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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)/^{75}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 ^{75}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^0-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 documented especially in the regions of the World with extremely selenium-poor soils (Rayman 2000). In contrast, based on epidemiologic studies, chronic exposure to raised selenium intake, especially in regions with elevated soil selenium concentrations, is associated with several adverse health effects. These include the impairment of the synthesis of thyroid hormones and metabolism of growth hormone as well as hepatotoxicity, gastrointestinal disturbances and nail and hair loss and dermatitis (Vinceti et al. 2001). Selenium supplementation may also increase risk for the type 2 diabetes (Stranges et al. 2010).

Human dietary intakes of selenium range from high to low according to geography and local soil conditions. The mean crustal abundance of selenium is 0.083mg/ kg (Antonyak et al. 2018). In most soils selenium concentrations range from 0.1 to 2 mg/kg (Oldfield 2002), but extremely high Se contents up to 1200 mg/kg have been reported e.g. from organic-rich soils in Ireland (Fleming and Walsh 1957). Understanding of these variations, and the biogeochemical factors affecting them, is essential for the improvement of health problems associated with selenium deficiency and toxicity. Certain regions of the world, including Finland, Sweden and Scotland, are deficient in selenium, while others (e.g. Japan, Greenland, USA, Venezuela and Canada) are Se-rich/Se-toxic due to natural and anthropogenic activities (Zhu et al. 2009, Yin and Yuan 2012, Fordyce 2005). Natural sources of environmental selenium include forest fires and soil erosion, whereas considerable amounts of selenium enter the environment via anthropogenic activities including coal combustion, mining, refining of sour crude oils and agricultural irrigation of seleniferous soils (Manceau et al. 1997, de Souza et al. 1999, Sharmasarkar and Vance 2002, Coppin et al. 2009). In addition, regarding the radiation protection point of view, the radioactive long-lived isotope of selenium, ^{79}Se, is found in spent nuclear fuel and is one of the high priority radionuclides when the biosphere safety of spent nuclear fuel disposal is to be considered (Helin et al. 2010).

Selenium enters the food chain through plants that take it up from the soil. In alkaline (agricultural) soils, selenium mostly exists as selenate (Se(VI), SeO_4^{2-}) whereas in acidic (forest) soils it exists as selenite (Se(IV), SeO_3^{2-}). The forms of selenium differ in terms of their absorption and mobility within the plant and are metabolized to form selenocompounds (Li et al., 2008). Selenite has been reported to be transported by a phosphate transport mechanism (Li et al., 2008) while selenate uptake is believed to occur through sulfate transporters (Feist and Parker, 2001, Zhang et al., 2003). However, only recently the involvement of a putative selenium-binding protein (SBP) gene family, composed of three members (SBP1–SBP3) has been described in *Arabidopsis thaliana* (e.g. Schild et al. 2014).

Plants are the main source of dietary selenium and plant selenium concentrations can vary from 0.005mg/kg in Se-deficient crops to 5500mg/kg in selenium hyper-accumulators (Fordyce 2005). The essentiality of selenium for plants is still debatable (Dumont et al. 2006, Gupta and Gupta 2017). At low doses, selenium may protect the plants from variety of abiotic stresses, such as cold, drought, and metal stress (Chu et al. 2010, Hasanuzzaman and Fujita 2011, Kumar et al. 2012). However, at higher doses selenium becomes toxic also for plants and its toxicity is caused by two different mechanisms; 1) the formation of malformed proteins due to the misincorporation of selenocysteine (SeCys) or selenomethionine (SeMet) in place of cysteine (Cys) or methionine (Met) in a protein, or 2) by inducing oxidative stress (Gupta and Gupta 2017).

Plants are in continuous contact with soil microbiota (Srivastava et al. 2012), which can modify the soil chemical environment through e.g. oxidation and reduction reactions and secretion of metabolites, altering the availability of chemical elements for plants. Interactions between plants and microorganisms take place during different phases of the plant life cycle and many of these interactions can be beneficial for the plant (Schirawski and Perlin 2018). In fact, there are multiple interactions where the plant benefits either directly or indirectly of the associated microbes. Further, plants may act as protected habitats for bacteria occupying the surfaces of the plant, the apoplast or the soil surrounding the plant roots (Schirawski and Perlin 2018). In addition, plants may release compounds attracting the accompanying soil bacteria and the associated microbes may in turn secrete compounds that favour e.g. plant growth, increase the plant capacity to resist abiotic and biotic stress or protect the plant against malignant microbes. Soil bacteria (especially plant growth promoting rhizobacteria, PGPR, such as *Pseudomonas putida*) can e.g. promote plant growth through production of phytohormones (e.g. indole-3-acetic acid (IAA), auxin, gibberellins) (Joo et al. 2005, Idris et al. 2007, Srivastava et al. 2012). Previously, we found that bacteria affected selenium behavior and retention in boreal bog environment and that the *Pseudomonas* sp. strain (T5-6-I) (Lusa et al. 2016), which was also used in the present study, removed ^{75}Se(IV) from solutions under different nutrient conditions (Lusa et al. 2015). In experiments with stable Se isotopes, brick-red reduced elemental selenium (Se^0) was formed from both Se(IV) and Se(VI) in incubations with the *Pseudomonas* sp. strain T5-6-I under aerobic conditions (Lusa et al. 2017). However, the reduction was significantly more efficient for Se(IV) and for Se(VI) formation of elemental selenium was only barely detectable (Lusa et al. 2017). In addition, nitrate (NO_3^-), nitrite (NO_2^-) and sulfate (SO_4^{2-}) enhanced Se(IV) uptake in *Pseudomonas* sp. strain T5-6-I, and Se(IV) uptake continued also under sulphur and nitrogen starvation.

Previously, the uptake of selenium oxyanions Se(IV) and Se(VI) by plants has been studied e.g. in *A. thaliana* (e.g. Barickman et al. 2013, White et al. 2004), *Stanleya pinnata* (e.g. El Mehdawi et al. 2017) and *Brassica juncea* (e.g. El Mehdawi et al. 2017). In turn, the effects of bacteria, e.g. *P. putida* (Srivastava et al. 2012), *Azospirillum brasilense*, *Serratia plymuthica, Stenotrophomonas maltophilia* (Wenke et al. 2012), *Bacillus subtilis* and *Burkholderia cepacia* (Vespermann et al. 2007, Kai et al. 2008) have been reported on *Arabidopsis* growth. However, previous research on the simultaneous effects of selenium and soil dwelling bacteria on selenium plant uptake is to date scarce. Previously, bacteria belonging to the genera *Bacillus*, *Pseudomonas*, *Pantoea*, *Staphylococcus*, *Paenibacillus*, *Advenella*, *Arthrobacter* and *Variovorax* have been found as endophytes from selenium hyperaccumulators *S. pinnata* (*Brassicaceae*) and *Astragalus bisulcatus* (*Fabaceae*) (Sura-de Jong et al. 2015). These bacteria reduced Se(IV) to elemental Se^0 and additionally had plant growth promoting properties. However, they had no effect on the amount of selenium accumulated into the plant (Sura-de Jong et al. 2015).

