca Reyes Marquez (lab technician at Laboratory of Genetics) helped point out that the RNA isolation may likely have been going wrong during step 4 of the protocol. In this step, it was specifically stated to grind up the plant material with a mortar and pestle that was first cooled with liquid nitrogen. However, during previous trials, other items also touched the plant material without first being cooled, mainly a tweezer to transfer the sample over to the mortar, a 15 mL tube in which the ground up material is stored, and a metal spoon that was used to transfer the ground up sample to the 15 mL tube. Therefore, during this third trial, all items that would touch the samples during step 4 were first chilled with liquid nitrogen.

Next, the pipetting of 1 mL of homogenised sample suspension in step 6 was done this time, though it was still difficult due to the viscosity. For this reason, there was some slight inconsistency in the amount of sample suspension that was transferred to the new tube. Additionally, during step 13, the supernatant was further removed by pipetting after decanting, because quite some supernatant was still present after only decanting. The drying step in 18 again took longer than according to the protocol (roughly 35 minutes), so more supernatant was pipetted off during the drying of the samples to speed up the process. Finally, during step 18, the water and pellet was accidentally not mixed by pipetting before putting the samples in a heat block at 65 °C. Therefore, instead, the samples were stored at -20 °C overnight and thawed for about 15 minutes the next day. The pellets were then mixed with the water by pipetting and were subsequently put in a heat block set at 65 °C for 3 minutes. Afterwards, the samples were put on ice before measuring RNA concentration.

Finally, the third trial was least successful in isolating RNA, with only one sample having more than 10 ng/µL: a root sample of *N. amplexicaulis* with treatment -P/+Se (10.965 ng/µL) (Appendix C). Due

to all trials being considered unsuccessful, other than designing primers for genes of interest, no further steps were taken to measure expression levels of P transporter genes.

Primer design

Before the primers could be designed, Genes of Interest (GOI) had to be identified. The genomes of *N. amplexicaulis* and *N. heliophila* were previously sequenced and a selection was made of all phosphate transporter genes (Pinto-Irish et al., unpublished). In literature, members of the phosphate transporter Pht1 family, namely *OsPT2* and *OsPT8*, were found to take selenite up via the roots [10, 12, 42]. Additionally, expression data of *N. amplexicaulis* and *N. heliophila* became available after an RNA sequencing experiment performed at the University of Queensland (Van der Ent et al., unpublished). This expression data showed the expression of a gene relative to all other genes for root and shoot tissue of both plant species treated with increasing levels of selenate. From this data, *PHT1-1*, *PHT2-1*, and *PHT4-1* were chosen as GOIs. *PHT1-1* was chosen as the relative expression continuously increased in *N. amplexicaulis* when selenate concentration increased, and it did not in *N. heliophila*. Next, *PHT2-1* and *PHT4-1* were chosen as their relative expression levels increased in *N. amplexicaulis* when selenate concentration increased, while their expression decreased in *N. heliophila*. Lastly, for all three genes, a forward and reverse primer were designed for both *N. amplexicaulis* and *N. heliophila* (Table 1).

Table 1. Primers designed for RT-qPCR. The primers are written down from 5' end to 3' end.

Gene	Species	Forward primer	Reverse primer
PHT1-1	*N. amplexicaulis*	CCATTGGGTCTTGGCCAAATCG	TCCCCCTCCTCTCTTTCTTTCC
	N. heliophila	GGTACCATTTCACGGCCATTGT	GCAAAGAGCCTGGCTTTGGC
PHT2-1	*N. amplexicaulis*	AGGATACTCTGCTCTTCGCTGG	AGTGTGTGGTAGACACTGGCC
	N. heliophila	TGCTCCGACTATACAGTGTGTGG	CTGGATTGCTCTCTTCGCTTGC
PHT4-1	*N. amplexicaulis*	ACCTACTTCTCTCCGCTACCGG	CCTGTTGCTGCGAAACTCGG
	N. heliophila	CGCAGTTCAGGATCATCAAGAGG	ATTCATCGGTTTGGATGGCC
Actin	*N. amplexicaulis*	TGTATGTTGCTATCCAGGCCG	TGTGTGGCTCACACCATCACC
	N. heliophila	GCTGACGCTGAGGATATTCAACC	AGGTCGTCCAACAATGCTGG
UBC	*N. amplexicaulis*	CTGGCTCACTGAGAAGACGC	ACAAACCCTATTGCGTGATTTGC
	N. heliophila	CTGAAAGATAAGTGGAGCCCTGC	GGCAATGTTCTCAGAAAGCGG
β-tubulin	*N. amplexicaulis*	GACAACAGGTAACTCCACTCATGG	GCATGGTCTTGGACAATGAGGC
	N. heliophila	ATGTTGTGAGGAAGGAGGCCG	AGTGTTCCCATTCCTGAGCC

3 Discussion & Conclusion

Se is a vital trace element for humans, in which approximately 15% of the global population is deficient [1, 3, 5]. In an effort to increase Se content in crops for human consumption [5], the methods in which plants take up Se has been studied in several plants [10–12, 31–33, 35]. One of the forms in which Se is mostly taken up is selenite, which is taken up by phosphate transporters [10, 12]. Many papers have described the influence of phosphate on selenite uptake, where an adequate application of phosphate increases selenite uptake, but higher phosphate concentrations decrease selenite uptake [31, 34–37]. However, this is not the case for all plants, as increasing phosphate supply increased selenite uptake in berseem [33]. Though, fewer studies have been done on the influence of selenite application on phosphate uptake, it is suggested that higher selenite application decreases phosphate uptake [31, 33, 43].

This interaction between phosphate and selenite uptake has hardly been studied in Se hyperaccumulators such as *N. amplexicaulis*, a species that originates from a region where P bioavailability is low and Se content is high [25–29]. If the antagonistic relationship between phosphate and selenite uptake hypothesised in other studies is true for *N. amplexicaulis*, the low P bioavailability would significantly increase selenite uptake and the high selenite content in the soil would decrease phosphate uptake. When it is better understood how phosphate and selenite application influence each other's uptake in a Se hyperaccumulator, that knowledge can be applied to how these plants can best be cultivated to increase their Se content. Therefore, the aim of this research was to study the effects of changing phosphate and selenite supply concentrations on the uptake of both elements in *N. amplexicaulis*.

During this study, two hydroponics experiments were performed with the plant species *N. amplexicaulis* and *N. heliophila*. *N. heliophila* is a non-accumulator and close relative to *N. amplexicaulis* and thus functioned as a control. In the first experiment, the plants were treated with either 100 µM or 1,000 µM phosphate and all plants were also provided with 25 µM selenite. This experiment allowed to study how changing supply concentrations of phosphate influenced selenite uptake. In the second experiment, the plants were treated with either only 25 µM phosphate, 25 µM phosphate and 25 µM selenite, or 5 µM phosphate and 25 µM selenite. With this experiment, the influence of changing selenite supply concentrations on phosphate uptake could be studied. Additionally, the second experiment had a much lower phosphate supply concentration than the first experiment, likely resulting in a stronger P-deficiency in the plants [34, 40]. Finally, three attempts were made at isolating RNA from *N. amplexicaulis* and *N. heliophila* in an effort to measure expression levels of the phosphate transporter genes *PHT1-1*, *PHT2-1*, and *PHT4-1*.

