

## The reduction of selenium(IV) by boreal *Pseudomonas* sp. strain T5-6-I – effects on selenium(IV) uptake in *Brassica oleracea*

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### Abstract

*Selenium (Se) is an essential micronutrient but toxic when taken in excessive amounts. Therefore, understanding the metabolic processes related to selenium uptake and bacteria-plant interactions coupled with selenium metabolism are of high importance. We cultivated Brassica oleracea with the previously isolated heterotrophic aerobic Se(IV)-reducing Pseudomonas sp. T5-6-I strain to better understand the phenomena of bacteria-mediated Se(IV) reduction on selenium availability to the plants. B. oleracea grown on Murashige and Skoog medium (MS-salt agar) with and without of Pseudomonas sp. were amended with Se(IV)/<sup>75</sup>Se(IV), and selenium transfer into plants was studied using autoradiography and gamma spectroscopy. XANES was in addition used to study the speciation of selenium in the B. oleracea plants. In addition, the effects of Se(IV) on the protein expression in B. oleracea was studied using HPLC-SEC. TEM and confocal microscopy were used to follow the bacterial/Se-aggregate accumulation in plants and the effects of bacterial inoculation on root-hair growth. In the tests using <sup>75</sup>Se(IV) on average 130% more selenium was translocated to the B. oleracea plants grown with Pseudomonas sp. compared to the plants grown with selenium, but without Pseudomonas sp.. In addition, these bacteria notably increased root hair density. Changes in the protein expression of B. oleracea were observed on the ~30 – 58 kDa regions in the Se(IV) treated samples, probably connected e.g. to the oxidative stress induced by Se(IV) or expression of proteins connected to the Se(IV) metabolism. Based on the XANES measurements, selenium appears to accumulate in B. oleracea mainly in organic C-Se-H and C-Se-C bonds with and without bacteria inoculation. We conclude that the Pseudomonas sp. T5-6-I strain seems to contribute positively to the selenium accumulation in plants, establishing the high potential of Se<sup>0</sup>-producing bacteria in the use of phytoremediation and biofortification of selenium.*

**Keywords: Bacteria, Pseudomonas, Selenium, Plant uptake, Bacteria-plant interactions**

### 1. Introduction

Selenium (Se) is an essential micronutrient for humans and animals (Rayman 2000, Hartikainen 2005, Gupta and Gupta 2017), but is characterized by a very narrow range between deficient (<40 µg/day for human diet) and toxic (>400 µg/day for human diet) (WHO 1996) concentrations for most living organisms. In low concentrations selenium has antioxidant properties and in animals it is essential e.g. in immune responses and thyroid hormone metabolism (Reid et al. 2008). Selenium deficiency induces heart and joint related diseases, such as endemic cardiomyopathy and arthritis that have been

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4 documented especially in the regions of the World with extremely selenium-poor soils (Rayman 2000).  
5 In contrast, based on epidemiologic studies, chronic exposure to raised selenium intake, especially in  
6 regions with elevated soil selenium concentrations, is associated with several adverse health effects.  
7 These include the impairment of the synthesis of thyroid hormones and metabolism of growth hormone  
8 as well as hepatotoxicity, gastrointestinal disturbances and nail and hair loss and dermatitis (Vinceti et  
9 al. 2001). Selenium supplementation may also increase risk for the type 2 diabetes (Stranges et al.  
10 2010).  
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14 Human dietary intakes of selenium range from high to low according to geography and local soil  
15 conditions. The mean crustal abundance of selenium is 0.083mg/ kg (Antonyak et al. 2018). In most  
16 soils selenium concentrations range from 0.1 to 2 mg/kg (Oldfield 2002), but extremely high Se  
17 contents up to 1200 mg/kg have been reported e.g. from organic-rich soils in Ireland (Fleming and  
18 Walsh 1957). Understanding of these variations, and the biogeochemical factors affecting them, is  
19 essential for the improvement of health problems associated with selenium deficiency and toxicity.  
20 Certain regions of the world, including Finland, Sweden and Scotland, are deficient in selenium, while  
21 others (e.g. Japan, Greenland, USA, Venezuela and Canada) are Se-rich/Se-toxic due to natural and  
22 anthropogenic activities (Zhu et al. 2009, Yin and Yuan 2012, Fordyce 2005). Natural sources of  
23 environmental selenium include forest fires and soil erosion, whereas considerable amounts of  
24 selenium enter the environment via anthropogenic activities including coal combustion, mining,  
25 refining of sour crude oils and agricultural irrigation of seleniferous soils (Manceau et al. 1997, de  
26 Souza et al. 1999, Sharmasarkar and Vance 2002, Coppin et al. 2009). In addition, regarding the  
27 radiation protection point of view, the radioactive long-lived isotope of selenium, <sup>79</sup>Se, is found in  
28 spent nuclear fuel and is one of the high priority radionuclides when the biosphere safety of spent  
29 nuclear fuel disposal is to be considered (Helin et al. 2010).  
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35 Selenium enters the food chain through plants that take it up from the soil. In alkaline (agricultural)  
36 soils, selenium mostly exists as selenate (Se(VI), SeO<sub>4</sub><sup>2-</sup>) whereas in acidic (forest) soils it exists as  
37 selenite (Se(IV), SeO<sub>3</sub><sup>2-</sup>). The forms of selenium differ in terms of their absorption and mobility within  
38 the plant and are metabolized to form selenocompounds (Li et al., 2008). Selenite has been reported to  
39 be transported by a phosphate transport mechanism (Li et al., 2008) while selenate uptake is believed to  
40 occur through sulfate transporters (Feist and Parker, 2001, Zhang et al., 2003). However, only recently  
41 the involvement of a putative selenium-binding protein (SBP) gene family, composed of three members  
42 (SBP1–SBP3) has been described in *Arabidopsis thaliana* (e.g. Schild et al. 2014).  
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46 Plants are the main source of dietary selenium and plant selenium concentrations can vary from  
47 0.005mg/kg in Se-deficient crops to 5500mg/kg in selenium hyper-accumulators (Fordyce 2005). The  
48 essentiality of selenium for plants is still debatable (Dumont et al. 2006, Gupta and Gupta 2017). At  
49 low doses, selenium may protect the plants from variety of abiotic stresses, such as cold, drought, and  
50 metal stress (Chu et al. 2010, Hasanuzzaman and Fujita 2011, Kumar et al. 2012). However, at higher  
51 doses selenium becomes toxic also for plants and its toxicity is caused by two different mechanisms; 1)  
52 the formation of malformed proteins due to the misincorporation of selenocysteine (SeCys) or  
53 selenomethionine (SeMet) in place of cysteine (Cys) or methionine (Met) in a protein, or 2) by  
54 inducing oxidative stress (Gupta and Gupta 2017).  
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58 Plants are in continuous contact with soil microbiota (Srivastava et al. 2012), which can modify the soil  
59 chemical environment through e.g. oxidation and reduction reactions and secretion of metabolites,  
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4 altering the availability of chemical elements for plants. Interactions between plants and  
5 microorganisms take place during different phases of the plant life cycle and many of these interactions  
6 can be beneficial for the plant (Schirawski and Perlin 2018). In fact, there are multiple interactions  
7 where the plant benefits either directly or indirectly of the associated microbes. Further, plants may act  
8 as protected habitats for bacteria occupying the surfaces of the plant, the apoplast or the soil  
9 surrounding the plant roots (Schirawski and Perlin 2018). In addition, plants may release compounds  
10 attracting the accompanying soil bacteria and the associated microbes may in turn secrete compounds  
11 that favour e.g. plant growth, increase the plant capacity to resist abiotic and biotic stress or protect the  
12 plant against malignant microbes. Soil bacteria (especially plant growth promoting rhizobacteria,  
13 PGPR, such as *Pseudomonas putida*) can e.g. promote plant growth through production of  
14 phytohormones (e.g. indole-3-acetic acid (IAA), auxin, gibberellins) (Joo et al. 2005, Idris et al. 2007,  
15 Srivastava et al. 2012). Previously, we found that bacteria affected selenium behavior and retention in  
16 boreal bog environment and that the *Pseudomonas* sp. strain (T5-6-I) (Lusa et al. 2016), which was  
17 also used in the present study, removed <sup>75</sup>Se(IV) from solutions under different nutrient conditions  
18 (Lusa et al. 2015). In experiments with stable Se isotopes, brick-red reduced elemental selenium (Se<sup>0</sup>)  
19 was formed from both Se(IV) and Se(VI) in incubations with the *Pseudomonas* sp. strain T5-6-I under  
20 aerobic conditions (Lusa et al. 2017). However, the reduction was significantly more efficient for  
21 Se(IV) and for Se(VI) formation of elemental selenium was only barely detectable (Lusa et al. 2017).  
22 In addition, nitrate (NO<sub>3</sub><sup>-</sup>), nitrite (NO<sub>2</sub><sup>-</sup>) and sulfate (SO<sub>4</sub><sup>2-</sup>) enhanced Se(IV) uptake in *Pseudomonas*  
23 sp. strain T5-6-I, and Se(IV) uptake continued also under sulphur and nitrogen starvation.  
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32 Previously, the uptake of selenium oxyanions Se(IV) and Se(VI) by plants has been studied e.g. in *A.*  
33 *thaliana* (e.g. Barickman et al. 2013, White et al. 2004), *Stanleya pinnata* (e.g. El Mehdawi et al. 2017)  
34 and *Brassica juncea* (e.g. El Mehdawi et al. 2017). In turn, the effects of bacteria, e.g. *P. putida*  
35 (Srivastava et al. 2012), *Azospirillum brasilense*, *Serratia plymuthica*, *Stenotrophomonas maltophilia*  
36 (Wenke et al. 2012), *Bacillus subtilis* and *Burkholderia cepacia* (Vespermann et al. 2007, Kai et al.  
37 2008) have been reported on *Arabidopsis* growth. However, previous research on the simultaneous  
38 effects of selenium and soil dwelling bacteria on selenium plant uptake is to date scarce. Previously,  
39 bacteria belonging to the genera *Bacillus*, *Pseudomonas*, *Pantoea*, *Staphylococcus*, *Paenibacillus*,  
40 *Advenella*, *Arthrobacter* and *Variovorax* have been found as endophytes from selenium  
41 hyperaccumulators *S. pinnata* (*Brassicaceae*) and *Astragalus bisulcatus* (*Fabaceae*) (Sura-de Jong et al.  
42 2015). These bacteria reduced Se(IV) to elemental Se<sup>0</sup> and additionally had plant growth promoting  
43 properties. However, they had no effect on the amount of selenium accumulated into the plant (Sura-de  
44 Jong et al. 2015).  
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49 Nutritional intake is the most important source of selenium for humans and the biogeochemical  
50 components, including soil microbiota, are in a key role when assessing the transfer of environmental  
51 selenium into the human diet and its associated health effects (Fordyce 2005). Because of bacterial  
52 synergy and interactions with plants, soil bacteria may have vital, yet unidentified, roles in plant macro-  
53 and micronutrient metabolism as well as in overcoming toxic effects of e.g. selenium in different  
54 regions of the world. Therefore understanding the metabolic processes related to the toxic Se(IV) stress  
55 and bacteria-plant interactions in selenium accumulation is of high importance. To better understand  
56 the effects of bacteria-mediated selenium oxyanion reduction (that changes the speciation of selenium  
57 from Se(IV) available for plants to insoluble Se<sup>0</sup>) on the subsequent availability and transfer of  
58 selenium to plants, we cultivated *Brassica oleracea* with above mentioned, previously isolated  
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4 heterotrophic aerobic T5-6-I strain (Lusa et al. 2016). This strain was especially interesting, as it  
5 represents *Pseudomonas*, bacterial genus commonly found in many different types of environments,  
6 having versatile metabolism. In addition, previously this strain was shown to remove especially Se(IV)  
7 from growth solutions (Lusa et al. 2015, 2017). Therefore, this strain is suspect to affect particularly the  
8 overall transfer of Se(IV), a highly toxic species of selenium, from soil solutions into plants. Se(IV) is  
9 very reactive and reacts in particular with thiol groups found in glutathione (GSH), producing several  
10 Se-containing compounds, including selenodiglutathione, glutathioselenol, hydrogen selenide (H<sub>2</sub>Se)  
11 and elemental selenium (Se<sup>0</sup>) (Tarze et al. 2007). This reaction produces also highly toxic oxygen  
12 species like H<sub>2</sub>O<sub>2</sub> and O<sup>2-</sup> (Kramer and Ames 1988). *B. oleracea* was chosen as *Brassica* spp. are  
13 known secondary accumulators (i.e. are typically able to accumulate up to 1000 mg Se/kg DW) of  
14 selenium (Gupta and Gupta 2017).  
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## 18 2. Materials and methods