Nutritional intake is the most important source of selenium for humans and the biogeochemical components, including soil microbiota, are in a key role when assessing the transfer of environmental selenium into the human diet and its associated health effects (Fordyce 2005). Because of bacterial synergy and interactions with plants, soil bacteria may have vital, yet unidentified, roles in plant macro- and micronutrient metabolism as well as in overcoming toxic effects of e.g. selenium in different regions of the world. Therefore understanding the metabolic processes related to the toxic Se(IV) stress and bacteria-plant interactions in selenium accumulation is of high importance. To better understand the effects of bacteria-mediated selenium oxyanion reduction (that changes the speciation of selenium from Se(IV) available for plants to insoluble Se^0) on the subsequent availability and transfer of selenium to plants, we cultivated *Brassica oleracea* with above mentioned, previously isolated heterotrophic aerobic T5-6-I strain (Lusa et al. 2016). This strain was especially interesting, as it represents *Pseudomonas*, bacterial genus commonly found in many different types of environments, having versatile metabolism. In addition, previously this strain was shown to remove especially Se(IV) from growth solutions (Lusa et al. 2015, 2017). Therefore, this strain is suspect to affect particularly the overall transfer of Se(IV), a highly toxic species of selenium, from soil solutions into plants. Se(IV) is very reactive and reacts in particular with thiol groups found in glutathione (GSH), producing several Se-containing compounds, including selenodiglutathione, glutathioselenol, hydrogen selenide (H_2Se) and elemental selenium (Se^0) (Tarze et al. 2007). This reaction produces also highly toxic oxygen species like H_2O_2 and O^{2-} (Kramer and Ames 1988). *B. oleracea* was chosen as *Brassica* spp. are known secondary accumulators (i.e. are typically able to accumulate up to 1000 mg Se/kg DW) of selenium (Gupta and Gupta 2017).

2. Materials and methods

Bacteria. The isolation and characterization of the *Pseudomonas* sp. strain T5-6-I used in this study, has been described in Lusa et al., 2016. The 16S rRNA gene sequence has been deposited in GenBank under accession number KP100424. The stock cultures of this bacterium were grown aerobically on sterile PCA growth plates (PCA, Merckoplate®) at 20 °C in the dark and the strain was re-cultivated on new plates weekly.

Plant material. Seeds of *B. oleracea* were bought from a garden store. Surface sterilized (0.1 % Triton X in 95% ethanol) seeds were sown on MS-agar (Murashige and Skoog Basal Salt Mixture, Sigma M5524) (Xi et al. 2016) plates (50 mL) containing 1% sucrose (w/v) and 0.7% (w/v) agar (Sigma A1296). *B. oleracea* plates were transferred to the growth chamber set at 23°C in 16/8 h light and dark cycles immediately after sowing. Selenium (125 µM of selenite ($Na_2Se(IV)O_3$ (AlfaAesar®)) or 100 Bq of radioisotopes of selenium $^{75}[Se(IV)O_3^{2-}]$ with 2.7×10^{-8} M stable Se(IV) carrier) and bacterial suspensions (0 or $5 \cdot 10^7$ CFU of exponential growth phase bacteria) were added on one week old seedlings by spreading in total 5 mL of the suspensions on the surface of the plant agar. Plants were thereafter grown for two (XANES, gammaspectrometry and confocal experiments) or three weeks (autoradiography, CT, and protein/HPLC experiments) depending on the set of experiments. Of all plates 3 – 7 replicates were prepared depending on the set of experiments. For subsequent experiments, leaves and roots of plants were harvested. Control samples without selenium or bacterium inoculation were used in all experiments to ensure no contaminations occurred.

The effect of Se(IV) on bacterial growth. The effect of Se(IV) on the growth of *Pseudomonas* sp. T5-6-I was tested by growing the *Pseudomonas* sp. T5-6-I with and without Se(IV) amendment. In these experiments, the cells were grown in 1 % Tryptone solution at +20°C. $Na_2Se(IV)O_3$ (AlfaAesar®) was added to the growth medium to a final concentration of 6 mM of Se(IV). The cells were incubated in the dark on an orbital shaker (120 rpm) for up to 360 hours and sampled regularly. The absorbance of the cell solutions without Se(IV) was recorded at the wavelength of 600 nm to estimate the overall CFU/mL. Se(IV) amended samples were not used in the absorbance determinations due to the strong formation of Se precipitates interfering with the measurements. However, viable cell counts (CFU/mL) were determined for both Se(IV) treated and control samples without Se(IV). The samples were serially diluted in 10-fold steps to a dilution of 10^{-6}. Aliquots of 100 µL from dilutions 10^{-4} and 10^{-6} were spread on Plate Count Agar (PCA, Merckoplate®) and the plates were further incubated aerobically at 20°C for up to 408 hours in the dark and the CFUs were counted.

Speciation of selenium in bacterial cells and in plant material. The speciation of selenium in the *Pseudomonas* sp. cells and in the differentially treated roots and leaves of *B. oleracea* plants was examined using X-ray absorption near-edge structure (XANES) spectroscopy. The XANES measurements were performed at Se K edge (12.7 keV) using a table top Johann-type spectrometer detailed in (Honkanen et al. 2019). For the XANES experiments bacterial cultures were treated with 3 or 6 mM Se(IV) and incubated under aerobic conditions at 20 °C for 7 days. After incubation, the cells were separated by centrifugation at 5000×g for 15 min at room temperature followed by washing of the remaining bacterial pellet twice with 0.01 M (pH 7) phosphate buffer. Thereafter, bacterial pellets pre-frozen at -18 °C were lyophilized using an Alpha 1-4 LSCbasic lyophilizer (Christ). *B. oleracea* seeds were grown as described above for the plant material, with 125 µM of Se(IV) and with or without $5{\cdot}10^7$ CFU of *Pseudomonas* sp. T5-6-I amendment. After 14 days growth period, fresh leafs and roots were harvested, pre-frozen at -18 °C and lyophilized as described for the bacterial cells.

The lyophilized pellets were homogenised with a mortar and pestle and bacterial samples were prepared as such in M8 steel washers with a thickness of 1.4 mm and an inner diameter of 8.6 mm. Leaf samples were prepared as such in M5 steel washers with a thickness of 1.9 mm and inner diameter of 5.4 mm. For the root samples, potato starch was used as a filler. The washers were covered with Scotch tape. Elemental (black, Se^0) selenium, selenium salts $Na_2Se(IV)O_3$ (AlfaAesar®), $Na_2Se(VI)O_4$ (AnalaR®) and PbSe (AlfaAesar®) were used as reference compounds for the bacterial samples. For plant material in addition L-selenocysteine (Acros organics) and L-selenomethionine (BioVision) were used. The reference compounds were weighed to optimize the change in the absorption coefficient, mixed with potato starch to make the samples more homogeneous and to increase their total volume. M8 steel washers (thickness 1.4 mm, inner diameter 8.6 mm) were used for the reference compounds.