3.1 Hydroponics experiments

Change in phosphate uptake due to changing phosphate supply concentrations

The first hydroponics experiment showed that when phosphate supply concentrations increased, there was a much larger increase in P concentration in both root and shoot tissue for *N. amplexicaulis* than for *N. heliophila*. Moreover, the P concentration in tissue required by most plants is 2,000 ppm

[13], and with a median of 15,000 ppm P in the root and a median of 7,000 ppm P in the shoot, *N. amplexicaulis* took up excessive amounts of phosphate. In usual cases, when plants experience excessive amounts of phosphate, phosphate uptake is suppressed [44]. It is possible that this response of down-regulating phosphate transporters under high phosphate conditions has been lost through evolution due to *N. amplexicaulis* naturally never experiencing excessive levels of phosphate [45–47]. Additionally, it is often the case in hyperaccumulator species that the transporters responsible for taking up and translocating the hyperaccumulated element are overexpressed due to gene duplications [48]. Subsequently, this allows for higher transport activity and possible increase in affinity of one of those gene copies to the element that is hyperaccumulated [48]. *N. amplexicaulis* contains eleven more phosphate transporter gene copies than *N. heliophila*, which could explain this excessive accumulation of phosphate under high phosphate conditions (Pinto-Irish et al., unpublished).

In the second hydroponics experiment, decreasing phosphate supply concentration did not result in significant difference in phosphate uptake, though *N. amplexicaulis* did have higher phosphate uptake than *N. heliophila*, independent of treatments. What could have caused these different results is the phosphate supply concentrations that were used in both experiments. In the first experiment, phosphate was supplied at either 100 or 1,000 µM, while in the second experiment, phosphate was supplied at either 5 or 25 µM. There is a ten-fold difference in the first experiment and a five-fold difference in the second experiment, and significant differences caused by treatments are more easily found when the difference between treatments is larger as well.

Change in selenite uptake due to changing phosphate supply concentrations

In the first experiment, increasing phosphate supply concentration only resulted in a significant decrease in Se concentration in the root of *N. amplexicaulis*. This is in line with the often hypothesised antagonistic relationship [31, 34–37]. However, a higher phosphate treatment caused no significant decrease in Se concentration in the shoot of *N. amplexicaulis*. A possible explanation for this could be that selenite is a relatively immobile form of Se and often accumulates in the roots [49]. Notably, the treatments had no significant effects on *N. heliophila* for both root and shoot Se concentrations. Considering the treatments caused a much larger difference in phosphate uptake for *N. amplexicaulis* than *N. heliophila*, it could be possible that *N. amplexicaulis* has continuously high phosphate transporter activity [44]. However, in the case of high phosphate application, Se concentration in *N. amplexicaulis* is significantly lower than with low phosphate application. Thus, perhaps the highly active phosphate transporters are more preferential towards phosphate than selenite. A similar mechanism can be found in Se non-accumulators, where sulphate transporters have higher affinity for sulphate than selenate [48, 50]. If this is the case in *N. amplexicaulis*, high phosphate supply concentration would decrease selenite uptake.

In the second experiment, there was a significant increase in Se concentration in the shoot of *N. amplexicaulis* when decreasing phosphate supply from 25 µM to 5 µM. The fact that phosphate uptake was not significantly different for both plant species in the second experiment when changing phosphate supply concentrations could be an explanation for selenite uptake also not

being significantly different for in the root of both species and in the shoot of *N. heliophila*. However, the significant differences in Se concentrations due to changing phosphate supply concentrations are again in line with the hypothesised antagonistic relationship [31, 34–37].

Influence of different selenite supply concentrations on phosphate uptake

The effect of changing selenite supply concentrations on phosphate uptake was only researched with the second hydroponics experiment. From this, the data showed that adding selenite to the growing medium only had a significant, positive effect on root P concentration in *N. heliophila*. Limited research has previously been done on the effect of selenite application on phosphate uptake, most of which reported higher selenite application to decrease phosphate uptake [31, 33, 43]. In berseem, however, higher selenite application increased phosphate uptake, similar to current findings [33]. A possible explanation is that, in low doses, Se application can increase photosynthetic rate, which also increases sugar production, a process that requires P [16, 18–21]. Thus, Se application increasing photosynthetic rate would create a larger demand for phosphate uptake.

Photosynthetic rate was measured for this experiment in Φ_{PSII}, but no significant effects by treatments were found. These measurements were taken for only a single plant per bucket, and the median Φ_{PSII} is higher for *N. heliophila* when treated with 25 µM selenite on top of 25 µM phosphate. Therefore, perhaps with more measurements, this would have been significantly different.

Notably, phosphate uptake by *N. amplexicaulis* was not influenced by the application of selenite. Nevertheless, when grown without selenite, *N. amplexicaulis* had significantly higher root P concentration that *N. heliophila*, suggesting that perhaps *N. amplexicaulis* was already taking up the maximum amount of phosphate from the growing medium. This would not leave enough room to increase phosphate uptake in other growing conditions.

Indication of P toxicity

One of the signs of P toxicity in plants is the inhibition of growth [32, 47]. During both hydroponics experiments, plant growth was quantified as relative growth in projected leaf area (PLA). The relative growth was calculated per plant by dividing its final PLA measurement by its first PLA measurement. For the first experiment, the different phosphate treatments had no effect on relative growth in PLA for *N. heliophila*. Contrastingly, higher phosphate application resulted in a significantly lower relative growth in PLA for *N. amplexicaulis* compared to the low phosphate treatment. Under the same conditions, *N. amplexicaulis* had excessively high P concentrations in the roots and shoot (on average 16,102 ppm and 6,519 ppm, respectively) compared to the 2,000 ppm P that most plants require in their tissues [13]. Taken together, this suggests that *N. amplexicaulis* did experience P toxicity under high phosphate conditions.

Plant growth was also quantified by measuring the final root and shoot dry weight (DW). For the first hydroponics experiment, root DW of *N. amplexicaulis* similarly showed that *N. amplexicaulis* receiving 1,000 µM phosphate may have experienced P toxicity as there was a significant decrease in root DW. Contrastingly, for *N. heliophila*, treatments had no significant effects on root DW and increasing phosphate supply concentration increased shoot DW.

In regular 1x Hoagland solution, phosphate has a concentration of 2,000 µM, which is calculated to be the maximum concentration that can be given before without becoming toxic to a plant [13]. In this experiment, plants were given 0.5x Hoaglands, meaning the plants received 1,000 µM. This would not have been an excessively high concentration for most plants, and receiving 1,000 µM phosphate instead of 100 µM phosphate should increase plant growth, resulting in higher root and shoot DW. This response to the treatments was visible for *N. heliophila*, a Se non-accumulator, as a higher phosphate supply concentration either had no effect or a positive effect on plant DW compared to the low phosphate treatment. The fact that receiving 1,000 µM phosphate instead of 100 µM phosphate either had no effect or a negative effect on plant DW of *N. amplexicaulis* strongly suggests that *N. amplexicaulis* experienced P toxicity under high phosphate conditions.