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21 **Bacteria.** The isolation and characterization of the *Pseudomonas* sp. strain T5-6-I used in this study,  
22 has been described in Lusa et al., 2016. The 16S rRNA gene sequence has been deposited in GenBank  
23 under accession number KP100424. The stock cultures of this bacterium were grown aerobically on  
24 sterile PCA growth plates (PCA, Merckoplate®) at 20 °C in the dark and the strain was re-cultivated on  
25 new plates weekly.  
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29 **Plant material.** Seeds of *B. oleracea* were bought from a garden store. Surface sterilized (0.1 % Triton  
30 X in 95% ethanol) seeds were sown on MS-agar (Murashige and Skoog Basal Salt Mixture, Sigma  
31 M5524) (Xi et al. 2016) plates (50 mL) containing 1% sucrose (w/v) and 0.7% (w/v) agar (Sigma  
32 A1296). *B. oleracea* plates were transferred to the growth chamber set at 23°C in 16/8 h light and dark  
33 cycles immediately after sowing. Selenium (125 µM of selenite (Na<sub>2</sub>Se(IV)O<sub>3</sub> (AlfaAesar®)) or 100 Bq  
34 of radioisotopes of selenium <sup>75</sup>[Se(IV)O<sub>3</sub><sup>2-</sup>] with 2.7 × 10<sup>-8</sup> M stable Se(IV) carrier) and bacterial  
35 suspensions (0 or 5·10<sup>7</sup> CFU of exponential growth phase bacteria) were added on one week old  
36 seedlings by spreading in total 5 mL of the suspensions on the surface of the plant agar. Plants were  
37 thereafter grown for two (XANES, gammaspectrometry and confocal experiments) or three weeks  
38 (autoradiography, CT, and protein/HPLC experiments) depending on the set of experiments. Of all  
39 plates 3 – 7 replicates were prepared depending on the set of experiments. For subsequent experiments,  
40 leaves and roots of plants were harvested. Control samples without selenium or bacterium inoculation  
41 were used in all experiments to ensure no contaminations occurred.  
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46 **The effect of Se(IV) on bacterial growth.** The effect of Se(IV) on the growth of *Pseudomonas* sp. T5-  
47 6-I was tested by growing the *Pseudomonas* sp. T5-6-I with and without Se(IV) amendment. In these  
48 experiments, the cells were grown in 1 % Tryptone solution at +20°C. Na<sub>2</sub>Se(IV)O<sub>3</sub> (AlfaAesar®) was  
49 added to the growth medium to a final concentration of 6 mM of Se(IV). The cells were incubated in  
50 the dark on an orbital shaker (120 rpm) for up to 360 hours and sampled regularly. The absorbance of  
51 the cell solutions without Se(IV) was recorded at the wavelength of 600 nm to estimate the overall  
52 CFU/mL. Se(IV) amended samples were not used in the absorbance determinations due to the strong  
53 formation of Se precipitates interfering with the measurements. However, viable cell counts (CFU/mL)  
54 were determined for both Se(IV) treated and control samples without Se(IV). The samples were serially  
55 diluted in 10-fold steps to a dilution of 10<sup>-6</sup>. Aliquots of 100 µL from dilutions 10<sup>-4</sup> and 10<sup>-6</sup> were  
56 spread on Plate Count Agar (PCA, Merckoplate®) and the plates were further incubated aerobically at  
57 20°C for up to 408 hours in the dark and the CFUs were counted.  
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4 **Speciation of selenium in bacterial cells and in plant material.** The speciation of selenium in the  
5 *Pseudomonas* sp. cells and in the differentially treated roots and leaves of *B. oleracea* plants was  
6 examined using X-ray absorption near-edge structure (XANES) spectroscopy. The XANES  
7 measurements were performed at Se K edge (12.7 keV) using a table top Johann-type spectrometer  
8 detailed in (Honkanen et al. 2019). For the XANES experiments bacterial cultures were treated with 3  
9 or 6 mM Se(IV) and incubated under aerobic conditions at 20 °C for 7 days. After incubation, the cells  
10 were separated by centrifugation at 5000×g for 15 min at room temperature followed by washing of the  
11 remaining bacterial pellet twice with 0.01 M (pH 7) phosphate buffer. Thereafter, bacterial pellets pre-  
12 frozen at -18 °C were lyophilized using an Alpha 1-4 LSCbasic lyophilizer (Christ). *B. oleracea* seeds  
13 were grown as described above for the plant material, with 125 µM of Se(IV) and with or without 5·10<sup>7</sup>  
14 CFU of *Pseudomonas* sp. T5-6-I amendment. After 14 days growth period, fresh leafs and roots were  
15 harvested, pre-frozen at -18 °C and lyophilized as described for the bacterial cells.  
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20 The lyophilized pellets were homogenised with a mortar and pestle and bacterial samples were  
21 prepared as such in M8 steel washers with a thickness of 1.4 mm and an inner diameter of 8.6 mm.  
22 Leaf samples were prepared as such in M5 steel washers with a thickness of 1.9 mm and inner diameter  
23 of 5.4 mm. For the root samples, potato starch was used as a filler. The washers were covered with  
24 Scotch tape. Elemental (black, Se<sup>0</sup>) selenium, selenium salts Na<sub>2</sub>Se(IV)O<sub>3</sub> (AlfaAesar®), Na<sub>2</sub>Se(VI)O<sub>4</sub>  
25 (AnalaR®) and PbSe (AlfaAesar®) were used as reference compounds for the bacterial samples. For  
26 plant material in addition L-selenocysteine (Acros organics) and L-selenomethionine (BioVision) were  
27 used. The reference compounds were weighed to optimize the change in the absorption coefficient,  
28 mixed with potato starch to make the samples more homogeneous and to increase their total volume.  
29 M8 steel washers (thickness 1.4 mm, inner diameter 8.6 mm) were used for the reference compounds.  
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33 For the bacteria, the spectra were recorded over the energy range of 12.538-12.986 keV divided evenly  
34 into 450 steps using Si(953) monochromator in the transmission mode. The acquisition time for the  
35 direct beam was 12.5 h and the transmitted beams through bacterial pellets from the treatments with 3  
36 mM and 6 mM selenium for 16.5 h and 5.5 h, respectively. The transmission through the reference  
37 samples were in the range of 5.5 – 9.0 h per sample. The X-ray tube acceleration voltage was set to 20  
38 kV and the current was 10 mA. Samples were positioned at the tube exit and a 20 µm thick Al filter  
39 was placed in the beam path before the sample to absorb low energy photons and thus reduce the  
40 unnecessary radiation dose to the samples. For the plant material, the spectra were recorded over the  
41 energy range of 12.624-12.713 keV divided evenly into 100 steps using Si(953) monochromator in the  
42 simultaneous transmission and fluorescence mode. The acquisition time for the direct beam was 14.0 h  
43 and in the range of 19.6 – 56.1 h per plant sample. The wide range in the measurement times was due  
44 to variation in signal strength between the samples. The X-ray tube acceleration voltage was 20 kV and  
45 the current 40 mA. Samples were positioned in front of the detector. The calculation of spectra from  
46 the raw instrument data and final visualizations were performed with custom Python scripts. The  
47 details of spectra calculation is presented in (Honkanen et al., 2019).  
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53 The selenium content of the plant samples was estimated by determining magnitude of Se K edge step  
54 in the transmission signal and calculating the corresponding amount of selenium. The compositions of  
55 obtained spectra from plants were estimated by fitting linear combinations of measured reference  
56 spectra with an ordinary least-squares fit using a Python implementation available at  
57 <https://github.com/aripekka/bayesfit>. Utilizing the Bayes' theorem, the (posterior) probability p(H|D)  
58 for the hypothesis H to be true when given the data D is  
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4  $p(H|D) \propto p(D|H)p(H)$   
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7 where  $P(D|H)$  is the probability of obtaining  $D$  when  $H$  is true and  $p(H)$  is the a priori probability of  $H$   
8 to be true. This allows us to compare the relative probability of competing models to each other as  
9 follows:  
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$$p(A|D)/p(B|D) = p(D|A, \lambda_A) / p(D|B, \lambda_B) \sqrt{\det(\Sigma_A)} / \sqrt{\det(\Sigma_B)},$$
  