For the bacteria, the spectra were recorded over the energy range of 12.538-12.986 keV divided evenly into 450 steps using Si(953) monochromator in the transmission mode. The acquisition time for the direct beam was 12.5 h and the transmitted beams through bacterial pellets from the treatments with 3 mM and 6 mM selenium for 16.5 h and 5.5 h, respectively. The transmission through the reference samples were in the range of 5.5 – 9.0 h per sample. The X-ray tube acceleration voltage was set to 20 kV and the current was 10 mA. Samples were positioned at the tube exit and a 20 µm thick Al filter was placed in the beam path before the sample to absorb low energy photons and thus reduce the unnecessary radiation dose to the samples. For the plant material, the spectra were recorded over the energy range of 12.624-12.713 keV divided evenly into 100 steps using Si(953) monochromator in the simultaneous transmission and fluorescence mode. The acquisition time for the direct beam was 14.0 h and in the range of 19.6 – 56.1 h per plant sample. The wide range in the measurement times was due to variation in signal strength between the samples. The X-ray tube acceleration voltage was 20 kV and the current 40 mA. Samples were positioned in front of the detector. The calculation of spectra from the raw instrument data and final visualizations were performed with custom Python scripts. The details of spectra calculation is presented in (Honkanen et al., 2019).

The selenium content of the plant samples was estimated by determining magnitude of Se K edge step in the transmission signal and calculating the corresponding amount of selenium. The compositions of obtained spectra from plants were estimated by fitting linear combinations of measured reference spectra with an ordinary least-squares fit using a Python implementation available at https://github.com/aripekka/bayesfit. Utilizing the Bayes' theorem, the (posterior) probability p(H|D) for the hypothesis H to be true when given the data D is

$$p(H|D) \propto p(D|H)p(H)$$

where P(D|H) is the probability of obtaining D when H is true and p(H) is the a priori probability of H to be true. This allows us to compare the relative probability of competing models to each other as follows:

$$p(A|D)/p(B|D) = p(D|A,\lambda_A)/p(D|B,\lambda_B) \quad \sqrt{(\det(\Sigma_A))}/\sqrt{(\det(\Sigma_B))},$$

where p(A|D) is the (posterior) probability of model A to be true when the data D is known, p(D | A, λ_A) is the probability to obtain D when model A is true with parameters fitted model parameter λ_A and det(Σ_A) is the determinant of the covariance matrix of λ_A. Definitions are similar for the model B. The given equation is a straightforward generalization of Eq. (4.9) in (Sivia and Skilling 2006) when a priori probabilities are taken equal and the number of fit parameters is the same. To compare how two models explain a group of independent fits, the relative probabilities of single fits are simply multiplied together.

Protein expression in plant roots and leafs. The effect of *Pseudomonas* sp. T5-6-I and Se(IV) amendment in the expressed protein profile of *B. oleracea* was examined to identify potentially important proteins involved in Se(IV) metabolism in the studied plants. Total plant protein extracts were examined using HPLC-SEC. The plants were grown with 125 µM Se(IV) amendment as described above for plant material and proteins from untreated control, *Pseudomonas* sp. T5-6-I treated, Se(IV) and *Pseudomonas* sp. T5-6-I + Se(IV) treated plants were isolated using the TPE Kit (G-Biosciences®) method according to the manufacturer's instructions. A protease inhibitor cocktail (G-Bioscieces® ProteaseArrest-kit) was used in the TPE-I buffer according to the manufacturer's instructions. The extracted protein concentrations were quantified using RED 660™ Protein Assay (G-Biosciences®) according to the manufacturer's instructions. 1:3 diluted total protein extracts in TBE-buffer (TPE Buffer-I, G-Bioscieces® ProteaseArrest-kit) were used for HPLC-SEC analysis (Symmetry semiprep C18 7.8x300 mm 7 µm). HPLC-SEC was run in 1M phosphate buffered saline (pH 7.9), with an injection volume of 5 µL and 20 minutes acquisition time. The UV-VIS spectra were recorded at 234 nm, 209 nm and 254 nm.

Quantification of Se(IV) transfer into the plants. Batch experiments with ^{75}[Se(IV)O$_3^{2-}$] radioisotope tracer and gammaspectrometry were used to quantify the transfer and accumulation of selenium into the roots and leafs of *B. oleracea* plants from the growth medium. For these experiments *B. oleracea* plants were grown on MS-salt agar as described above for plant material with 100 Bq of ^{75}[Se(IV)O$_3^{2-}$] (with 2.7×10^{-8} M stable Se(IV) carrier) and with or without *Pseudomonas* sp. T5-6-I inoculation. After a growing period of two weeks the leaves and roots were harvested and weighed and the plant material was digested using 5 mL (1:50 m/V) of 65% HNO$_3$ (suprapur). The samples were incubated at room temperature for 2 h after which the samples were centrifuged at 12 000 g for 10 minutes and filtered through a 0.2 µm membrane filter. ^{75}Se concentration was measured from the resulting solutions using NaI(Tl)-gammaspectrometer (Wizard® automatic gammacounter, PerkinElmer).

Visualization of Se accumulation in the plants. Digital autoradiography was used to visualize the location and accumulation density of selenium in dried, ^{75}[Se(IV)O$_3^{2-}$] treated *B. oleracea* leafs and roots with or without *Pseudomonas* sp. T5-6-I amendments. For these experiments, the plants were dried at room temperature, placed on a Fuji FLA imaging plate and exposed for six days in a lead shielded film cassette. The exposed FLA plate was thereafter scanned using Fujifilm FLA-5100 scanner with Aida Image analysis software. In addition, X-ray microtomography (µCT) and X-ray nanotomography (nanoCT) imaging methods were used to visualize changes in tissue density properties (absorption contrast) in response to applications with stable selenium (Se(IV), 125 µM) in the lyophilized (Alpha 1-4 LSCbasic lyophilizer, Christ) *B. oleracea* leaves (µCT) and roots (nanoCT). This enabled a higher image resolution in tissues in comparison to the digital autoradiography. Confocal microscopy was used to examine the changes in the root morphology of *B. oleracea* in response to Se(IV) (125µM) and bacteria treatments from fresh samples dyed with propidium iodide (PI) just prior to imaging. Transmission electron microscopy (TEM) was in addition used to examine the possible accumulation of bacteria and selenium in the leaves of plants treated with 125 µM Se(IV) and *Pseudomonas* sp. T5-6-I. For the TEM experiments the plants were grown in Se(IV) containing MS-agar plates as described above. Freshly collected leaves were fixed in 2% glutaraldehyde for 2 hours and cut into 1cm x 1cm pieces. Cells were dehydrated through an ethanol series and then embedded in Taab hard epon and polymerized at 60 °C. Thin sections were cut using Leica ultracut UCT ultramicrotome (Leica Mikrosysteme GmbH, Austria) and collected on single-slot copper grids. Section thickness of 60 nm was used for the morphological examinations. After double staining with uranyl acetate and lead citrate, the sections were examined using FEI Tecnai F20 at 200 kV under standard operating conditions.