In the second experiment, a lower phosphate supply resulted in significantly lower root and shoot DW for *N. amplexicaulis* and significantly lower shoot DW for *N. heliophila*. This experiment had much lower phosphate concentrations in its treatments than the first experiment, the highest (25 µM) being four times lower than the lowest phosphate concentration in the first experiment (100 µM). Thus, it is understandable that no signs of P toxicity were visible in root and shoot DW measurements for the second experiment. Furthermore, when *N. amplexicaulis* was treated with 25 µM phosphate, it had a root and shoot P concentration close to the 2,000 ppm that is to be expected in plant tissues [13]. Seeing as P is a vital element for plant growth [26], decreasing the phosphate supply concentration from 25 µM to 5 µM led to even lower root and shoot P concentrations in *N. amplexicaulis* and is likely what caused the decrease in plant growth.

Indication of selenite toxicity

Se toxicity can be caused by an excessive accumulation of selenite within the plant, which gives oxidative stress [3, 9, 22, 23]. Additionally, Se is very similar to sulphur (S) in terms of physical properties, causing Se to sometimes be mistakenly incorporated into cysteine or methionine instead of S [3, 9, 22, 23]. If those selenoamino acids are incorporated into proteins, it could cause misfolding and loss-of-function. Se hyperaccumulators have found ways to prevent these issues, mainly by storing Se in organic forms that do not cause oxidative stress and will not be incorporated into proteins [51, 52]. Ultimately, among other symptoms, Se toxicity limits plant growth [22].

In the second experiment, an indication of Se toxicity was only visible in the shoot DW data for *N. heliophila*. When *N. heliophila* received 25 µM selenite on top of 25 µM phosphate, shoot DW decreased significantly. Furthermore, this decrease in shoot DW could not have been caused by a lower shoot P concentration as this was not significantly different for *N. heliophila* across the different treatments. *N. amplexicaulis* showed no change in root or shoot DW in response to added selenite, suggesting that *N. amplexicaulis* did not experience Se toxicity. This is to be expected considering *N. amplexicaulis* can accumulate Se up to 13,600 ppm under experimental conditions [9, 25].

3.2 RNA isolation

During this project, three attempts were made to isolate RNA from *N. amplexicaulis* and *N. heliophila* root and shoot tissues, with the final goal being to measure expression levels of the P transporters *PHT1-1*, *PHT2-1*, and *PHT4-1*. For this, a protocol was used that was provided by Subash Rai from the University of Queensland, which uses a CTAB extraction method from Acosta-Maspons et al. [41] and a TRIzol method in the final steps (Appendix B). According to this protocol, an isolated RNA concentration of 146-164 ng/µL was to be expected, however, none of the trials delivered such results. To summarize the results, in the first trial, two out of sixteen samples had some isolated RNA (30-40 ng/µL), in the second trial, three out of twelve samples had some isolated RNA (20-27 ng/µL) (Appendix C), and in the third trial, the highest concentration of isolated RNA out of all twenty-four samples was 10.965 ng/µL (Appendix C).

There are many steps during the protocol where something could have gone wrong. Considering this protocol has resulted in successful isolation of RNA for Subash Rai, there is no reason to believe the protocol itself is incorrect. Thus, the low isolated RNA concentrations from the three trials must be, at least mainly, due to improper execution of the protocol.

First, something that went wrong for all trials was the speed at which the centrifuge was set. The protocol uses the unit *g*, which is interchangeable with RCF (relative centrifugal force). However, during all trials, the values in *g* were put in as RPM (revolutions per minute), and these units are not interchangeable. As a result, instead of 10,000*g*, the samples were centrifuged at 10,000 RPM (= 10,621*g*), and instead of 17,000*g*, the samples were centrifuged at 14,000 RPM (= 20,817*g*), which was the maximum RPM of the centrifuge that was used. For the steps that the centrifuge should have ran at 10,000*g*, there is only a small increase in speed, but for the steps that the centrifuge should have ran at 17,000*g*, there is a larger increase. While no literature was found that suggests that 20,817*g* would have damaged the RNA, it cannot yet be ruled out.

Next, the inconsistent results of the first and second trial can be due to the speed at which the protocol was executed. If the supernatant had to be pipetted off and put in a new tube, the first few samples would sometimes be waiting for 10 minutes until the next step while the other samples still had to be done. If RNase was present in the solution at that moment, whether endogenous RNase that had not been fully inhibited previously or newly introduced exogenous RNase, the RNA in the samples that were done first could have started degrading.

Additionally, in the first and second trial, the samples were not properly kept cool during the grinding step. While the mortar and pestle were cooled with liquid nitrogen before they were used, other items that touched the samples were not. This allowed for the samples to thaw before the RNA extraction buffer was added, which could have led to rapid degradation of RNA by endogenous RNase [53]. For the third trial, this step was changed accordingly. This time, not only the mortar and pestle, but also the 15 mL tubes, tweezers, and spoons were cooled with liquid nitrogen prior to touching the samples. Nevertheless, the third trial had the lowest success in RNA isolation.

The most likely explanation for the consistently low results from the third trial is the mistake made during step 18 of the protocol (Appendix B). The pellet containing RNA was not mixed with the water before putting the samples in a heat block. Due to time constraints, the samples had to be stored at -20 °C and thawed the next day to then mix the pellet with the water and again place it in a heat block. RNA is relatively unstable and the protocol advices not to freeze and thaw isolated RNA too many times. However, this was a single freezing and thawing of the isolated RNA and a previous study found that even after being kept at 24 hours at room temperature, total RNA was preserved from isolated RNA samples that were previously kept at -80 °C [54]. Yet, the reason for incubating isolated RNA mixed with water at 65 °C for 10 minutes at the end of the protocol is to resolubilise the RNA, and as it was not first properly mixed before incubation, this step may have gone wrong. Additionally, the protocol says to let the pellet dry for 5-10 minutes before resolubilising the pellet in nuclease-free water, but during each trial, the drying period was over 25 minutes. This could have resulted in the pellet not fully resolubilising during incubation, decreasing the measured RNA concentration at the end [55].

Furthermore, another explanation for the poor results of the third trial could be that the RNase free water was contaminated. Before the first trial, a 50 mL tube was filled with RNase free water, which turned out to be enough for all three trials. It is possible that it got contaminated with RNase during one of the trials, which would then degrade RNA [56]. If another attempt will be made with this protocol, it is advised to prepare a new tube with RNase free water for each new attempt in order to eliminate the possibilities of previous contaminations. Also, a very high 260/280 ratio can be the result of a poor quality blank [57]. This was the case for multiple samples (Appendix C) and further suggests that the nuclease-free water was contaminated. However, abnormal 260/280 values (< 0 or > 10) could also have been due to the very low concentrations of nucleic acids (< 10 ng/µL) [57].

Lastly, Acosta-Maspons et al. [41] states that the LiCl step should take 12-15 hours and that longer periods could result in the accumulation of unwanted proteins and DNA. During the execution of the protocol, the LiCl step often took approximately 20 hours. The protocol that was used for this report did state to let the LiCl step take 19 hours, but it is worth testing whether a shorter time period would improve the results.

