AGRICULTURAL AND FOOD SCIENCE

M.M. Seppänen et al. (2018) 27: 38–49

Dynamics of selenium uptake and metabolism of organic selenium species in the leaves and seeds of *Brassica napus* L.

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The essential micronutrient selenium (Se) is retained better in animal and human tissues in its organic forms while nonprotein selenoamino acids, such as selenomethylselenocysteine (SeMetSeCys), are considered as functional organic Se species. We studied the ability of oilseed rape *Brassica napus* to metabolize inorganic selenate/selenite (SeVI/SeIV) into various organic Se species, including SeMetSeCys. At 14 d after the inorganic Se application, 33% of the Se had accumulated as selenomethionine (SeMet) and 60% as SeVI, whereas no SeMetSeCys was detected. SeMet was the main organic Se species (53–94%) in seeds. *Brassica napus* selenocysteine methyltransferase (SMT) protein sequence revealed a substitution typical of nonaccumulators explaining the low SeMetSeCys accumulation. *Brassica napus* absorbs rapidly inorganic Se and converts it into organic Se forms, mainly SeMet, that are suitable for augmenting animal feed and thereby supplementing the human food chain in Se-deficient countries. In contrast, Se biofortification did not result in accumulation of the more valuable SeMetSeCys.

Key words: selenomethionine, selenomethylselenocysteine, Se biofortification, Adenosine triphosphate sulphurylase, selenocysteine methyltransferase

Introduction

Selenium (Se) is an essential microelement for humans and animals. It is incorporated into the food chain as inorganic or organic Se compounds via crop plants. In regions of deficiency, such as Finland, the adequate intake of Se (55 µg d^{-1}) by humans is ensured by the biofortification of crops by Se-augmented inorganic fertilizers.

Higher daily intake of Se (up to 200 µg d^{-1}) may furnish additional health benefits, such as reduced cancer risk and an improved immune system (Rayman 2008b). Organic Se species are less toxic and better retained in human and animal tissues than inorganic Se (Rayman 2008b). Selenomethionine (SeMet) is the most abundant organic Se form in most plants and plant –derived food and, is the main species found in the seeds of Se-biofortified turnip *Brassica rapa* L. and oilseed rape *B. napus* L. subsp. *oleifera* (DC.) Metzg. plants (Seppänen et al. 2010). The nonprotein Se compound selenomethylselenocysteine (SeMetSeCys) and its γ-glutamyl derivative have antitumour properties in animals and are therefore considered to be valuable organic Se species (Rayman 2008a).

Due to their chemical similarity, selenate (SeVI) and sulphur (S) in the form of sulphate (SO$_4$) are taken up and assimilated by plants through the same Se/S pathway. SeVI is taken up by high-affinity SO$_4$ transporters in the roots (Sors et al. 2005a, White and Broadley 2009), whereas phosphate transporters are active in selenite (SeIV) transport (Li et al. 2008). SeVI biotransformation is activated by adenosine triphosphate sulphurylase (ATPS), after which SeVI is reduced to SeIV by ATPS reductase. This reduction reaction may be the rate-limiting step of Se assimilation in plants (Pilon-Smits et al. 1999) that controls the flux throughout the Se/S assimilation pathway (Vauclare et al. 2002). SeIV is further reduced to selenide by sulphite reductase and then incorporated into serine by cysteine synthetase to form selenocysteine (SeCys). SeMet is synthesized from SeCys and *O*-phosphohomoserine in multiple chemical reactions that involve enzymes, including cystathione γ-synthase, cystathione β-lyase and methionine synthase (Terry et al. 2000).

Plant species differ in their ability to take up and assimilate Se and can be divided into nonaccumulators, accumulators and an intermediate group of secondary accumulators that are also known as indicators (Bañuelos et al. 1997, Terry et al. 2000). The brassica species Chinese mustard *Brassica juncea* (L.) Czern. and *B. napus* belong to the group of Se secondary accumulators that can accumulate up to 1 mg Se g^{-1} DM. The current understanding is that the Se-sensitive species incorporate SeCys and SeMet into proteins, whereas the Se-tolerant species have mechanisms to methylate these species into small nonprotein-forming amino acids such as SeMetSeCys (Brown and Shrift 1981, Sors et al. 2005b). The enzyme responsible for SeMetSeCys synthesis in the Se hyperaccumulator twogrooved milk-vetch *Astragalus bisulcatus* (Hook.) A. Gray is selenocysteine methyltransferase (SMT) (Neuhierl and Bock 1996, Ellis and Salt 2003). This enzyme shows a high degree of homology in accumulator and