Statistical analyses. To study the statistical difference in the Se-accumulation between the differentially treated *B. oleracea* plants using $^{75}[\mathrm{Se(IV)O_3}^{2-}]$ radioisotope tracers, the analysis of variance was performed using OriginPro 8.6 (OriginLab®) and one-way ANOVA at the $p < 0.05$ level. Prior to analysis, Shapiro-Wilk test ($p < 0.05$) was used to examine the normality of the data, and as the normality hypotheses were rejected, log transformed data was used for the one-way ANOVA tests.

3. Results

3.1.Bacterial growth under Se(IV) stress

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

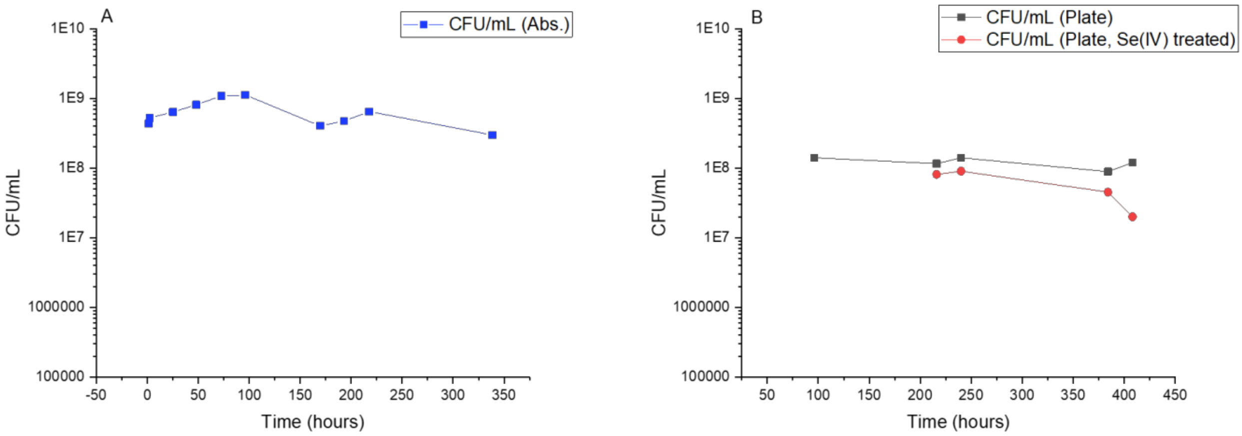

Figure 1. The effect of Se(IV) on bacterial growth of Pseudomonas sp. T5-6-I on 6 mM Se(IV) amended 1% Tryptone solution. A) 600 nm absorbance based CFU/mL obtained from the untreated control cells. B) Grey = Viable cell counts (CFU/mL) obtained from the PCA plates of untreated cells, Red = Viable cell 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^0) selenium reference. The spectrum recorded in 3 mM Se(IV) amended bacteria was similar within the statistical accuracy.

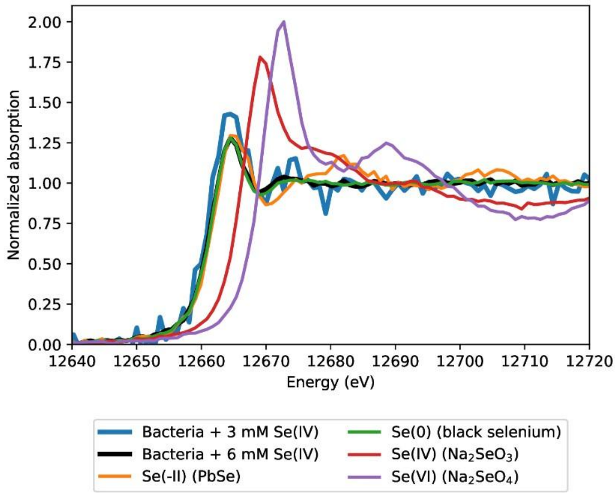

Figure 2. Se K edge of accumulated selenium in Pseudomonas sp. *T5-6-I after incubation in presence of Se(IV) (3 mM and 6 mM concentration) together with reference compound spectra. The signal from the bacteria samples follow closely that of elemental Se.*

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

B. oleracea showed increased transfer of ^{75}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}Se(IV) amendment but without *Pseudomonas* sp. T5-6-I inoculation seen as increased ^{75}Se(IV) signal (red colour in figure 3A) in the autoradiography images. Furthermore, ^{75}Se seemed rather evenly distributed throughout the *Pseudomonas* sp. T5-6-I treated plants (red colour in Figure 3A). In the µCT 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).

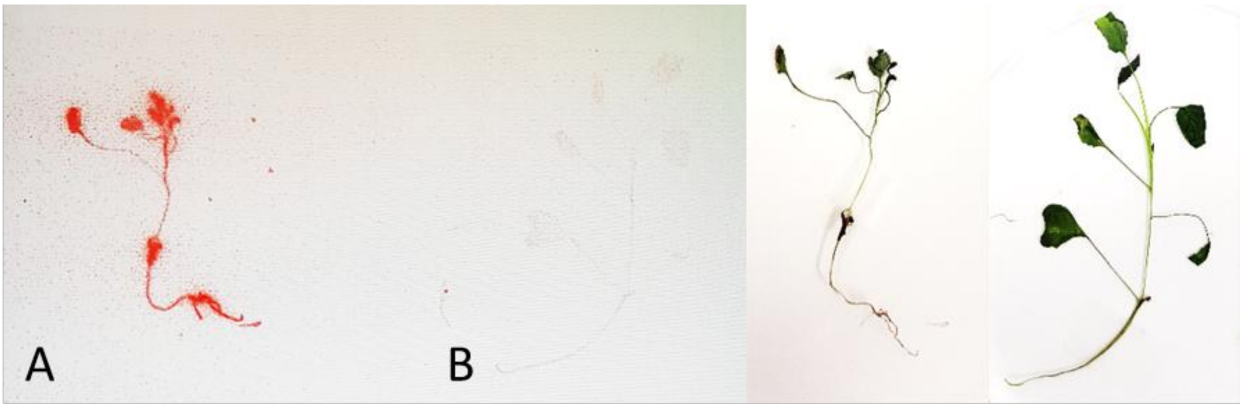

Figure 3. A) B. oleracea grown on MS-salt agar labelled with $^{75}[Se(IV)O_3^{2-}]$ *with Pseudomonas sp. T5-6-I inoculation. B) B. oleracea grown on MS-salt agar labelled with* $^{75}[Se(IV)O_3^{2-}]$ *without Pseudomonas sp. T5-6-I. On the right photographs of the plants used for autoradiograms. Data are representative of at least three biological replicates.*