Things that should be taken into consideration for a next trial:

- Decrease the LiCl step to 15 hours if possible as this could prevent the accumulation of unwanted proteins and DNA [41].
- Take leaf samples of younger leaves, seeing as the yield of RNA can decrease as leaves mature [53].
- When supernatant is transferred to a new tube, those tubes should be pre-cooled [53].
- Have a shorter drying period at the end of the protocol. The final wash could be done with 95-100% ethanol to have the drying process be done quicker [55]. Additionally, the drier the RNA pellet has become, the harder it is to resolubilise it, so it is advised to not allow the pellet to dry completely [55].

- After the RNA extraction buffer is fully homogenised with the ground up sample, incubate it at 65 °C for 10 minutes, while having the samples lightly mix [53, 58]. This should have the cells better lysed and result in higher RNA yield [53, 58].
- It is possible that the sample size was too large. Having a smaller sample size could lead to better homogenisation with the RNA extraction buffer and inhibition of endogenous RNases [59].
- Even when endogenous RNases are permanently inhibited, new RNases can be introduced to the samples through contamination [53]. Thus, more care should go into working clean, i.e., wearing gloves, and the step of grinding samples should be done fast so the extraction buffer can be introduced as soon as possible to inhibit RNases. Additionally, new tubes with solutions should be made for each trial to prevent possible previous contaminations from negatively affecting the new trial.

3.3 Conclusion & recommendations

Final conclusions

With this project, the aim was to research the effects of phosphate and selenite supply concentrations on the uptake of phosphate and selenite in *N. amplexicaulis*. First, *N. amplexicaulis* had on average 2.7 times higher phosphate uptake than *N. heliophila* when treated with 1,000 µM phosphate, a concentration that is also in 0.5x Hoaglands. The hypothesis is that *N. amplexicaulis* lacks the mechanisms to down-regulate phosphate transporter genes when there is an excess of phosphate. It is also possible that *N. amplexicaulis* has higher phosphate transporter activity due to the eleven additional gene copies compared to *N. heliophila*. When phosphate was supplied at 25 µM, the plant tissue of *N. amplexicaulis* had a P concentration of close to 2,000 ppm, which is the expected P concentration in plants [13].

Next, with higher phosphate treatment, *N. amplexicaulis* had a significant increase in selenite uptake. Considering this species likely has consistently active phosphate transporters, a possibility is that these transporters are all or mostly preferential towards phosphate. This would mean that when phosphate concentration is low, selenite can be abundantly taken up, and when phosphate concentration is high, this gets preference over selenite and selenite is taken up much less. This aligns with the fact that the different selenite treatments did not influence phosphate uptake in *N. amplexicaulis*. This mechanism would also be advantageous for *N. amplexicaulis* in its natural habitat, seeing as P bioavailability there is very low and the high Se concentration in that region would further decrease phosphate uptake, which is already limited.

Lastly, one of the aims of this project was to investigate which growing condition is best for *N. amplexicaulis*, both in terms of high selenite uptake and good plant growth. The data in relative growth in PLA showed that under high phosphate conditions, *N. amplexicaulis* had significantly lower relative growth in PLA than under low phosphate condition. This is hypothesised to be due to P toxicity. Additionally, while *N. heliophila* likely experienced Se toxicity, seeing as it had significantly

lower shoot DW when selenite was added to the treatment, *N. amplexicaulis* did not show the same response.

Ultimately, one of the major applications of this research topic would be to increase Se concentration in crops in regions where humans and other mammals are Se-deficient. To reach this goal, it is vital to understand the dynamics between phosphate and selenite uptake and take that into account when fertilising crops. The data from this research have shown that careful consideration should go into the amount of phosphate that is applied to plants if the desire is to increase selenite uptake. Though phosphate is needed for proper plant growth, higher phosphate concentrations does decrease selenite uptake. In the case of *N. amplexicaulis*, that would mean giving much lower concentrations of phosphate than most plants would require, given that this species takes up much more phosphate relative to non-accumulators under the same conditions.

Future research

To further understand the mechanisms behind phosphate and selenite uptake in *N. amplexicaulis*, expression levels of phosphate transporter genes can be measured via RT-qPCR. Most interesting phosphate transporters to study with this would be *PHT1-1*, *PHT2-1*, and *PHT4-1* as those are known in literature to be highly expressed during P-deficiency in rice. Additionally, in rice, *PHT2-1* is shown to facilitate selenite uptake. Furthermore, considering *N. amplexicaulis* has eleven more phosphate transporter genes than *N. heliophila*, it would be valuable to investigate whether one or several of the phosphate transporter genes of *N. amplexicaulis* have neofunctionalised, making them more preferential towards selenite.

During the first experiment, changing phosphate supply concentrations did not result in a significant change in shoot Se concentration, while it did in the second experiment. Further research could be done in the influence of phosphate supply concentrations on selenite mobility within *N. amplexicaulis*.

Finally, a new attempt could be made at isolating RNA from *N. amplexicaulis* and *N. heliophila*. Three samples are still available for each unique treatment-species combination from the second hydroponics experiment. With the possible improvements mentioned in this report, perhaps better results will be obtained in a future trial than were obtained in this study.

4 Materials & Methods

4.1 Conditions in climate room

The climate room in which the experiments took place was B6 in Radix Klima, Wageningen University & Research (Wageningen, the Netherlands). In here, the lights were on for 12 hours per day, during which the temperature was 24 °C. Outside of those hours, the temperature dropped to 20 °C. Additionally, the humidity was permanently set to 70% and the plants received light at approximately 700 PAR.

4.2 Germinating seeds

For the two separate hydroponics experiments, seeds of both *N. amplexicaulis* and *N. heliophila* were sterilised, scarified, and germinated. First, for both species, a 50 mL tube was filled with approximately 100 seeds. These seeds were washed by adding a drop of liquid soap and demiwater to each tube and then rinsing three times with demiwater. For the first hydroponics experiment, after the seeds were washed, they were sterilised with bleach and a small cut was made in the seed coat. This allowed the seeds to take up water and, later, start germinating. For the second hydroponics experiment, the washed seeds were sterilised and scarified with sulfuric acid (95-98%). In a 50 mL tube containing the seeds, just enough sulfuric acid was added to cover all seeds. The sulfuric acid was removed after 30 minutes and the seeds were washed thrice with sterilised water.

After sterilisation and scarification, the seeds were put in 50 mL tubes with sterilised water, which were covered with aluminium foil and placed in the refrigerator at max. 8 °C for at least one day. Next, the seeds were placed on petridishes with ½ MS agar (2.2 g L^{-1} Murashige and Skoog medium, 0.5 g L^{-1} MES, 10 g L^{-1} agarose, 10 g L^{-1} saccharose, pH 5.8). A drop of sterilised water was put on each seed, after which the petridishes were closed and sealed with parafilm. The sealed petridishes were then placed in a larger container, which had the bottom lined with water-soaked paper tissues to keep the humidity high surrounding the petridishes. The container was closed and kept in a growing room (24 °C and constant light) for at least four days.