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14 where  $p(A|D)$  is the (posterior) probability of model  $A$  to be true when the data  $D$  is known,  $p(D | A,$   
15  $\lambda_A)$  is the probability to obtain  $D$  when model  $A$  is true with parameters fitted model parameter  $\lambda_A$  and  
16  $\det(\Sigma_A)$  is the determinant of the covariance matrix of  $\lambda_A$ . Definitions are similar for the model  $B$ . The  
17 given equation is a straightforward generalization of Eq. (4.9) in (Sivia and Skilling 2006) when a  
18 priori probabilities are taken equal and the number of fit parameters is the same. To compare how two  
19 models explain a group of independent fits, the relative probabilities of single fits are simply multiplied  
20 together.  
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23 **Protein expression in plant roots and leafs.** The effect of *Pseudomonas* sp. T5-6-I and Se(IV)  
24 amendment in the expressed protein profile of *B. oleracea* was examined to identify potentially  
25 important proteins involved in Se(IV) metabolism in the studied plants. Total plant protein extracts  
26 were examined using HPLC-SEC. The plants were grown with 125  $\mu$ M Se(IV) amendment as  
27 described above for plant material and proteins from untreated control, *Pseudomonas* sp. T5-6-I  
28 treated, Se(IV) and *Pseudomonas* sp. T5-6-I + Se(IV) treated plants were isolated using the TPE Kit  
29 (G-Biosciences<sup>®</sup>) method according to the manufacturer's instructions. A protease inhibitor cocktail  
30 (G-Biosciences<sup>®</sup> ProteaseArrest-kit) was used in the TPE-I buffer according to the manufacturer's  
31 instructions. The extracted protein concentrations were quantified using RED 660<sup>™</sup> Protein Assay (G-  
32 Biosciences<sup>®</sup>) according to the manufacturer's instructions. 1:3 diluted total protein extracts in TBE-  
33 buffer (TPE Buffer-I, G-Biosciences<sup>®</sup> ProteaseArrest-kit) were used for HPLC-SEC analysis (Symmetry  
34 semiprep C18 7.8x300 mm 7  $\mu$ m). HPLC-SEC was run in 1M phosphate buffered saline (pH 7.9), with  
35 an injection volume of 5  $\mu$ L and 20 minutes acquisition time. The UV-VIS spectra were recorded at  
36 234 nm, 209 nm and 254 nm.  
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42 **Quantification of Se(IV) transfer into the plants.** Batch experiments with  $^{75}[\text{Se(IV)O}_3^{2-}]$  radioisotope  
43 tracer and gamma spectrometry were used to quantify the transfer and accumulation of selenium into  
44 the roots and leafs of *B. oleracea* plants from the growth medium. For these experiments *B. oleracea*  
45 plants were grown on MS-salt agar as described above for plant material with 100 Bq of  
46  $^{75}[\text{Se(IV)O}_3^{2-}]$  (with  $2.7 \times 10^{-8}$  M stable Se(IV) carrier) and with or without *Pseudomonas* sp. T5-6-I  
47 inoculation. After a growing period of two weeks the leaves and roots were harvested and weighed and  
48 the plant material was digested using 5 mL (1:50 m/V) of 65%  $\text{HNO}_3$  (suprapur). The samples were  
49 incubated at room temperature for 2 h after which the samples were centrifuged at 12 000 g for 10  
50 minutes and filtered through a 0.2  $\mu$ m membrane filter.  $^{75}\text{Se}$  concentration was measured from the  
51 resulting solutions using NaI(Tl)-gamma spectrometer (Wizard<sup>®</sup> automatic gamma counter,  
52 PerkinElmer).  
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56 **Visualization of Se accumulation in the plants.** Digital autoradiography was used to visualize the  
57 location and accumulation density of selenium in dried,  $^{75}[\text{Se(IV)O}_3^{2-}]$  treated *B. oleracea* leafs and  
58 roots with or without *Pseudomonas* sp. T5-6-I amendments. For these experiments, the plants were  
59 dried at room temperature, placed on a Fuji FLA imaging plate and exposed for six days in a lead  
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4 shielded film cassette. The exposed FLA plate was thereafter scanned using Fujifilm FLA-5100  
5 scanner with Aida Image analysis software. In addition, X-ray microtomography ( $\mu$ CT) and X-ray  
6 nanotomography (nanoCT) imaging methods were used to visualize changes in tissue density  
7 properties (absorption contrast) in response to applications with stable selenium (Se(IV), 125  $\mu$ M) in  
8 the lyophilized (Alpha 1-4 LSCbasic lyophilizer, Christ) *B. oleracea* leaves ( $\mu$ CT) and roots (nanoCT).  
9 This enabled a higher image resolution in tissues in comparison to the digital autoradiography.  
10 Confocal microscopy was used to examine the changes in the root morphology of *B. oleracea* in  
11 response to Se(IV) (125 $\mu$ M) and bacteria treatments from fresh samples dyed with propidium iodide  
12 (PI) just prior to imaging. Transmission electron microscopy (TEM) was in addition used to examine  
13 the possible accumulation of bacteria and selenium in the leaves of plants treated with 125  $\mu$ M Se(IV)  
14 and *Pseudomonas* sp. T5-6-I. For the TEM experiments the plants were grown in Se(IV) containing  
15 MS-agar plates as described above. Freshly collected leaves were fixed in 2% glutaraldehyde for 2  
16 hours and cut into 1cm x 1cm pieces. Cells were dehydrated through an ethanol series and then  
17 embedded in Taab hard epon and polymerized at 60 °C. Thin sections were cut using Leica ultracut  
18 UCT ultramicrotome (Leica Mikrosysteme GmbH, Austria) and collected on single-slot copper grids.  
19 Section thickness of 60 nm was used for the morphological examinations. After double staining with  
20 uranyl acetate and lead citrate, the sections were examined using FEI Tecnai F20 at 200 kV under  
21 standard operating conditions.  
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27 **Statistical analyses.** To study the statistical difference in the Se-accumulation between the  
28 differentially treated *B. oleracea* plants using  $^{75}[\text{Se(IV)O}_3^{2-}]$  radioisotope tracers, the analysis of  
29 variance was performed using OriginPro 8.6 (OriginLab®) and one-way ANOVA at the  $p < 0.05$  level.  
30 Prior to analysis, Shapiro-Wilk test ( $p < 0.05$ ) was used to examine the normality of the data, and as the  
31 normality hypotheses were rejected, log transformed data was used for the one-way ANOVA tests.  
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### 34 3. Results

#### 35 3.1. Bacterial growth under Se(IV) stress

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39 The effect of Se(IV) on growth of *Pseudomonas* sp. T5-6-I was tested using 1% Tryptone growth broth  
40 amended with 6 mM Se(IV) and viable cells were counted using PCA plate count agar and measuring  
41 the absorbance of the cultures at 600 nm (Figure 1). In these experiments, Se(IV) was found to  
42 decrease the growth rate of the *Pseudomonas* sp. T5-6-I (Figure 1B). In the cultures treated with Se(IV)  
43 the plateau phase was reached before 216 h while the untreated control cultures reached the plateau  
44 phase already before 96 h.  
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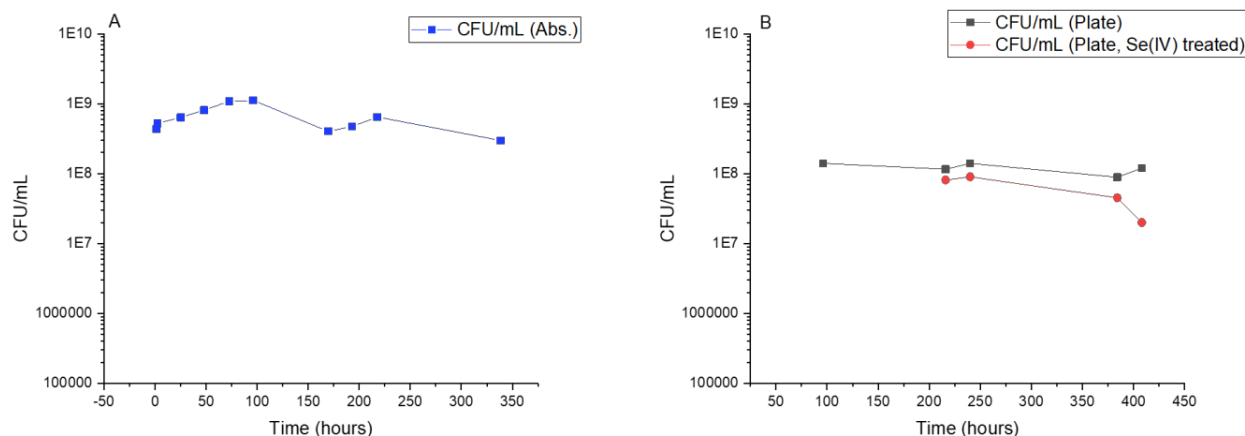