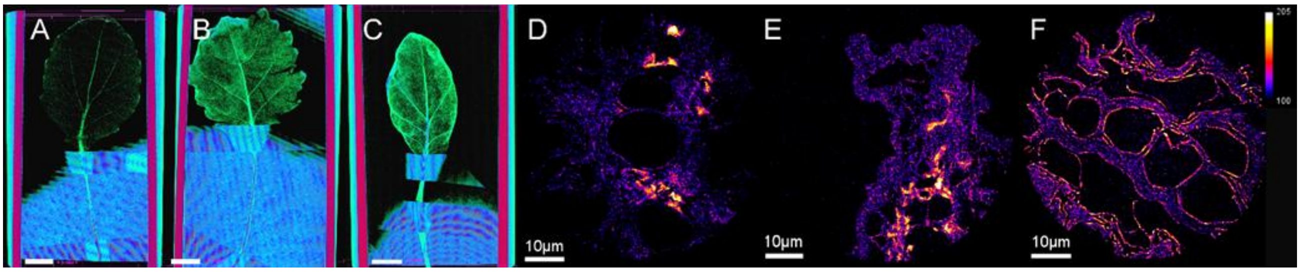

Figure 4. Leaves (A, B, C) and roots (D, E and F) of vacuum dried B. oleracea visualized using µCT (A, B and C) and nanoCT (D - F). In (A) a leaf of a plant without Se(IV) amendment grown on the MS-salt agar is shown (control). In (B) a leaf of a plant grown in Se(IV) amended MS-salt agar. In (C) leaf 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}[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 $5\cdot10^7$ 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}Se had no evident effect on the phenotype of *B. oleracea*.

Based on the analysis of variance (ANOVA), the inoculation of ^{75}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 ($F_{crit} = 4.7$, $F = 10.5$, $p = 0.007$). However, in the case of leaves, statistically significant difference between *Pseudomonas* sp. treated plants and plants without bacteria inoculation could not be shown.

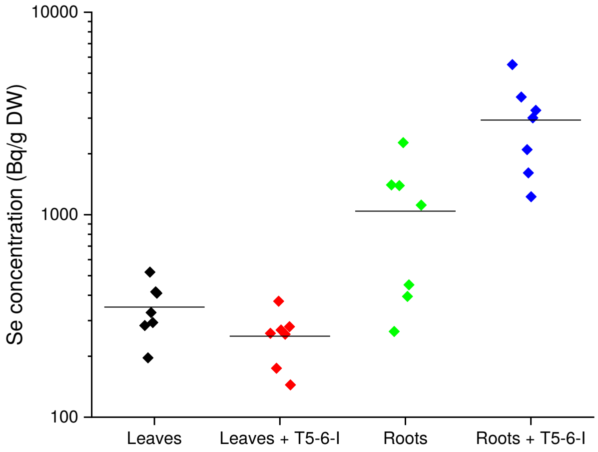

Figure 5. Selenium concentration in the leaves and roots of B. oleracea grown in $^{75}[Se(IV)O_3^{2-}]$ *amended MS-agar without bacteria (black and green) and with Pseudomonas sp. T5-6-I inoculation (red and blue).*

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 125µM 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).

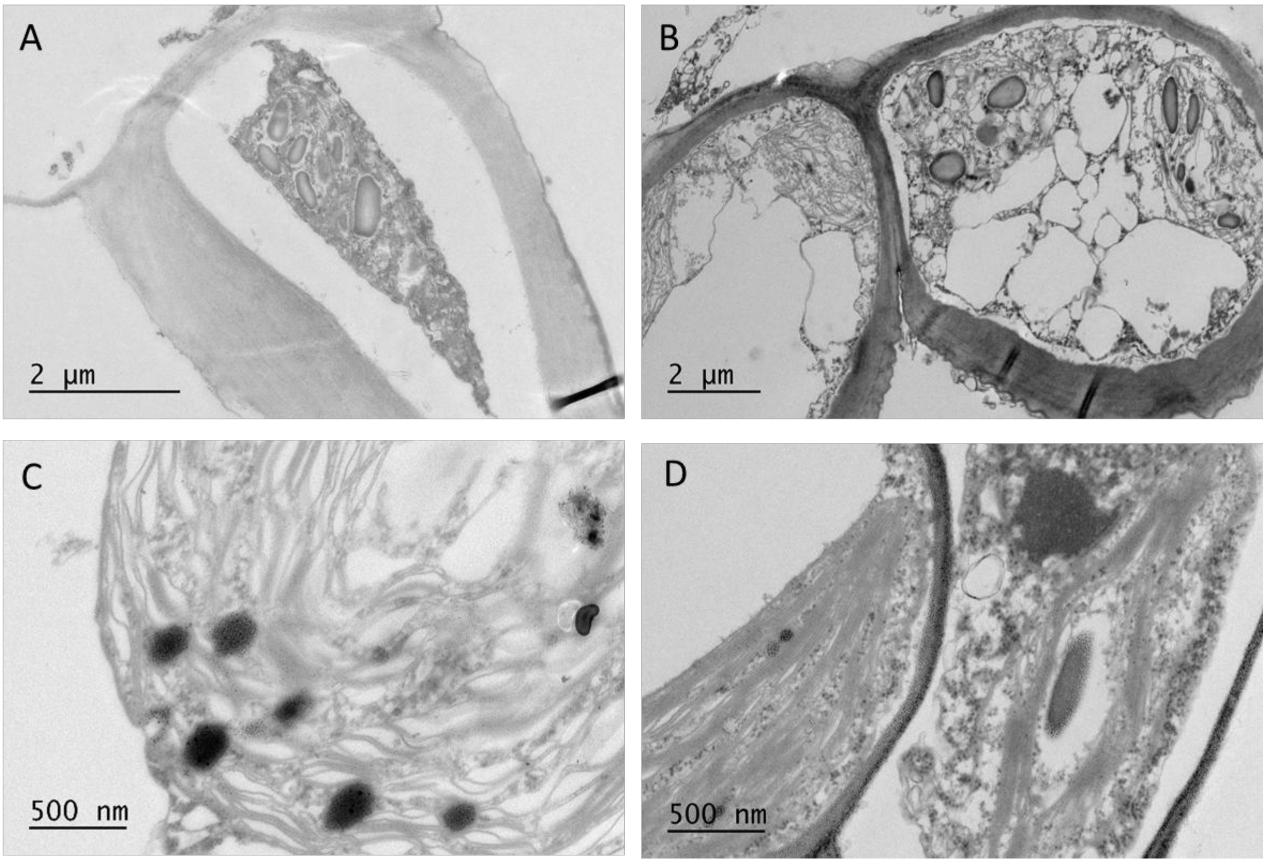

Figure 6. Leaves of B. oleracea grown with 125 µM Se(IV) (A), and 125 µM Se(IV) and 5·10^{7} CFU Pseudomonas sp. T5-6-I (B – D), visualized using transmission electron microscopy (TEM). Data are representatives of three biological replicates.