4.3 Hoagland nutrient solution

For the first hydroponics experiment, the plants first grew for two weeks on a 0.5x Hoagland nutrient solution ('Start' in Table 2) [60]. This solution was made in the lab. Here, 0.43 g L^{-1} MES monohydrate was added and the pH was adjusted to 5.5. The Hoagland solution was made with tap water. After the initial two weeks, the plants received the first treatment for six days, where $NH_4H_2PO_4$ was given at either 0.05, 1 or 5 mM, and Na_2SeO_3 was given at either 5 or 25 µM ('Treatment 1' in Table 2). Finally, for the last 25 days, the plants received the second treatment, where $NH_4H_2PO_4$ was given at either 0.1 or 1 mM, and Na_2SeO_3 at only 25 µM ('Treatment 2' in Table 2).

Table 2. Composition of 0.5x Hoagland solution for hydroponics experiment 1. The concentrations are in µM. In blue, the concentrations of elements within a treatment are highlighted that differ from the start condition. The column 'Start' contains the concentrations at which elements were given to the plants before the treatments began. Column 'Treatment 1' contains the first treatment the plants were given for the first six days. Column 'Treatment 2' contains the second

treatment the plants were given after those initial first six days. If multiple concentrations are given in a cell, that means that plants were given one of those concentrations.

Element	Start	Treatment 1	Treatment 2
KNO_3	3,000	3,000	3,000
$Ca(NO_3)_2 \cdot 4\ H_2O$	2,000	2,000	2,000
$NH_4H_2PO_4$	1,000	50/1,000/5,000	100/1,000
$MgSO_4 \cdot 7\ H_2O$	500	500	500
Fe(Na)EDTA	20	20	20
KCl	0.5	0.5	0.5
H_3BO_3	12.5	12.5	12.5
$MnSO_4 \cdot 4\ H_2O$	1	1	1
$ZnSO_4 \cdot 7\ H_2O$	1	1	1
$CuSO_4 \cdot 5\ H_2O$	0.05	0.05	0.05
$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	0.05	0.05	0.05
Na_2SeO_4	2.5	-	-
Na_2SeO_3	2.5	5/25	25

For the second hydroponics experiment, the 0.5x Hoagland nutrient solution was provided by Radix Klima (Table 3). This solution had a pH of 5.8 and no MES monohydrate was added. Additionally, this Hoagland solution was provided at 1x strength and was diluted to 0.5x with tap water. During the first week of the experiment, the plants grew on this 0.5x Hoagland solution. After this, the plants were randomly assigned to one of three treatments: 5 µM $NH_4H_2PO_4$ and 25 µM Na_2SeO_3, 25 µM $NH_4H_2PO_4$ and 25 µM Na_2SeO_3, or 25 µM $NH_4H_2PO_4$ and 0 µM Na_2SeO_3 (Table 3). The nutrient solution was refreshed every week. Furthermore, due to the plants showing Fe-deficiency within the first few weeks of the experiment, starting from the beginning of week three of the experiment, all plants received an additional 40 µM Fe(EDDHA). The plants grew for five weeks with the treatments before they were harvested.

Table 3. Composition of 0.5x Hoagland solution for hydroponics experiment 2. The concentrations are in µM. In blue, the concentrations of elements within a treatment are highlighted that differ from the start condition. The 40 µM Fe(EDDHA) was only added to the treatment starting from the beginning of week three of the experiment.

Element	Start	Treatment
NH_4	500	500
K	3,000	3,000
Ca	1,800	1,800
Mg	1,000	1,000
NO_3	5,500	5,500
SO_4	1,800	1,800
Fe	12.5	12.5

Mn	5.5	5.5
Zn	0.875	0.875
B	22.0	22.0
Cu	0.0625	0.0625
Mo	0.26	0.26
$NH_4H_2PO_4$	25	5/25
Na_2SeO_3	-	0/25
Fe(EDDHA)	-	40

4.4 Experimental set-up for hydroponics

Both hydroponics experiments were performed in climate room B6 in Radix Klima, Wageningen University & Research. There, 48 buckets with a volume of 4.5 L were set up, each topped with a lid containing three holes (Figure 10). Through the smaller hole, a tube from an aeration pump was pulled, to which an air stone was attached on the end that was within the bucket. The two bigger holes were made for one plant each, both within the same bucket being from the same species, which was either *N. amplexicaulis* or *N. heliophila*.

When placing the plants in the hydroponics system, the root system of a seedling was first packed in rockwool, which was soaked in 0.5x Hoagland nutrient solution. Next, the seedling covered in rockwool was put in a plastic bucket-shaped net that fit in one of the two bigger holes in the lid. The plants within a bucket were labelled with either “A” or “B”. Additionally, in the second hydroponics system, a thin rubber circle was placed on top of the nets with a small hole in the middle from which the plant could emerge, to minimise algae growth on the rockwool.

The separate plants within a bucket were considered pseudo replicates, while the bucket itself was considered a true replicate. The location of the buckets within the climate room were randomised.

Figure 10. Experimental set-up of the second hydroponics experiment. Buckets of 4.5 L contained two plants of one plant species, either *N. amplexicaulis* or *N. heliophila*. The plants were placed within a plastic net with rockwool soaked in

0.5x Hoagland nutrient solution. These nets were then placed within holes in the lid that is placed on top of the buckets. Additionally, a tube from an aeration pump was pulled through a smaller hole in the lid and an air stone was attached to the tube on the end that is in the bucket. The plants within a bucket were labelled with either “A” or “B”.

4.5 Obtaining data from the hydroponics experiments

4.5.1 Measuring Projected Leaf Area (PLA)

To obtain the PLA, in the first hydroponics experiment, photos were taken of each plant on the first day the treatments were given and on the day the plants were harvested. In the second hydroponics experiment, photos were taken on the first day the treatments were given and the last day the treatments were refreshed. These pictures were processed in ImageJ (Version 2.16.10). The scale was set by using the width of the bucket (24 cm) or the rubber circle (6 cm). Next, a colour threshold was set, with approximately the following settings: Hue 38-255, Saturation 47-255, and Brightness 95-255. The colour threshold was adjusted until only the plants were selected in the photo. Finally, the area of the selected regions were measured in ImageJ.

4.5.2 Photosynthesis efficiency (Φ_{PSII})

Φ_{PSII} was measured for both hydroponics experiments during the last week of the treatments. For this, the MultispeQ from PhotosynQ was used with the protocol ‘Photosynthesis RIDES 2.0’ [61]. For each plant, a single measurement was taken of one of the youngest fully-developed leaves.

4.5.3 Weighing fresh weight (FW) and dry weight (DW)

On the day the plants from the hydroponics experiments were harvested, each plant was cut in two, so that the root and shoot were separated, and then the plant material was patted dry with tissue paper. With the first hydroponics experiment, the plant material was next weighed on a scale to get the FW. During the second hydroponics experiment, this step was skipped. After this, the shoot of each plant was separately put in a paper envelope and the root in a 15 mL screw cap tube. These were then placed in an oven at 60 °C for several days, with the paper enveloped opened and the tubes without the cap on. Once the plant material was fully dried, the material was weighed again to obtain the dry weight DW.