Figure 1. The effect of Se(IV) on bacterial growth of *Pseudomonas sp. T5-6-I* on 6 mM Se(IV) amended 1% Tryptone solution. A) 600 nm absorbance based CFU/mL obtained from the untreated control cells. B) Grey = Viable cell counts (CFU/mL) obtained from the PCA plates of untreated cells, Red = Viable cell counts (CFU/mL) obtained from the PCA plates of Se(IV) treated cells. At time = 72h, the cell counts both on Se(IV) treated plates as well as on plates without Se(IV) amendment were 0 CFU/mL, At time = 96 h, the cell counts on Se(IV) treated plates was 0 CFU/mL

### 3.2. Selenium speciation in *Pseudomonas sp. T5-6-I*

The Se K edge XANES spectra were recorded for *Pseudomonas sp. T5-6-I* cells grown in 1% Tryptone amended with 3mM and 6 mM Se(IV) together with the reference compounds (Figure 2) to study the speciation of selenium in the bacterial cells. The absorption coefficient from 6 mM Se(IV) bacteria samples followed closely that of the black (vitreous, Se<sup>0</sup>) selenium reference. The spectrum recorded in 3 mM Se(IV) amended bacteria was similar within the statistical accuracy.

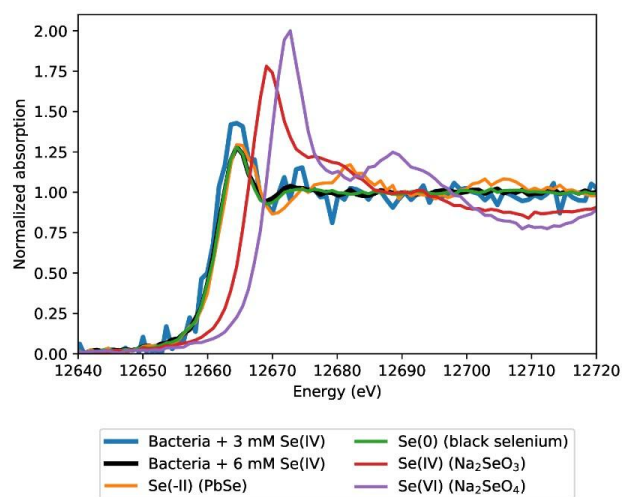


Figure 2. Se K edge of accumulated selenium in *Pseudomonas sp. T5-6-I* after incubation in presence of Se(IV) (3 mM and 6 mM concentration) together with reference compound spectra. The signal from the bacteria samples follow closely that of elemental Se.



### 3.3. Effect of *Pseudomonas* sp. T5-6-I on Se uptake by *B. oleracea*

*B. oleracea* showed increased transfer of  $^{75}\text{Se(IV)}$  to the roots, leaves and stems in the *Pseudomonas* sp. T5-6-I treated samples (Figure 3A) compared to the plants with only  $^{75}\text{Se(IV)}$  amendment but without *Pseudomonas* sp. T5-6-I inoculation seen as increased  $^{75}\text{Se(IV)}$  signal (red colour in figure 3A) in the autoradiography images. Furthermore,  $^{75}\text{Se}$  seemed rather evenly distributed throughout the *Pseudomonas* sp. T5-6-I treated plants (red colour in Figure 3A). In the  $\mu\text{CT}$  studies, leaves of Se supplemented plants with (Figure 4C) and without (Figure 4B) of *Pseudomonas* sp. T5-6-I inoculation showed increased absorption contrast attenuation (visualized in green signal) compared to the control leaf (Figure 4A). The green signal appeared to be evenly distributed throughout the leaves of both Se and Se+bacteria supplemented plants. In contrast, only the leaf veins of control leaves showed higher density (likely due to lignified vascular cells). In the roots of *B. oleracea*, visualized using nanoCT, (Figures 4D – E), increased tissue density (pink and yellow signal) was seen mainly in the *Pseudomonas* sp. T5-6-I inoculated and Se(IV) treated sample (F), compared to the control root (D) and Se supplemented sample without *Pseudomonas* sp. inoculation (E).

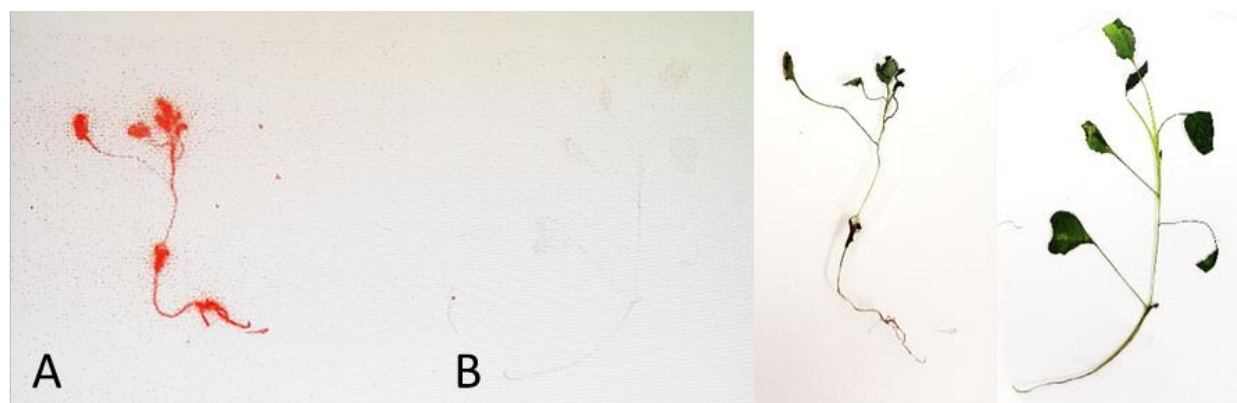


Figure 3. A) *B. oleracea* grown on MS-salt agar labelled with  $^{75}[\text{Se(IV)}\text{O}_3^{2-}]$  with *Pseudomonas* sp. T5-6-I inoculation. B) *B. oleracea* grown on MS-salt agar labelled with  $^{75}[\text{Se(IV)}\text{O}_3^{2-}]$  without *Pseudomonas* sp. T5-6-I. On the right photographs of the plants used for autoradiograms. Data are representative of at least three biological replicates.

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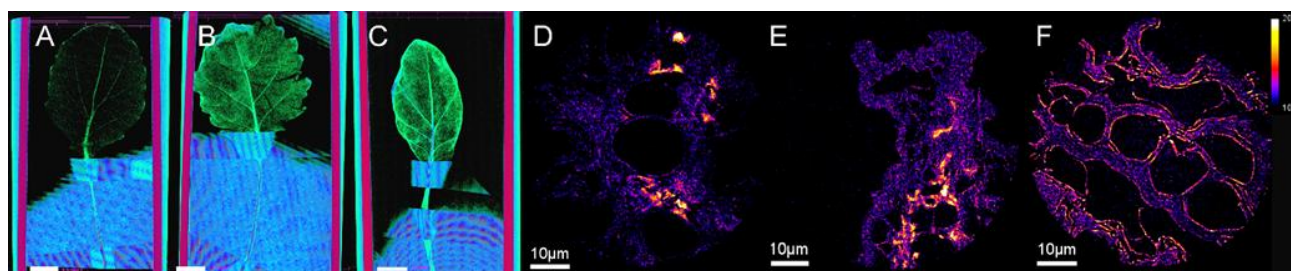


Figure 4. Leaves (A, B, C) and roots (D, E and F) of vacuum dried *B. oleracea* visualized using  $\mu$ CT (A, B and C) and nanoCT (D - F). In (A) a leaf of a plant without Se(IV) amendment grown on the MS-salt agar is shown (control). In (B) a leaf of a plant grown in Se(IV) amended MS-salt agar. In (C) leaf grown with Se+*Pseudomonas* sp. T5-6-I inoculation. Adsorption contrast attenuation visualized in green signal. The light blue colour under the leaves comes from the paper on which the samples were attached to. Red vertical lines are plastic of the tubes in which the samples were imaged. In (D) a control root without Se(IV) amendment, (E) Se(IV) treated *B. oleracea* root and in (F) Se+*Pseudomonas* sp. T5-6-I inoculated root sample.

In the tests using  $^{75}\text{[Se(IV)O}_3^{2-}]$  and gamma spectroscopy on average 130% (2.3-fold) more selenium was translocated to the *B. oleracea* plants grown with *Pseudomonas* sp. T5-6-I, compared to the plants grown without *Pseudomonas* sp. T5-6-I (Figure 5). Selenium was especially accumulated to the roots. In the plants grown without *Pseudomonas* sp. amendment, on average 3-fold more selenium was found in the roots, compared to the leaves. Even more notably difference between selenium concentrations found in the roots and leaves was found in the samples inoculated with  $5 \cdot 10^7$  CFU *Pseudomonas* sp. T5-6-I strain. In these samples on average 12-fold more selenium was found in the roots, compared to the concentrations observed in the leaves. However, it is impossible to distinguish whether selenium was accumulated onto the bacteria residing on the root surfaces, adsorbed onto root surfaces or translocated inside the root tissue. Nevertheless, since the roots were rinsed several times before extraction of selenium, selenium was either; i) tightly bound on root surfaces, or ii) translocated inside the root tissue. Addition of 100 Bq of  $^{75}\text{Se}$  had no evident effect on the phenotype of *B. oleracea*.