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_3, Se-Cys:Se-Met, Se:Se-Met, Se:Se-Cys, Se-Cys:SeO_3 and Se-Met:SeO_3 were fitted (Appendix 1, Table A1 and A2). Of the fitted pairs Se:Se-Cys and Se-Met:SeO_3 lead to negative fractions for Se and SeO_3, respectively, indicating non-physical fits and were rejected from the subsequent analysis. Se:SeO_3 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 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.

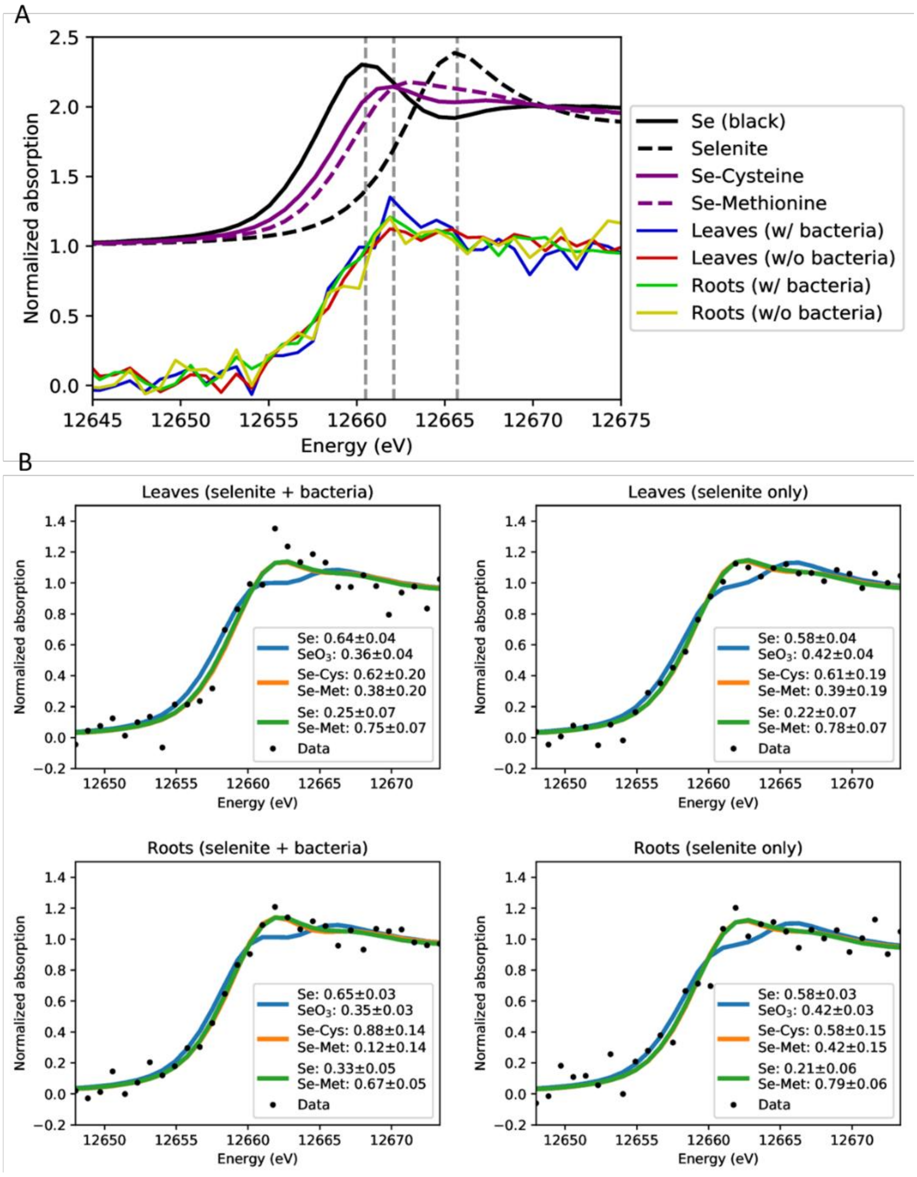

Figure 7. Fitted XANES spectra of B. oleracea leaves and roots with 125 µM Se(IV) amendment and with or without Pseudomonas sp. T5-6-I inoculation. A) Combined absorption spectra of Se^0 *(Se(Black)), Se(IV)(selenite), Se-Cysteine, Se-methionine, and the leaves and roots of B. oleracea*

grown with 125µM Se(IV) amendment and with/without Pseudomonas sp. T5-6-I inoculation. B) Fit with Se:SeO$_2$ pair, with Se-Cys:Se-Met pair and with Se:Se-Met pair.

To better understand the effects of Se(IV) amendment and *Pseudomonas* sp. T5-6-I inoculation in *B. oleracea,* root hair formation in Se(IV) and bacteria treated samples was investigated using confocal microscopy. No clear differences were observed in the root hair density in the plants grown with 125 µM Se(IV) amendment (Figure 8B) compared to the plants with no Se(IV) present (Figure 8A) in the MS-growth agar. However, a notably increased root hair density was seen in *Pseudomonas* sp. T5-6-I amended plants (Figure 8C) compared to the control plants without bacteria (Figure 8A). Similarly, in Se(IV) amended plants with *Pseudomonas* sp. T5-6-I inoculation (Figure 8D) a clear increase in the root hair density was observed, when the root morphology was compared with plain Se(IV) amended plants (Figure 8B).

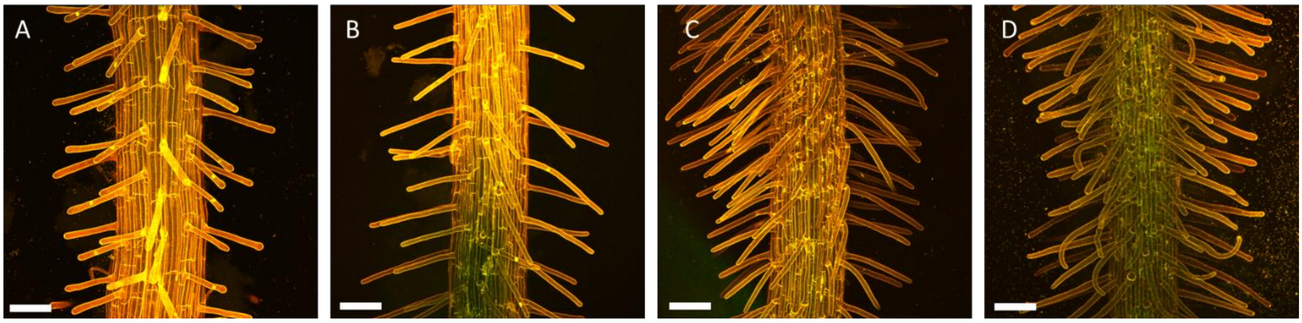

Figure 8. Confocal microscopy images of roots of B. oleracea grown on MS-salt agar, Control (no additions) (A), with Se(IV) addition (B), with Pseudomonas sp. T5-6-I addition (C) and Se(IV) + Pseudomonas sp. T5-6-I additions (D). A significantly increased root hair density is seen in Pseudomonas sp. 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 50µm.