4.5.4 X-ray fluorescence (XRF)

With XRF, the concentrations of P and Se, among many other elements, were measured for all plant samples. For this, the dried plant material, i.e., the roots and shoots that were dried to obtain DW measurements, were ground into a powder. This was done by putting the plant material in a mortar with liquid nitrogen, and grinding it to a powder with a pestle. The powder (100-300 mg DW) was then transferred to a sample holder that is provided with the machines JP500 and E-lite from Z-Spec. For all samples, the following settings were used on the machines: ‘Test Time’ was set to ‘30’, ‘Calibration’ was set to ‘Default’, and ‘Material’ was set to ‘Plant’. Each sample was measured at two different angles, and these measurements were averaged per plant.

4.6 Quantifying the expression levels of phosphate transporter genes

4.6.1 Taking plant material samples for RNA isolation

To isolate RNA, root and leaf samples of 200-800 mg FW were taken from the largest plant of each bucket of the second hydroponics experiment. This was done on day 37, one day after the last nutrient solution refreshment. For a leaf sample, three leaves were taken in the case of *N. amplexicaulis*, and five leaves were taken in the case of *N. heliophila*. These leaf samples were always fully developed leaves. For a root sample, approximately 20-25 cm was cut. Each plant sample went into a separate 2 mL screw cap tube, which was then immediately put in liquid nitrogen to snap freeze the sample. After collecting all samples, they were stored at -80 °C until the next step.

4.6.2 Isolation of RNA

To isolate the RNA from *N. amplexicaulis* and *N. heliophila*, a protocol from Subash Rai was followed (Appendix B). This protocol uses a CTAB extraction method as described by Acosta-Maspons et al. [41], with the final steps modified based on a TRIzol method.

4.6.3 Identifying Genes Of Interest (GOI)

The genomes of *N. amplexicaulis* and *N. heliophila* were previously sequenced and a selection was made of all phosphate transporter genes (Pinto-Irish et al., unpublished). In literature, members of the phosphate transporter Pht1 family, namely *OsPT2* and *OsPT8*, were found to take selenite up via the roots [10, 12, 42]. Additionally, expression data of *N. amplexicaulis* and *N. heliophila* became available after an RNA sequencing experiment performed at the University of Queensland (Van der Ent et al., unpublished). This expression data showed the expression of a gene relative to all other genes for root and shoot tissue of both plant species treated with increasing levels of selenate. From this data, *PHT1-1*, *PHT2-1*, and *PHT4-1* were chosen as GOIs. *PHT1-1* was chosen as the relative expression continuously increased in *N. amplexicaulis* when selenate concentration increased, and it did not in *N. heliophila*. Next, *PHT2-1* and *PHT4-1* were chosen as their relative expression levels increased in *N. amplexicaulis* when selenate concentration increased, while their expression decreased in *N. heliophila*.

4.6.4 Primer design

Sequences of the GOIs from the genomes of both *N. amplexicaulis* and *N. heliophila* were imported into SnapGene software [62]. This was aligned with the cDNA sequence of the same gene to find the introns. The primers were made to flank an intron (forward on one side and reverse on the other side) so that genomic DNA will not result in a product and cDNA will when running a PCR. The primers are designed to have a melting temperature (Tm) of at least 58 °C and 50-60% GC content. The end of the primer also ends with at least two Gs or Cs. The primers are 20-24 bp long. The reverse and forward primers of the same gene did not differ for more than 3 °C in Tm. The resulting amplicon was 75-200 basepairs long and the goal was to keep 50-60% GC content.

Table 4. Primers used for each gene in RT-qPCR. The gene codes for *N. amplexicaulis* start with "Na" and the gene codes for *N. heliophila* start with "Ng". The primers are written from 5' end to 3' end.

Gene	Gene code	Forward primer	Reverse primer
PHT1-1	Na_s05_g07800	CCATTGGGTCTTGGCCAAATCG	TCCCCCTCCTCTCTTTCTTTCC
	Ng_s011_g11734	GGTACCATTTCACGGCCATTGT	GCAAAGAGCCTGGCTTTGGC
PHT2-1	Na_s08_g12503	AGGATACTCTGCTCTTCGCTGG	AGTGTGTGGTAGACACTGGCC
	Ng_s005_g05918	TGCTCCGACTATACAGTGTGTGG	CTGGATTGCTCTCTTCGCTTGC
PHT4-1	Na_s24_g23933	ACCTACTTCTCTCCGCTACCGG	CCTGTTGCTGCGAAACTCGG
	Ng_s016_g15096	CGCAGTTCAGGATCATCAAGAGG	ATTCATCGGTTTGGATGGCC
Actin	Na_s33_g25959	TGTATGTTGCTATCCAGGCCG	TGTGTGGCTCACACCATCACC
	Ng_s026_g19943	GCTGACGCTGAGGATATTCAACC	AGGTCGTCCAACAATGCTGG
UBC	Na_s20_g21811	CTGGCTCACTGAGAAGACGC	ACAAACCCTATTGCGTGATTTGC
	Ng_s014_g13797	CTGAAAGATAAGTGGAGCCCTGC	GGCAATGTTCTCAGAAAGCGG
β-tubulin	Na_s33_g26272	GACAACAGGTAACTCCACTCATGG	GCATGGTCTTGGACAATGAGGC
	Ng_s103_g25772	ATGTTGTGAGGAAGGAGGCCG	AGTGTTCCCATTCCTGAGCC

4.7 Statistical analysis

For both hydroponics experiments, a data frame was created in RStudio (R 4.2.0) in which each row represented a singular plant. Considering a full replicate was one bucket, the phenotypic data for both plants in each bucket were averaged. This data was used in further statistical analysis and data visualisation.

All dependent variables were tested on homogeneity of variance and normal distribution. Homogeneity of variance was tested using the function *leveneTest* from the R library *car*. Normal distribution was tested using the function *shapiro.test* from the R library *stats*. If the data had both homogeneity of variance and normal distribution, a two-way ANOVA was performed. If one or both requirements were not met, the data was transformed using either a square root transformation or a $\log_{10}$ transformation. If homogeneity of variance and normal distribution were still not met, a two-factor Aligned Rank Transform (ART) ANOVA was performed using the R library *ARTool* [63]. After a two-way ANOVA or two-factor ART ANOVA, a Tukey HSD was performed to test which groups were significantly different from each other. All statistical results can be found in Appendix A.

Data visualisation was done with the R package ggplot2.

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Appendix A: Statistical analysis

Table 5. Results of statistical analyses of all response variables for both hydroponics experiments. DW = dry weight; FW = fresh weight; Se = selenium; P = phosphate; PLA = projected leaf area. For the response variable 'Growth in PLA', the last PLA measurement was divided by the first PLA measurement for each plant. Significant p-values are given a bold font.