Based on the analysis of variance (ANOVA), the inoculation of  $^{75}\text{Se(IV)}$  treated plants with *Pseudomonas* sp. T5-6-I, significantly enhanced selenium accumulation in the roots of *B. oleracea* plants. The differences in the Se accumulation on the roots between *Pseudomonas* sp. T5-6-I inoculated plants, and plants without *Pseudomonas* sp. addition was found statistically significant at the  $p < 0.05$  level ( $F_{\text{crit}} = 4.7$ ,  $F = 10.5$ ,  $p = 0.007$ ). However, in the case of leaves, statistically significant difference between *Pseudomonas* sp. treated plants and plants without bacteria inoculation could not be shown.

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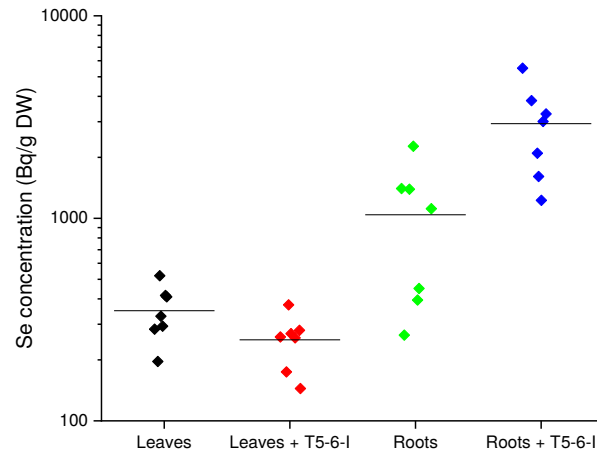


Figure 5. Selenium concentration in the leaves and roots of *B. oleracea* grown in  $^{75}\text{[Se(IV)O}_3^{2-}]$  amended MS-agar without bacteria (black and green) and with *Pseudomonas* sp. T5-6-I inoculation (red and blue).

Transmission electron microscopy (TEM) showed small, <50 nm, dense granules in the leaf cells of *B. oleracea* grown with *Pseudomonas* sp. T5-6-I and 125 $\mu\text{M}$  of Se(IV) (Figures 6B-D). Similar structures were not seen in plants with Se(IV) but without *Pseudomonas* sp. T5-6-I inoculation (Figure 6A).

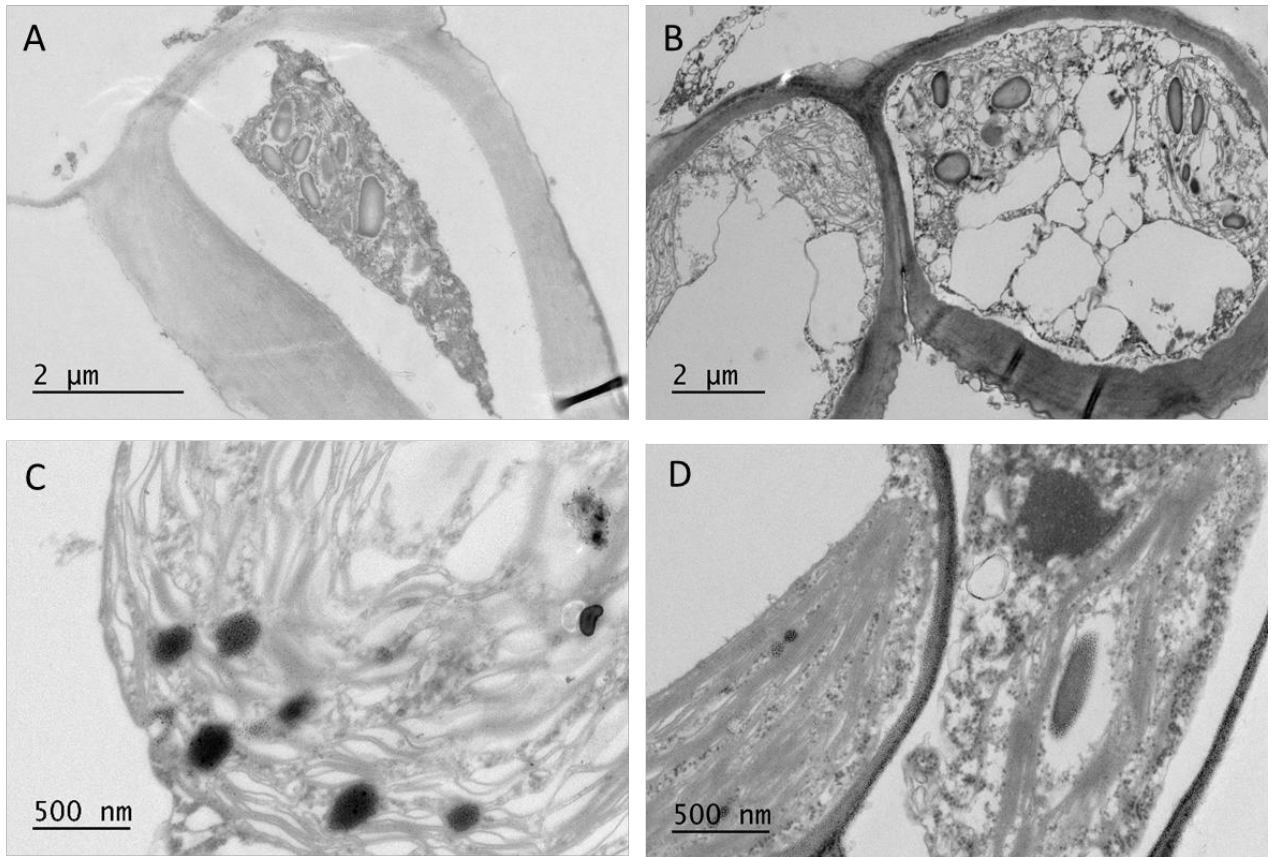


Figure 6. Leaves of *B. oleracea* grown with 125  $\mu\text{M}$  Se(IV) (A), and 125  $\mu\text{M}$  Se(IV) and  $5 \cdot 10^7$  CFU *Pseudomonas* sp. T5-6-I (B – D), visualized using transmission electron microscopy (TEM). Data are representatives of three biological replicates.

X-ray absorption near edge spectroscopy (XANES) was used to further examine the speciation of selenium in the *B. oleracea* leaves and roots. The Se signals from the samples fall between the elemental Se and Se(IV) and followed closely the seleno-amino acid references (Figure 7A). The details of Se speciation were investigated by making two component least squares linear combination fits of the reference compounds to the sample spectra. Reference pairs Se:SeO<sub>3</sub>, Se-Cys:Se-Met, Se:Se-Met, Se:Se-Cys, Se-Cys:SeO<sub>3</sub> and Se-Met:SeO<sub>3</sub> were fitted (Appendix 1, Table A1 and A2). Of the fitted pairs Se:Se-Cys and Se-Met:SeO<sub>3</sub> lead to negative fractions for Se and SeO<sub>3</sub>, respectively, indicating non-physical fits and were rejected from the subsequent analysis. Se:SeO<sub>3</sub> fits were found to fit the data very poorly, which was indicated by extremely small relative probabilities and visual mismatch around 12663 eV. Of tested pairs, the data was best explained by Se-Cys:Se-Met (Figure 7B), representing 2.4 times more probable explanation to the bacteria-inoculated data than Se:Se-Met pair. As XANES is most sensitive to the local structure of the absorbing element, the compounds containing selenium in the structural motif C-Se-C, such as selenomethionine, methylselenocysteine, or selenocystathione, are spectroscopically nearly indistinguishable (Pickering et al. 1999; Lindblom et al. 2012; Wang et al. 2015). The results thus indicate that selenium found in leaves and roots is mainly in organic C-Se-H and C-Se-C bonds. Together with the fact that in the presence of the bacteria, the fit values tended to give more emphasis on components with absorption edge at lower energies, the

Bayesian analysis suggests that while most of the observations can be sufficiently explained with the seleno-amino acids only, the data cannot confidently reject the possible presence of small amounts of more reduced Se compounds, such as  $\text{Se}^0$  granules, when grown in the presence of *Pseudomonas* sp. T5-6-I.

Based on XANES experiments, Se levels of 300-400 mg/kg DW were found in the roots, compared to 40-50 mg/kg DW in leaves (Appendix 1, Table A1 and A2). However, no significant differences in the concentrations were observed between the samples grown with or without *Pseudomonas* sp. T5-6-I.