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)**	***Pseud.*** **MW (kDA)**	**Se+*Pseud.*** **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 NO_3^-, NO_2^- or 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* *metallidurans* (CH34) (Roux et al. 2001). However, in our study after the increased lag phase, the cell numbers in the cultures exposed to Se(IV) reached the number of stationary phase cells in the control cultures without Se(IV), after a rapid exponential growth phase. Previously we observed 6 mM Se(IV) amendment to decrease ^{75}Se accumulation in the bacterial cells to approximately one tenth of the accumulation observed after 0.3 mM Se(IV) amendment using 7 days incubation (Lusa et al. 2017). Based on the previous results and the results obtained in the present study, the decrease in the ^{75}Se accumulation is most probably caused by the increase in the lag phase after increased Se(IV) exposure. This is in good line with the results of Sarret et al. (2005), which reported that the accumulation of selenite was minimal in *R. metallidurans* during the prolonged lag phase after increased Se(IV) exposure and that selenium was not accumulated until the late exponential and stationary phases of the bacterial growth. The growth of *Bifidobacterium animalis* was also significantly reduced by increasing Se(IV) concentrations (0.06 mM) compared to unsupplemented cultures (Zhang et al. 2008). In contrast, the biomass of *Enterococcus* species has been reported to increase with increasing Se(IV) concentrations of 0.06 to 0.36 mM (Pieniz et al. 2011). Concurrently, Se(IV) bioaccumulation was reported to increase (Pieniz et al. 2011). Selenium may accumulate in bacterial cells either in organic, inorganic or elemental form or as a mixture of these forms, and the varying accumulation pathways may further affect cell growth and ultimate cell density in the growth solutions (Zhang et al. 2009, Pieniz et al. 2011). In our study, the XANES analyses showed that *Pseudomonas* sp. T5-6-I reduced Se(IV) into elemental Se^0. In e.g. *E. coli*, Se(IV) reduction can follow a nonenzymatic pathway, which comprises of several organoselenium intermediates (Turner et al. 1998). The fact that Se^0 dominated in the *Pseudomonas* sp. T5-6-I after Se(IV) introduction, suggests that selenite is accumulated mainly through the dissimilatory detoxification pathway in this bacterium. Certain microorganisms are able to obtain metabolic energy from dissimilatory reduction processes, and in the environment, microbial Se(IV) reduction is an important process through which toxic soluble selenium oxyanionic species are removed from water and soil solutions (Eswayah et al. 2016, Nancharaiah and Lens 2015). Various electron donors, including sugars, alcohols and organic acids can be utilized in the dissimilatory reactions (Astratinei et al 2006, Zhang et al. 2008, Kashiwa et al. 2000, Chung et al. 2006). From the bioremediation point of view, dissimilatory selenium reduction is expected to be more important in contrast to the assimilatory reduction. This is because of the higher selenium concentrations passing through the dissimilatory pathway (Eswayah et al. 2016). For this reason, especially the microorganisms utilizing the dissimilatory reduction of selenium could be used as profitable means for the remediation of selenium polluted areas (Eswayah et al. 2016). In the environment, the reduced insoluble Se^0 is considered less harmful compared to the soluble oxyanionic forms of Se(IV) and Se(VI). The aqueous Se(IV) and Se(VI) species could, however, be immobilized using Se-oxyanion-reducing bacterial strains, especially suitable for bioremediation of aquatic environments. The separation and removal of the bacteria containing the reduced Se^0 from solid soils and sediments would however be more challenging. Therefore, we also tested the effect of the Se-oxyanion-reducing *Pseudomonas* sp. T5-6-I strain on selenium uptake by plants, in order to evaluate its potential use in the bacteria-based phytoremediation of toxic selenium oxyanionic species from soils. Generally, phytoremediation for soil cleaning is considered very beneficial as it does not reduce the fertility of the soil, which is a typical problem with other engineered methods (e.g. thermal soil remediation, encapsulation and air sparcing) used for soil clean-up (Robinson et al. 2000, Pilon-Smits and Freeman 2006). Phytoremediation can be coupled to biofortification, in which the selenium enriched plant material will be decomposed in agricultural soil and used further for the enrichment of food products with Se (Schiavon and Pilon-Smits 2017).

In the plant-uptake experiments, we observed *Pseudomonas* sp. T5-6-I to promote root hair development in *B. oleracea*, and in addition, this bacterium affected selenium uptake in the plants after Se(IV) exposure. Gammaspectrometry showed that 130% more activity was translocated to the *B. oleracea* plants treated with ^{75}Se(IV) and *Pseudomonas* sp. T5-6-I compared to the samples receiving only ^{75}Se(IV) (n=14). The increase in the ^{75}Se concentrations after *Pseudomonas* sp. T5-6-I inoculation were most notable in the roots, compared to the leaves in young seedlings after a two weeks growth period. A 7.5 – 12-fold higher concentration of selenium was found accumulated to the roots compared to the leaves in the *Pseudomonas* sp. inoculated samples. Previously, rhizospheric bacteria have been reported to enhance selenium accumulation e.g. in *Brassica juncea* (de Souza et al. 1999).

Selenium may have beneficial effects on plants and several studies have shown that at low doses Se protects the plants from variety of abiotic stresses including cold (Chu et al., 2010), drought (Hasanuzzaman and Fujita, 2011), and metal stress (Kumar et al., 2012, Pandey and Gupta, 2015). However, selenium toxicity (selenosis) arises in plants after protective concentrations are exceeded (Gupta and Gupta 2017). Selenosis is caused by two distinctive mechanisms, i) by inducing oxidative stress and ii) by formation of malformed selenoproteins. Malformed Se-proteins are caused by the misincorporation of SeCys/SeMet in place of Cys/Met in an amino acid chain. As compared to SeMet, substitution of SeCys is more detrimental for the protein function, due to the important roles of cysteine residues in the protein structure (Gupta and Gupta 2017). Cysteine residues are essential for the formation of disulphide bridges, enzyme catalysis and metal binding sites. The substitution of cysteine by selenocysteine distorts the tertiary structure of a protein due to the formation of diselenide bridges instead of disulphide ones (Hondal et al., 2012). In addition, the enzyme kinetics can be altered because of changes in the redox potential. Previously, e.g. Se-MeSeCys (Selenomethylselenocysteine) has been described in SeMet enriched *Brassica* plants (Gupta and Gupta 2017). In addition, Se-MeSeCys and SeMet and selenate were detected in shoots, whereas selenate, selenite and SeMet were found in the roots of these plants (Gupta and Gupta 2017). In our study, the XANES analyses showed that Se was most likely present in H-Se-C and C-Se-C bonds in the *B. oleracea* leaves and roots after Se(IV) amendment and the presence of selenite, selenate or Se^0 was not supported by the data. Furthermore, in our study, statistically significant differences in the selenium speciation, which could explain the increased accumulation in the roots compared to the leaves, could not be observed in the XANES spectra obtained from the differently treated plants.