Response variable	Transformation	Statistical test	Variable	F-statistic	P-value
First hydroponics experiment					
Shoot FW	-	Two-way ANOVA	Treatment	$F(1, 44) = 0.591$	$p = 0.446$
			Species	$F(1, 44) = 0.109$	$p = 0.742$
			Treatment:Species	$F(1, 44) = 20.300$	**$p < 0.001$**
Root FW	-	Two-way ANOVA	Treatment	$F(1, 44) = 0.213$	$p = 0.646$
			Species	$F(1, 44) = 2.592$	$p = 0.115$
			Treatment:Species	$F(1, 44) = 22.371$	**$p < 0.001$**
Shoot DW	-	Two-way ANOVA	Treatment	$F(1, 44) = 0.164$	$p = 0.688$
			Species	$F(1, 44) = 1.927$	$p = 0.172$
			Treatment:Species	$F(1, 44) = 15.286$	**$p < 0.001$**
Root DW	Square root transformation	Two-way ANOVA	Treatment	$F(1, 42) = 5.017$	**$p = 0.030$**
			Species	$F(1, 42) = 0.470$	$p = 0.497$
			Treatment:Species	$F(1, 42) = 16.006$	**$p < 0.001$**
Φ_{PSII}	-	Two-factor ART ANOVA	Treatment	$F(1, 44) = 0.432$	$p = 0.432$
			Species	$F(1, 44) = 63.003$	**$p < 0.001$**
			Treatment:Species	$F(1, 44) = 0.772$	$p = 0.384$
Growth in PLA	Log_{10} transformation	Two-way ANOVA	Treatment	$F(1, 44) = 1.240$	$p = 0.271$
			Species	$F(1, 44) = 18.004$	**$p < 0.001$**
			Treatment:Species	$F(1, 44) = 7.120$	**$p = 0.011$**
Shoot Se concentration	-	Two-factor ART ANOVA	Treatment	$F(1, 44) = 0.061$	$p = 0.806$
			Species	$F(1, 44) = 5.197$	**$p = 0.028$**

			Treatment:Species	F(1, 44) = 0.392	p = 0.534
Root Se concentration	-	Two-way ANOVA	Treatment	F(1, 25) = 12.232	**p = 0.002**
			Species	F(1, 25) = 10.706	**p = 0.003**
			Treatment:Species	F(1, 25) = 0.361	p = 0.553
Shoot P concentration	-	Two-factor ART	Treatment	F(1, 44) = 43.097	**p < 0.001**
		ANOVA	Species	F(1, 44) = 24.583	**p < 0.001**
			Treatment:Species	F(1, 44) = 15.765	**p = 0.003**
Root P concentration	Log_{10} transformation	Two-way ANOVA	Treatment	F(1, 26) = 101.478	**p < 0.001**
			Species	F(1, 26) = 9.796	**p < 0.001**
			Treatment:Species	F(1, 26) = 27.376	**p < 0.001**
Second hydroponics experiment					
Shoot DW	-	Two-way ANOVA	Treatment	F(2, 42) = 38.484	**p < 0.001**
			Species	F(1, 42) = 44.596	**p < 0.001**
			Treatment:Species	F(2, 42) = 7.348	**p = 0.002**
Root DW	-	Two-way ANOVA	Treatment	F(2, 42) = 19.034	**p < 0.001**
			Species	F(1, 42) = 31.113	**p < 0.001**
			Treatment:Species	F(2, 42) = 2.230	p = 0.120
Φ_{PSII}	-	Two-way ANOVA	Treatment	F(2, 41) = 0.395	p = 0.676
			Species	F(1, 41) = 0.419	p = 0.521
			Treatment:Species	F(2, 41) = 2.730	p = 0.077
Growth in PLA	-	Two-way ANOVA	Treatment	F(2, 42) = 11.519	**p < 0.001**
			Species	F(1, 42) = 1.627	p = 0.209
			Treatment:Species	F(2, 42) = 1.651	p = 0.204
Shoot Se concentration	Log_{10} transformation	Two-way ANOVA	Treatment	F(2, 42) = 775.78	**p < 0.001**
			Species	F(1, 42) = 503.78	**p < 0.001**

			Treatment:Species	F(2, 42) = 188.58	**p < 0.001**
Root Se concentration	-	Two-factor ART ANOVA	Treatment	F(2, 42) = 54.965	**p < 0.001**
			Species	F(1, 42) = 77.248	**p < 0.001**
			Treatment:Species	F(2, 42) = 11.889	**p < 0.001**
Shoot P concentration	Log_{10} transformation	Two-way ANOVA	Treatment	F(2, 42) = 0.137	p = 0.872
			Species	F(1, 42) = 38.677	**p < 0.001**
			Treatment:Species	F(2, 42) = 0.695	p = 0.505
Root P concentration	Log_{10} transformation	Two-way ANOVA	Treatment	F(2, 42) = 3.112	p = 0.055
			Species	F(1, 42) = 35.858	**p < 0.001**
			Treatment:Species	F(2, 42) = 6.515	**p = 0.003**
Shoot to root ratio	-	Two-way ANOVA	Treatment	F(2, 42) = 7.135	**p = 0.002**
			Species	F(1, 42) = 0.006	p = 0.939
			Treatment:Species	F(2, 42) = 3.335	**p = 0.045**

Appendix B: RNA isolation protocol

Information on the protocol

The following protocol was provided by Subash Rai. The steps that were added for this report are included in the procedures description in light blue, bold font.

The protocol is a modified version of Acosta-Maspons et al. [41] and TRIzol (Invitorgen) methods. The extraction part is taken from Acosta-Maspons et al. [41] and final steps are modified based on TRIzol method. High quality Total RNA can be extracted from plant roots and leaves sample using this protocol.

Reagents

- UltraPure 1 M Tris-HCl, pH 8.0
- CTAB
- EDTA (0.5 M), pH 8.0, Nuclease-free
- Sodium chloride
- Chloroform:Isoamyl alcohol (24:1)
- SDS, 20% solution, RNase-free
- Ethanol (> 98% undenatured)
- Lithium chloride, 8 M, for molecular biology
- 2-propanol
- β-mercaptoethanol
- Spermidine trihydrochloride
- RNaseZap solution
- TURBO DNA-free™ kit (optional)
- Distilled water ultra pure DNase/RNase free Gibco 500 mL

Recipes for the buffers

RNA extraction buffer: 100 mM Tris-HCl, 25 mM EDTA, 2% CTAB (w/v), 2 M NaCl, 0.75 g/L spermidine trihydrochloride, 4% β-mercaptoethanol

Combine 5 mL of 1 M Tris-HCl (pH 8.0), 2.5 mL of 0.5 M EDTA (pH 8.0), 0.0375 g spermidine trihydrochloride, 1 g of CTAB powder and 5.844 g of NaCl. Complete to 50 mL with lab grade water. Add 4% β-mercaptoethanol just before use.

Resuspension buffer SSTE: 1 M NaCl, 0.5% SDS (w/v), 10 mM Tris-HCl (pH 8.0), 1 mM EDTA

Combine 0.25 mL SDS (20%), 0.2 mL of 1 M Tris-HCl (pH 8.0), 0.04 mL of 0.5 M EDTA (pH 8.0) in 8 mL nuclease free water and add 1.167 g NaCl and adjust final volume to 10 mL with nuclease free water.