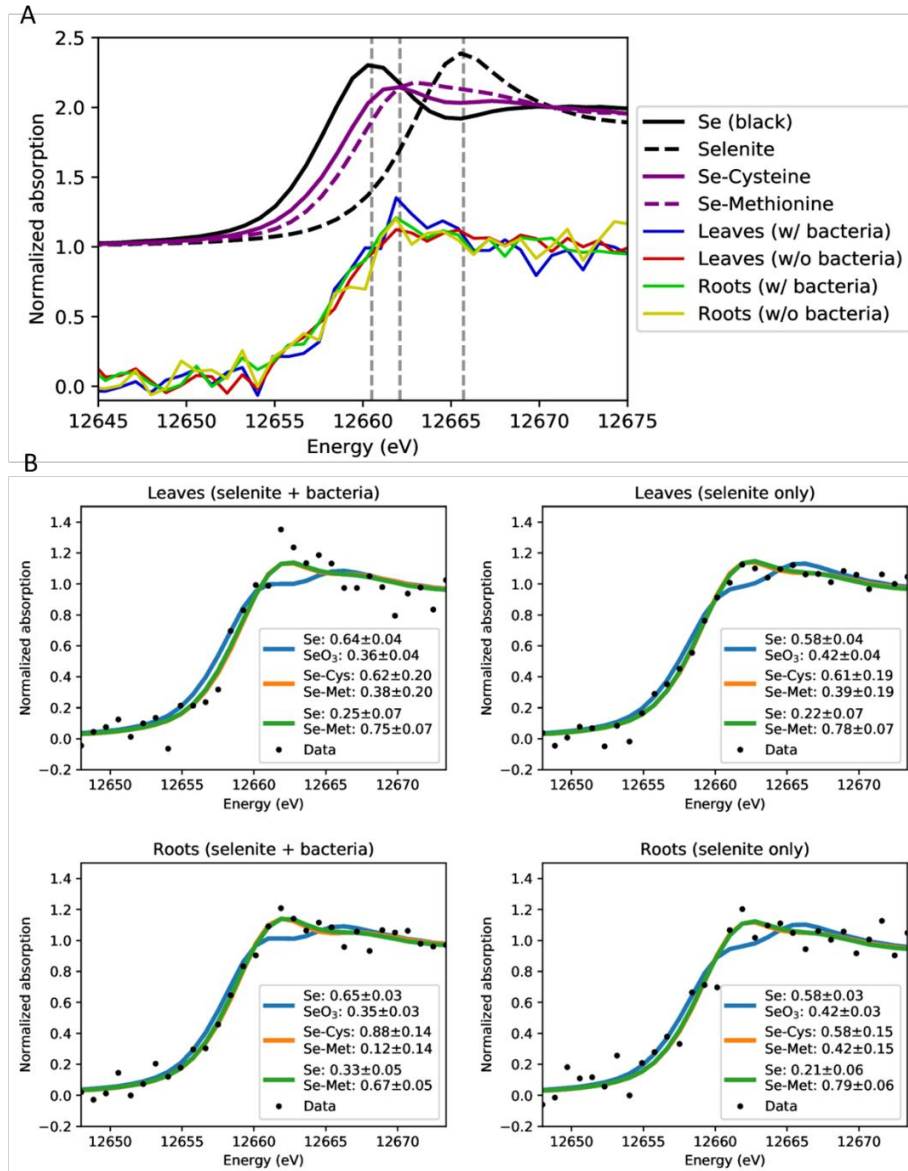
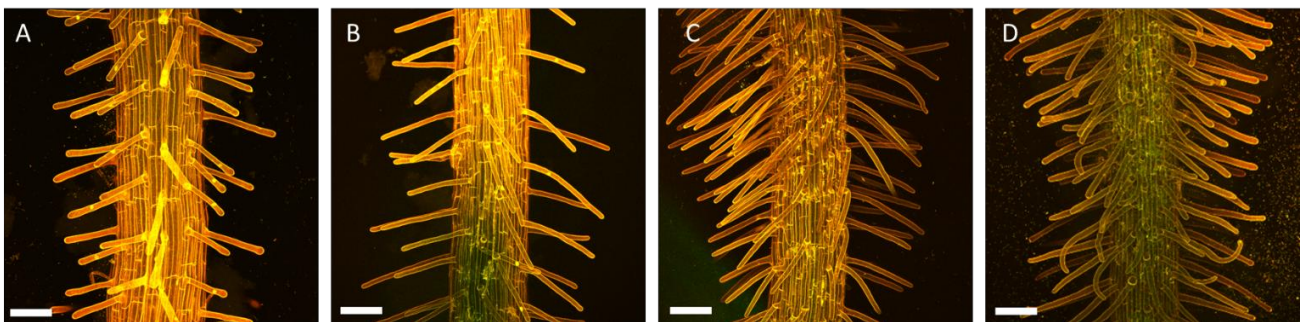


Figure 7. Fitted XANES spectra of *B. oleracea* leaves and roots with 125  $\mu\text{M}$   $\text{Se(IV)}$  amendment and with or without *Pseudomonas* sp. T5-6-I inoculation. A) Combined absorption spectra of  $\text{Se}^0$  (Se(Black)),  $\text{Se(IV)}$ (selenite), Se-Cysteine, Se-methionine, and the leaves and roots of *B. oleracea*



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4 grown with 125 $\mu$ M Se(IV) amendment and with/without *Pseudomonas* sp. T5-6-I inoculation. B) Fit  
5 with Se:SeO<sub>2</sub> pair, with Se-Cys:Se-Met pair and with Se:Se-Met pair.

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7 To better understand the effects of Se(IV) amendment and *Pseudomonas* sp. T5-6-I inoculation in *B.*  
8 *oleracea*, root hair formation in Se(IV) and bacteria treated samples was investigated using confocal  
9 microscopy. No clear differences were observed in the root hair density in the plants grown with 125  
10  $\mu$ M Se(IV) amendment (Figure 8B) compared to the plants with no Se(IV) present (Figure 8A) in the  
11 MS-growth agar. However, a notably increased root hair density was seen in *Pseudomonas* sp. T5-6-I  
12 amended plants (Figure 8C) compared to the control plants without bacteria (Figure 8A). Similarly, in  
13 Se(IV) amended plants with *Pseudomonas* sp. T5-6-I inoculation (Figure 8D) a clear increase in the  
14 root hair density was observed, when the root morphology was compared with plain Se(IV) amended  
15 plants (Figure 8B).  
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Figure 8. Confocal microscopy images of roots of *B. oleracea* grown on MS-salt agar, Control (no additions) (A), with Se(IV) addition (B), with *Pseudomonas* sp. T5-6-I addition (C) and Se(IV) + *Pseudomonas* sp. T5-6-I additions (D). A significantly increased root hair density is seen in *Pseudomonas* sp. T5-6-I amended sample (C), compared to control plant (A). Similarly in a Se(IV) amended sample with *Pseudomonas* sp. T5-6-I (D) a clear increase in the root hair density is seen, when comparing with plain Se(IV) amended sample (B). Scale bars 50 $\mu$ m.

### 3.4. Protein profiles in Se(IV) and *Pseudomonas* sp. T5-6-I treated plants

The profile of expressed proteins in response to Se(IV) and *Pseudomonas* sp. T5-6-I amendment in the *B. oleracea* plants was investigated to identify potentially important proteins involved in Se(IV) metabolism. Total protein fractions were isolated from the plants grown with Se(IV) and/or *Pseudomonas* sp. T5-6-I and the proteome was analyzed using HPLC-SEC (Table 1). Several differences in the HPLC-SEC protein profiles generated by Se(IV) and *Pseudomonas* sp. T5-6-I inoculation were observed in *B. oleracea*. A protein of ~31 kDa, was observed in the plants treated with Se(IV) with or without *Pseudomonas* sp. T5-6-I inoculation. In the *B. oleracea* plants treated with only *Pseudomonas* sp. T5-6-I a 28 kDa protein was also expressed. In addition, in the *Pseudomonas* sp. T5-6-I inoculated *B. oleracea* plants treated with Se(IV) additional low molecular weight proteins, corresponding to a molecular weight of ~8.5 kDa were observed. Interestingly, higher molecular weight proteins ~240 kDa, ~150 kDa and ~85 kDa were only recorded for the untreated control *B. oleracea*.

Table 1. Proteins (calculated molecular weight (MW), kDa) expressed in *B. oleracea* in response to Se(IV) and *Pseudomonas* sp. T5-6-I treatments obtained using HPLC-SEC and total protein fractions.

Peak position	CTRL MW (kDa)	Se MW (kDa)	<i>Pseud.</i> MW (kDa)	Se+ <i>Pseud.</i> MW (kDa)
1	239.8			
2	149.6			
3	85.0			
4	77.3	77.5	77.3	77.0
5	62.3	62.1		
6	52.5	51.9	52.9	52.8
7	48.3	48.0	48.2	48.2
8	38.0	38.0	37.8	37.5
9	34.3	34.2	34.2	33.9
10		31.5	31.9	30.8
11			28.0	
12	20.8	20.8	20.8	20.6
13	18.9	18.9	18.9	18.8
14	17.0	17.0	17.0	17.0
15				14.8
16	13.4	13.2	13.1	13.4
17	11.4	11.5	11.4	11.4
18	9.2			
19				8.5
20				8.3

#### 4. Discussion

We have previously shown that the *Pseudomonas* sp. strain T5-6-I used in this study was able to remove Se(IV) from nutrient solutions depending on incubation time and temperature (Lusa et al. 2015). We also observed intracellular Se-aggregates using TEM and EDX after Se(IV) exposure in this bacterium and concluded that accumulation and transport was likely metabolism-dependent and suggested two different transport mechanisms for Se(IV) uptake: (1) A low affinity transport system up-regulated by  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  or  $\text{SO}_4^{2-}$ , and (2) a Se(IV) regulated transport system (Lusa et al. 2017). In the present study, we tested this bacterium further in order to identify the speciation of selenium in the bacterium after Se(IV) introduction using XANES, as well as to test its effects on Se accumulation and speciation in *B. oleracea* plants.