Depending on the concentrations and form of selenium, its uptake varies among plant species and is affected by the activity of membrane transporters, the developmental phase of the plant, as well as physiological conditions and accompanying substances (Zhao et al., 2005, Li et al., 2008). Selenium is expected to enter the plant cells as selenite or selenate through sulphate transporters and to thereafter be translocated to leaves where it is metabolized in plastids via the sulfur assimilation pathway into SeCys or SeMet (Gupta and Gupta 2017). In our study, selenium was found to be most likely incorporated in C-Se-C and C-Se-H bonds (as in SeMet and SeCys) in the plant cells after Se(IV) introduction. This would well fit the sulfur assimilation pathway for Se(IV). As somewhat increased uptake of selenium (^{75}Se) was observed in the bacteria-inoculated *B. oleracea* plants, also other mechanisms could however be expected. This is because these bacteria were shown to reduce Se(IV) into Se^0 and based on previous studies, plants are known to be unable to take up colloidal elemental Se^0 (White and Broadley 2009). The changes observed in the *B. oleracea* protein profiles after Se(IV) and

Pseudomonas sp. treatments could be generated by these mechanisms. However, further characterization of the proteins is needed, to confirm their role in selenium accumulation. Previously high fractions (up to 35% of all selenium) of elemental reduced Se^0 has been reported in roots of *Astragalus bisulcatus* (*Fabaceae*), which was suggested to be produced by microbial endophytes (Lindblom et al. 2012, Lindblom et al. 2018). Reduced Se^0 was observed especially in the *A. bisulcatus* nodules and roots inoculated with Se^0 -producing fungi *Alternaria astragali* or *A. tenuissima*. Furthermore, e.g. bacteria belonging to the genera *Bacillus*, *Pseudomonas*, *Pantoea*, *Staphylococcus*, *Paenibacillus*, *Advenella*, *Arthrobacter* and *Variovorax* are known endophytes from Se hyperaccumulators *Stanleya pinnata* (*Brassicaceae*) and *A. bisulcatus* (Sura-de Jong et al. 2015). These bacteria are also able to reduce Se(IV) to elemental Se^0 and have plant growth promoting properties but their effect on plant Se accumulation has not been reported (Sura-de Jong et al. 2015). However, in our study Se^0 was not observed in the significant amounts in the *B. oleracea* plants after Se(IV) treatment nor Se(IV) + *Pseudomonas* sp. T5-6-I treatments using XANES. In addition, no structures resembling endophytic bacterial cells were found in the plant cells using TEM, although we observed small (<50 nm) dense granules in the leaf cells of *B. oleracea* grown with *Pseudomonas* sp. T5-6-I and Se(IV), which were not seen in plants without Se(IV) and *Pseudomonas* sp. T5-6-I amendments. At the same time, the root hair density was clearly increased in the *Pseudomonas* sp. inoculated plants, increasing the surface area available for selenium uptake by the plants. Thus, it appears that *Pseudomonas* sp. T5-6-I forms favorable symbiosis with the plants and contributes to the Se accumulation observed in *B. oleracea*, even though endophytic interactions could not be verified. Our results could not exhaustively conclude whether selenium is initially translocated to the plants as reduced Se^0 species inside the bacterial cells, or if the bacteria-plant interactions on the root surfaces change the initial selenium speciation enabling increased uptake to the plant through increased uptake surface area and increased expression of proteins involved in the selenium metabolism. Nevertheless, our findings demonstrate the potential for Se^0 -producing (symbiotic) bacteria to affect plant properties relevant for phytoremediation or biofortification. Facultative symbionts may also be beneficial in bioremediation and biofortification, due to their ability to turn toxic oxyanionic form of selenium, Se(IV), into less-toxic (or even metabolically beneficial) forms and to increase selenium(IV) accumulation in the plants.

5. Conclusions

The bacterial growth rate in *Pseudomonas* sp. T5-6-I was affected by Se(IV) amendment and Se(IV) increased the duration of the lag phase of the growth. However, following rapid exponential growth, growth rate promptly reached the growth rate observed in the control cells without Se(IV) amendment. Based on the following XANES measurements, *Pseudomonas* sp. T5-6-I effectively reduced Se(IV) into elemental Se^0 under oxic conditions. In addition, this *Pseudomonas* sp. strain increased selenium (^{75}Se(IV)) accumulation in *B. oleracea*. However, this bacterium was not found to affect the following selenium speciation in the *B. oleracea* plants. We conclude that the increase in selenium plant-uptake after *Pseudomonas* sp. T5-6-I inoculation may, at least partly, be caused by the increased root-hair growth, which was observed in the *B. oleracea* plants after *Pseudomonas* sp.T5-6-I inoculation.

This study shows that Se(IV) tolerant, Se^0 producing soil bacteria isolated from boreal, harsh bog environment, can influence Se accumulation in a common crop plant *B. oleracea* (kale). This feature could be highly beneficial for the further development of phytoremediation and biofortification applications, especially as these bacteria seem not to distinguish between their habitat or plant partner.

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_3 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^{-13}	2.3 x 10^{-7}	1.7 x 10^{-19}
SeO_3	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_3	0.02 ± 0.06	0.13 ± 0.06	0.02 ± 0.04	0.12 ± 0.04			

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Table 1

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)**	***Pseud.* MW (kDA)**	**Se+*Pseud.* 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 Appendix A1

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 Appendix A2

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_3 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 SeO_3	0.64 ± 0.04 0.36 ± 0.04	0.58 ± 0.04 0.42 ± 0.04	0.65 ± 0.03 0.35 ± 0.03	0.58 ± 0.03 0.42 ± 0.03	7.3×10^{-13}	2.3×10^{-7}	1.7×10^{-19}
Se-Cys Se-Met	0.6 ± 0.2 0.4 ± 0.2	0.6 ± 0.2 0.4 ± 0.2	0.88 ± 0.14 0.12 ± 0.14	0.58 ± 0.15 0.42 ± 0.15	1	1	1
Se Se-Met	0.25 ± 0.07 0.75 ± 0.07	0.22 ± 0.07 0.78 ± 0.07	0.33 ± 0.05 0.67 ± 0.05	0.21 ± 0.06 0.79 ± 0.06	0.42	0.011	0.048
Se-Cys SeO_3	0.98 ± 0.06 0.02 ± 0.06	0.87 ± 0.06 0.13 ± 0.06	0.98 ± 0.04 0.02 ± 0.04	0.88 ± 0.04 0.12 ± 0.04	0.011	0.013	0.0015