Other needed solutions: chloroform:isoamyl alcohol (24:1 v/v), 8 M LiCl, 2-propanol, and 80% ethanol

Procedures

1. Finish the RNA extraction buffer by adding the β-mercaptoethanol.

2. Incubate the RNA extraction buffer at 65 °C for at least 30 minutes.
3. Clean up the working bench, mortar-pestle, pipettes, and tube racks with RNAZap.
4. Chill the mortar and pestle **and any other material that will be touching the samples (e.g., 15 mL tubes and spatulas used for transferring the ground up samples)**, and grind 200-800 mg of snap frozen leaves in liquid nitrogen till finely ground (may require top up of liquid nitrogen).
5. Transfer the finely ground tissue powder to a 15 mL falcon tube and add 3,000 µL of preheated (65 °C) RNA extraction buffer, followed by vigorous homogenisation (vertex, followed by P1000 pipetting) after which samples are kept on ice. Perform following steps under fume hood.
6. Dispense 1,000 µL of homogenised sample suspension into a 2 mL tube.
7. Add 1,000 µL (equal volume) of chloroform:isoamyl alcohol (24:1), mix well and centrifuge for 10 minutes at 10,000*g* at 4 °C.
8. Carefully transfer supernatant to a new 2 mL tube (approximately 900 µL).
9. Repeat steps 6 and 7 **(but this time using a 1.5 mL tube instead of a 2 mL tube)**; then add the appropriate amount of LiCl to 2 M final concentration (from 8 M LiCl starting solution, add ½ v/v).
10. Leave precipitating overnight at 4 °C (19 hours).
11. Preheat SSTE buffer to 65 °C.
12. Centrifuge the samples at 17,000*g* for 30 minutes at 4 °C.
13. Decant the supernatant **and pipet off any leftover supernatant**. Resuspend the pellet in 100 µL of preheated (65 °C) SSTE buffer by shaking. To avoid undesired precipitates, do net set samples on ice.
14. Add equal volume chloroform:isoamyl alcohol, mix well, and centrifuge for 10 minutes at 10,000*g* at 4 °C. Transfer supernatant to a new 1.5 mL tube, and then keep on ice.
15. Add 1 volume of 2-propanol (v/v), mix gently, and incubate for 10 minutes at room temperature.
16. Centrifuge at 17,000*g* for 10 minutes at 4 °C, then pipette off the supernatant.
17. Wash pellet with 500 µL 80% ethanol, dislodge the pellet by 2 seconds of pulse vertexing, and then centrifuge at 17,000*g* for 5 minutes at 4 °C. Repeat this step once.
18. Dry pellet 5-10 minutes on fume hood and resuspend in 50 µL of nuclease free water and incubate for 10 minutes at 65 °C and put on ice. Perform QC in NanoDrop and qubit.
19. Treat sample with TURBO DNA-free™ kit according to manufacturer's instruction if DNA appears as contamination. This step requires while high amount of tissues (> 200 mg) are used in RNA extraction.
20. Store RNA at -80 °C and avoid multiple freezing and thawing.

Appendix C: Complete results RNA isolation

Table 6. Complete RNA isolation results from the second trial. The measurements were obtained with Nanodrop. P = phosphate; Se = selenite. Samples were taken from plants in the second hydroponics experiment. Replicate number represents which bucket the plant was harvested from. Sample was always taken from the largest plant in the bucket.

Species	Plant material	Replicate	Treatment	ng/µL	A260	260/230	260/280
N. amplexicaulis	Root	10	25 µL P	22.762	0.5691	2.565	1.870
N. amplexicaulis	Root	38	25 µL P + 25 µL Se	0.504	0.0126	1.803	3.830
N. amplexicaulis	Root	23	5 µL P + 25 µL Se	1.374	0.0344	2.347	-10.389
N. amplexicaulis	Shoot	10	25 µL P	2.295	0.0574	1.628	1.250
N. amplexicaulis	Shoot	38	25 µL P + 25 µL Se	2.421	0.0605	1.694	1.355
N. amplexicaulis	Shoot	23	5 µL P + 25 µL Se	2.723	0.0681	1.342	1.960
N. heliophila	Root	40	25 µL P	3.457	0.0864	2.188	1.872
N. heliophila	Root	16	25 µL P + 25 µL Se	26.862	0.6716	2.426	1.724
N. heliophila	Root	28	5 µL P + 25 µL Se	21.757	0.5439	2.360	1.794
N. heliophila	Shoot	40	25 µL P	0.668	0.0167	0.859	12.363
N. heliophila	Shoot	16	25 µL P + 25 µL Se	4.364	0.1091	1.750	1.577
N. heliophila	Shoot	28	5 µL P + 25 µL Se	0.158	0.0039	-1.218	1.868

Table 7. Complete RNA isolation results from the third trial. The measurements were obtained with Nanodrop. P = phosphate; Se = selenite. Samples were taken from plants in the second hydroponics experiment. Replicate number represents which bucket the plant was harvested from. Sample was always taken from the largest plant in the bucket.

Species	Plant material	Replicate	Treatment	ng/µL	A260	260/230	260/280
N. heliophila	Root	5	5 µL P + 25 µL Se	0.652	0.0163	2.489	2.498
N. heliophila	Root	8	5 µL P + 25 µL Se	0.330	0.0083	24.086	0.659
N. amplexicaulis	Root	11	5 µL P + 25 µL Se	10.965	0.2741	2.117	1.852
N. heliophila	Root	12	25 µL P	1.539	0.0385	2.633	1.420

N. heliophila	Root	14	25 µL P + 25 µL Se	0.842	0.0210	1.672	1.919
N. heliophila	Root	15	25 µL P	2.068	0.0517	0.950	1.233
N. amplexicaulis	Root	17	25 µL P + 25 µL Se	1.054	0.0263	1.333	2.242
N. amplexicaulis	Root	20	25 µL P	1.391	0.0348	1.225	2.212
N. amplexicaulis	Root	21	25 µL P	0.984	0.0246	1.076	1.063
N. heliophila	Root	24	25 µL P	0.954	0.0239	1.053	2.634
N. amplexicaulis	Root	26	25 µL P + 25 µL Se	1.303	0.0326	1.368	1.640
N. heliophila	Root	27	25 µL P + 25 µL Se	1.296	0.0324	1.242	2.637
N. heliophila	Root	30	25 µL P + 25 µL Se	1.563	0.0391	1.589	3.681
N. heliophila	Root	31	25 µL P	7.570	0.1892	2.274	1.831
N. heliophila	Root	32	5 µL P + 25 µL Se	1.247	0.0312	1.139	3.501
N. amplexicaulis	Root	34	25 µL P + 25 µL Se	0.607	0.0152	0.855	-39.591
N. amplexicaulis	Root	36	5 µL P + 25 µL Se	7.579	0.1895	2.209	1.781
N. amplexicaulis	Root	39	25 µL P	0.675	0.0169	1.022	2.170
N. heliophila	Root	41	5 µL P + 25 µL Se	1.115	0.0279	1.023	1.738
N. amplexicaulis	Root	43	5 µL P + 25 µL Se	1.107	0.0277	1.297	-5.301
N. amplexicaulis	Root	44	25 µL P + 25 µL Se	0.958	0.0240	1.351	1.089
N. amplexicaulis	Root	45	25 µL P	0.792	0.0198	1.054	1.610
N. amplexicaulis	Root	46	5 µL P + 25 µL Se	2.596	0.0649	0.721	0.967
N. heliophila	Root	48	25 µL P + 25 µL Se	0.376	0.0094	0.384	1.499