The effect of Se(IV) exposure on growth of *Pseudomonas* sp. T5-6-I was tested by adding 6 mM of selenite to the bacterial culture at the beginning of the growth phase and compared the viable cell counts on these cultures to the bacterial cultures grown without Se(IV) amendment. The presence of Se(IV) induced a notable increase in the lag phase duration by approximately 120 hours. Similar retardation on the lag phase after Se(IV) exposure has been previously described e.g. for *Ralstonia*

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4 *metallidurans* (CH34) (Roux et al. 2001). However, in our study after the increased lag phase, the cell  
5 numbers in the cultures exposed to Se(IV) reached the number of stationary phase cells in the control  
6 cultures without Se(IV), after a rapid exponential growth phase. Previously we observed 6 mM Se(IV)  
7 amendment to decrease <sup>75</sup>Se accumulation in the bacterial cells to approximately one tenth of the  
8 accumulation observed after 0.3 mM Se(IV) amendment using 7 days incubation (Lusa et al. 2017).  
9 Based on the previous results and the results obtained in the present study, the decrease in the <sup>75</sup>Se  
10 accumulation is most probably caused by the increase in the lag phase after increased Se(IV) exposure.  
11 This is in good line with the results of Sarret et al. (2005), which reported that the accumulation of  
12 selenite was minimal in *R. metallidurans* during the prolonged lag phase after increased Se(IV)  
13 exposure and that selenium was not accumulated until the late exponential and stationary phases of the  
14 bacterial growth. The growth of *Bifidobacterium animalis* was also significantly reduced by increasing  
15 Se(IV) concentrations (0.06 mM) compared to unsupplemented cultures (Zhang et al. 2008). In  
16 contrast, the biomass of *Enterococcus* species has been reported to increase with increasing Se(IV)  
17 concentrations of 0.06 to 0.36 mM (Pieniz et al. 2011). Concurrently, Se(IV) bioaccumulation was  
18 reported to increase (Pieniz et al. 2011). Selenium may accumulate in bacterial cells either in organic,  
19 inorganic or elemental form or as a mixture of these forms, and the varying accumulation pathways  
20 may further affect cell growth and ultimate cell density in the growth solutions (Zhang et al. 2009,  
21 Pieniz et al. 2011). In our study, the XANES analyses showed that *Pseudomonas* sp. T5-6-I reduced  
22 Se(IV) into elemental Se<sup>0</sup>. In e.g. *E. coli*, Se(IV) reduction can follow a nonenzymatic pathway, which  
23 comprises of several organoselenium intermediates (Turner et al. 1998). The fact that Se<sup>0</sup> dominated in  
24 the *Pseudomonas* sp. T5-6-I after Se(IV) introduction, suggests that selenite is accumulated mainly  
25 through the dissimilatory detoxification pathway in this bacterium. Certain microorganisms are able to  
26 obtain metabolic energy from dissimilatory reduction processes, and in the environment, microbial  
27 Se(IV) reduction is an important process through which toxic soluble selenium oxyanionic species are  
28 removed from water and soil solutions (Eswayah et al. 2016, Nancharaiyah and Lens 2015). Various  
29 electron donors, including sugars, alcohols and organic acids can be utilized in the dissimilatory  
30 reactions (Astratinei et al 2006, Zhang et al. 2008, Kashiwa et al. 2000, Chung et al. 2006). From the  
31 bioremediation point of view, dissimilatory selenium reduction is expected to be more important in  
32 contrast to the assimilatory reduction. This is because of the higher selenium concentrations passing  
33 through the dissimilatory pathway (Eswayah et al. 2016). For this reason, especially the  
34 microorganisms utilizing the dissimilatory reduction of selenium could be used as profitable means for  
35 the remediation of selenium polluted areas (Eswayah et al. 2016). In the environment, the reduced  
36 insoluble Se<sup>0</sup> is considered less harmful compared to the soluble oxyanionic forms of Se(IV) and  
37 Se(VI). The aqueous Se(IV) and Se(VI) species could, however, be immobilized using Se-oxyanion-  
38 reducing bacterial strains, especially suitable for bioremediation of aquatic environments. The  
39 separation and removal of the bacteria containing the reduced Se<sup>0</sup> from solid soils and sediments would  
40 however be more challenging. Therefore, we also tested the effect of the Se-oxyanion-reducing  
41 *Pseudomonas* sp. T5-6-I strain on selenium uptake by plants, in order to evaluate its potential use in the  
42 bacteria-based phytoremediation of toxic selenium oxyanionic species from soils. Generally,  
43 phytoremediation for soil cleaning is considered very beneficial as it does not reduce the fertility of the  
44 soil, which is a typical problem with other engineered methods (e.g. thermal soil remediation,  
45 encapsulation and air sparging) used for soil clean-up (Robinson et al. 2000, Pilon-Smits and Freeman  
46 2006). Phytoremediation can be coupled to biofortification, in which the selenium enriched plant



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4 material will be decomposed in agricultural soil and used further for the enrichment of food products  
5 with Se (Schiavon and Pilon-Smits 2017).  
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8 In the plant-uptake experiments, we observed *Pseudomonas* sp. T5-6-I to promote root hair  
9 development in *B. oleracea*, and in addition, this bacterium affected selenium uptake in the plants after  
10 Se(IV) exposure. Gammaspectrometry showed that 130% more activity was translocated to the *B.*  
11 *oleracea* plants treated with <sup>75</sup>Se(IV) and *Pseudomonas* sp. T5-6-I compared to the samples receiving  
12 only <sup>75</sup>Se(IV) (n=14). The increase in the <sup>75</sup>Se concentrations after *Pseudomonas* sp. T5-6-I  
13 inoculation were most notable in the roots, compared to the leaves in young seedlings after a two weeks  
14 growth period. A 7.5 – 12-fold higher concentration of selenium was found accumulated to the roots  
15 compared to the leaves in the *Pseudomonas* sp. inoculated samples. Previously, rhizospheric bacteria  
16 have been reported to enhance selenium accumulation e.g. in *Brassica juncea* (de Souza et al. 1999).  
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20 Selenium may have beneficial effects on plants and several studies have shown that at low doses Se  
21 protects the plants from variety of abiotic stresses including cold (Chu et al., 2010), drought  
22 (Hasanuzzaman and Fujita, 2011), and metal stress (Kumar et al., 2012, Pandey and Gupta, 2015).  
23 However, selenium toxicity (selenosis) arises in plants after protective concentrations are exceeded  
24 (Gupta and Gupta 2017). Selenosis is caused by two distinctive mechanisms, i) by inducing oxidative  
25 stress and ii) by formation of malformed selenoproteins. Malformed Se-proteins are caused by the  
26 misincorporation of SeCys/SeMet in place of Cys/Met in an amino acid chain. As compared to SeMet,  
27 substitution of SeCys is more detrimental for the protein function, due to the important roles of cysteine  
28 residues in the protein structure (Gupta and Gupta 2017). Cysteine residues are essential for the  
29 formation of disulphide bridges, enzyme catalysis and metal binding sites. The substitution of cysteine  
30 by selenocysteine distorts the tertiary structure of a protein due to the formation of diselenide bridges  
31 instead of disulphide ones (Hondal et al., 2012). In addition, the enzyme kinetics can be altered because  
32 of changes in the redox potential. Previously, e.g. Se-MeSeCys (Selenomethylselenocysteine) has been  
33 described in SeMet enriched *Brassica* plants (Gupta and Gupta 2017). In addition, Se-MeSeCys and  
34 SeMet and selenate were detected in shoots, whereas selenate, selenite and SeMet were found in the  
35 roots of these plants (Gupta and Gupta 2017). In our study, the XANES analyses showed that Se was  
36 most likely present in H-Se-C and C-Se-C bonds in the *B. oleracea* leaves and roots after Se(IV)  
37 amendment and the presence of selenite, selenate or Se<sup>0</sup> was not supported by the data. Furthermore, in  
38 our study, statistically significant differences in the selenium speciation, which could explain the  
39 increased accumulation in the roots compared to the leaves, could not be observed in the XANES  
40 spectra obtained from the differently treated plants.  
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47 Depending on the concentrations and form of selenium, its uptake varies among plant species and is  
48 affected by the activity of membrane transporters, the developmental phase of the plant, as well as  
49 physiological conditions and accompanying substances (Zhao et al., 2005, Li et al., 2008). Selenium is  
50 expected to enter the plant cells as selenite or selenate through sulphate transporters and to thereafter be  
51 translocated to leaves where it is metabolized in plastids via the sulfur assimilation pathway into SeCys  
52 or SeMet (Gupta and Gupta 2017). In our study, selenium was found to be most likely incorporated in  
53 C-Se-C and C-Se-H bonds (as in SeMet and SeCys) in the plant cells after Se(IV) introduction. This  
54 would well fit the sulfur assimilation pathway for Se(IV). As somewhat increased uptake of selenium  
55 (<sup>75</sup>Se) was observed in the bacteria-inoculated *B. oleracea* plants, also other mechanisms could  
56 however be expected. This is because these bacteria were shown to reduce Se(IV) into Se<sup>0</sup> and based  
57 on previous studies, plants are known to be unable to take up colloidal elemental Se<sup>0</sup> (White and  
58 Broadley 2009). The changes observed in the *B. oleracea* protein profiles after Se(IV) and  
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4 *Pseudomonas* sp. treatments could be generated by these mechanisms. However, further  
5 characterization of the proteins is needed, to confirm their role in selenium accumulation. Previously  
6 high fractions (up to 35% of all selenium) of elemental reduced Se<sup>0</sup> has been reported in roots of  
7 *Astragalus bisulcatus* (*Fabaceae*), which was suggested to be produced by microbial endophytes  
8 (Lindblom et al. 2012, Lindblom et al. 2018). Reduced Se<sup>0</sup> was observed especially in the *A. bisulcatus*  
9 nodules and roots inoculated with Se<sup>0</sup>-producing fungi *Alternaria astragali* or *A. tenuissima*.  
10 Furthermore, e.g. bacteria belonging to the genera *Bacillus*, *Pseudomonas*, *Pantoea*, *Staphylococcus*,  
11 *Paenibacillus*, *Advenella*, *Arthrobacter* and *Variovorax* are known endophytes from Se  
12 hyperaccumulators *Stanleya pinnata* (*Brassicaceae*) and *A. bisulcatus* (Sura-de Jong et al. 2015).  
13 These bacteria are also able to reduce Se(IV) to elemental Se<sup>0</sup> and have plant growth promoting  
14 properties but their effect on plant Se accumulation has not been reported (Sura-de Jong et al. 2015).  
15 However, in our study Se<sup>0</sup> was not observed in the significant amounts in the *B. oleracea* plants after  
16 Se(IV) treatment nor Se(IV) + *Pseudomonas* sp. T5-6-I treatments using XANES. In addition, no  
17 structures resembling endophytic bacterial cells were found in the plant cells using TEM, although we  
18 observed small (<50 nm) dense granules in the leaf cells of *B. oleracea* grown with *Pseudomonas* sp.  
19 T5-6-I and Se(IV), which were not seen in plants without Se(IV) and *Pseudomonas* sp. T5-6-I  
20 amendments. At the same time, the root hair density was clearly increased in the *Pseudomonas* sp.  
21 inoculated plants, increasing the surface area available for selenium uptake by the plants. Thus, it  
22 appears that *Pseudomonas* sp. T5-6-I forms favorable symbiosis with the plants and contributes to the  
23 Se accumulation observed in *B. oleracea*, even though endophytic interactions could not be verified.  
24 Our results could not exhaustively conclude whether selenium is initially translocated to the plants as  
25 reduced Se<sup>0</sup> species inside the bacterial cells, or if the bacteria-plant interactions on the root surfaces  
26 change the initial selenium speciation enabling increased uptake to the plant through increased uptake  
27 surface area and increased expression of proteins involved in the selenium metabolism. Nevertheless,  
28 our findings demonstrate the potential for Se<sup>0</sup>-producing (symbiotic) bacteria to affect plant properties  
29 relevant for phytoremediation or biofortification. Facultative symbionts may also be beneficial in  
30 bioremediation and biofortification, due to their ability to turn toxic oxyanionic form of selenium,  
31 Se(IV), into less-toxic (or even metabolically beneficial) forms and to increase selenium(IV)  
32 accumulation in the plants.  
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## 42 5. Conclusions

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44 The bacterial growth rate in *Pseudomonas* sp. T5-6-I was affected by Se(IV) amendment and Se(IV)  
45 increased the duration of the lag phase of the growth. However, following rapid exponential growth,  
46 growth rate promptly reached the growth rate observed in the control cells without Se(IV) amendment.  
47 Based on the following XANES measurements, *Pseudomonas* sp. T5-6-I effectively reduced Se(IV)  
48 into elemental Se<sup>0</sup> under oxic conditions. In addition, this *Pseudomonas* sp. strain increased selenium  
49 (<sup>75</sup>Se(IV)) accumulation in *B. oleracea*. However, this bacterium was not found to affect the following  
50 selenium speciation in the *B. oleracea* plants. We conclude that the increase in selenium plant-uptake  
51 after *Pseudomonas* sp. T5-6-I inoculation may, at least partly, be caused by the increased root-hair  
52 growth, which was observed in the *B. oleracea* plants after *Pseudomonas* sp. T5-6-I inoculation.  
53 This study shows that Se(IV) tolerant, Se<sup>0</sup> producing soil bacteria isolated from boreal, harsh bog  
54 environment, can influence Se accumulation in a common crop plant *B. oleracea* (kale). This feature  
55 could be highly beneficial for the further development of phytoremediation and biofortification  
56 applications, especially as these bacteria seem not to distinguish between their habitat or plant partner.  
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## Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

## Author Contributions

Merja Lusa: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft, Writing – Reviewing and Editing, Supervision, Project Administration. Hanna Help-Rinta-Rahko: Methodology, Formal Analysis, Investigation, Writing – Reviewing, Ari-Pekka Honkanen: Methodology, Formal Analysis, Investigation, Writing-Reviewing, Jenna Knuutinen: Investigation, Joni Parkkonen: Investigation, Dominika Kalasová: Investigation, Malin Bomberg: Conceptualization, Methodology, Writing- Reviewing and Editing.

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## Appendix 1

*Table A1: Weighed masses of samples and estimated Se concentrations. A conservative 20 % error based on the statistical accuracy of data was used in the estimation.*

Sample	Dry mass (mg)	Se K edge steps	Estimated Se mass (µg)	Se concentration (mg/kg DW)
Leaves + T5-6-I	74.5	0.0019	3.2 ± 0.6	43 ± 9
Leaves	75.4	0.0024	4.0 ± 0.8	53 ± 11
Roots + T5-6-I	12.5	0.0025	4.2 ± 0.8	340 ± 70
Roots	9.1	0.0022	3.7±0.7	410 ± 80

Table A2: Fitted fractions of two component reference fits and their relative likelihoods to explain the observed data. Se:Se-Cys and Se-Met:SeO<sub>3</sub> fits lead to unphysical fit parameters and are thus omitted from the table.

Fitted references	Leaves + T5-6-I	Leaves	Roots + T5-6-I	Roots	Relative probability to explain bacteria samples	Relative probability to explain non-bacteria samples	Relative probability to explain all the samples
Se	0.64 ± 0.04	0.58 ± 0.04	0.65 ± 0.03	0.58 ± 0.03	7.3 x 10 <sup>-13</sup>	2.3 x 10 <sup>-7</sup>	1.7 x 10 <sup>-19</sup>
SeO <sub>3</sub>	0.36 ± 0.04	0.42 ± 0.04	0.35 ± 0.03	0.42 ± 0.03			
Se-Cys	0.6 ± 0.2	0.6 ± 0.2	0.88 ± 0.14	0.58 ± 0.15	1	1	1
Se-Met	0.4 ± 0.2	0.4 ± 0.2	0.12 ± 0.14	0.42 ± 0.15			
Se	0.25 ± 0.07	0.22 ± 0.07	0.33 ± 0.05	0.21 ± 0.06	0.42	0.011	0.048
Se-Met	0.75 ± 0.07	0.78 ± 0.07	0.67 ± 0.05	0.79 ± 0.06			
Se-Cys	0.98 ± 0.06	0.87 ± 0.06	0.98 ± 0.04	0.88 ± 0.04	0.011	0.013	0.0015
SeO <sub>3</sub>	0.02 ± 0.06	0.13 ± 0.06	0.02 ± 0.04	0.12 ± 0.04			

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Table 1. Proteins (calculated molecular weight (MW), kDa) expressed in *B. oleracea* in response to Se(IV) and *Pseudomonas* sp. T5-6-I treatments obtained using HPLC-SEC and total protein fractions.

Peak position	CTRL MW (kDa)	Se MW (kDa)	<i>Pseud.</i> MW (kDa)	Se+ <i>Pseud.</i> MW (kDa)
1	239.8			
2	149.6			
3	85.0			
4	77.3	77.5	77.3	77.0
5	62.3	62.1		
6	52.5	51.9	52.9	52.8
7	48.3	48.0	48.2	48.2
8	38.0	38.0	37.8	37.5
9	34.3	34.2	34.2	33.9
10		31.5	31.9	30.8
11			28.0	
12	20.8	20.8	20.8	20.6
13	18.9	18.9	18.9	18.8
14	17.0	17.0	17.0	17.0
15				14.8
16	13.4	13.2	13.1	13.4
17	11.4	11.5	11.4	11.4
18	9.2			
19				8.5
20				8.3

*Table A1: Weighed masses of samples and estimated Se concentrations. A conservative 20 % error based on the statistical accuracy of data was used in the estimation.*

Sample	Dry mass (mg)	Se K edge steps	Estimated Se mass ( $\mu\text{g}$ )	Se concentration (mg/kg DW)
Leaves + T5-6-I	74.5	0.0019	$3.2 \pm 0.6$	$43 \pm 9$
Leaves	75.4	0.0024	$4.0 \pm 0.8$	$53 \pm 11$
Roots + T5-6-I	12.5	0.0025	$4.2 \pm 0.8$	$340 \pm 70$
Roots	9.1	0.0022	$3.7 \pm 0.7$	$410 \pm 80$

*Table A2: Fitted fractions of two component reference fits and their relative likelihoods to explain the observed data. Se:Se-Cys and Se-Met:SeO<sub>3</sub> fits lead to unphysical fit parameters and are thus omitted from the table.*

Fitted references	Leaves + T5-6-I	Leaves	Roots + T5-6-I	Roots	Relative probability to explain bacteria samples	Relative probability to explain non-bacteria samples	Relative probability to explain all the samples
Se	$0.64 \pm 0.04$	$0.58 \pm 0.04$	$0.65 \pm 0.03$	$0.58 \pm 0.03$	$7.3 \times 10^{-13}$	$2.3 \times 10^{-7}$	$1.7 \times 10^{-19}$
SeO <sub>3</sub>	$0.36 \pm 0.04$	$0.42 \pm 0.04$	$0.35 \pm 0.03$	$0.42 \pm 0.03$			
Se-Cys	$0.6 \pm 0.2$	$0.6 \pm 0.2$	$0.88 \pm 0.14$	$0.58 \pm 0.15$	1	1	1
Se-Met	$0.4 \pm 0.2$	$0.4 \pm 0.2$	$0.12 \pm 0.14$	$0.42 \pm 0.15$			
Se	$0.25 \pm 0.07$	$0.22 \pm 0.07$	$0.33 \pm 0.05$	$0.21 \pm 0.06$	0.42	0.011	0.048
Se-Met	$0.75 \pm 0.07$	$0.78 \pm 0.07$	$0.67 \pm 0.05$	$0.79 \pm 0.06$			
Se-Cys	$0.98 \pm 0.06$	$0.87 \pm 0.06$	$0.98 \pm 0.04$	$0.88 \pm 0.04$	0.011	0.013	0.0015
SeO <sub>3</sub>	$0.02 \pm 0.06$	$0.13 \pm 0.06$	$0.02 \pm 0.04$	$0.12 \pm 0.04$			