AGRICULTURAL AND FOOD SCIENCE

M.M. Seppänen et al. (2018) 27: 38–49

nonaccumulator plants, but it lacks SMT activity in nonaccumulator plants, which results in low or nondetectable levels of SeMetSeCys (Sors et al. 2009). In addition, a site-directed mutagenesis approach has shown that a crucial amino-acid substitution of alanine (Ala) to threonine (Thr) was responsible for the specific capacity to methylate SeCys (Sors et al. 2009). The seeds of *Brassica napus* are an important source of vegetable oil for humans, and the protein-rich meal is used as livestock feed. Our aim was to evaluate whether *Brassica napus* has the capacity to synthesize and accumulate SeMetSeCys together with other organic Se compounds and to reveal the bottlenecks in the accumulation of organic Se compounds in seeds.

Materials and methods

Plant material and experimental conditions

The winter genotype of oilseed rape plants (*B. napus* L. ssp. *oleifera*) were grown in a greenhouse (16/8-h day/night lighting regime) in 1.5-l pots that contained a sand:quartz sand mixture (900 g sand:400 g quartz sand) to which the following plant nutrients (concentrations in mM) NH_4NO_3 222.9, $CaHPO_4$ 50.3, KH_2PO_4 67.1, $MgSO_4$ 65.0, H_3BO_3 9.6, $(NH_4)_6Mo_7O_{24}$ 0.5, $CuSO_4$ 0.8, $ZnCl_2$ 1.6, ethylenediaminetetraacetic acid (EDTA) 3.7, $MnCl_2$ 4.7, $Ni(NO_3)_2$ 0.9 were added. The nutrients (totally 20 ml pot^{-1}) were given in two applications, 2/3 of the total quantity at sowing and the remaining (1/3) 2 weeks later. Five *B. napus* (cv. 4021B) seeds were sown in each pot, and after germination thinned to one plant per pot. Four weeks after sowing, two soil applications of Se (0 µM or 65 µM of sodium selenate Na_2SeO_4) and three leaf applications of Se (0 µM or 100 µM of either Na_2SeO_4 or sodium selenite Na_2SeO_3) were carried out. In all, 25 ml of water or Na_2SeO_4 solution equal to 0 µg and 130 µg Se per plant (65 µM Se) were added to the soil (Soil$_0$, Soil$_{VI}$); simultaneously, 3.75 ml of water, Na_2SeO_4 or Na_2SeO_3 solutions were sprayed onto plant leaves in amounts equal to 0 µg (Leaf$_0$) or 30 µg Se per plant (100 µM Se) of either Na_2SeO_4 (Leaf$_{VI}$) or Na_2SeO_3 (Leaf$_{IV}$). Thus, there were six treatments: Soil$_0$/Leaf$_0$, Soil$_0$/Leaf$_{VI}$, Soil$_0$/Leaf$_{IV}$, Soil$_{VI}$/Leaf$_0$, Soil$_{VI}$/Leaf$_{VI}$ and Soil$_{VI}$/Leaf$_{IV}$. The pots were arranged in randomized complete block design (RCBD) in four blocks with four replications. Due to the four harvesting times (6 h, 7 d, 14 d after inorganic Se application and matured plants), at each sampling time, one of the blocks was used. For the gene expression study, the newly developed leaves (fourth or fifth leaf) from two individual plants were harvested. For the Se speciation analysis, leaves of the same age from three individual plants were harvested. The remaining shoots were harvested for total Se analysis. Except for the last harvest, all plants were in the vegetative growth phase. The Se application levels were selected, based on previous studies, to obtain optimal Se accumulations without toxicity to the plants (Seppänen et al. 2010). SeIV was applied only to the leaves, due to its low mobility in plants and its tendency to accumulate in the roots (Terry et al. 2000).

Plant growth and harvest

Plant development was recorded according to the Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (BBCH) scale, and the changes in leaf chlorophyll concentration of the marked leaves (*n* = 4 or 5 per plant) were monitored weekly, using a SPAD-502 chlorophyll meter (Minolta Co. Ltd. (now Konica Minolta), Osaka, Japan). The uniformly aged upper leaves (fourth or fifth leaf) were harvested and rinsed for RNA and Se analysis 6 h, 7 d and 14 d after inorganic Se application and at harvest. At harvest, the plant height was measured and the main yield components (number of side branches and the number of siliques in the main branch and side branches) were counted.

Gene expression analysis

The RNA was extracted using TRI REAGENT® (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Any possible DNA contamination was removed with DNase I digestion (SuperScript™ III Cells-Direct complementary DNA (cDNA) Synthesis System; Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized according to the manufacturer's instructions (SuperScript™ III reverse transcriptase, Invitrogen), using 1 µg of total RNA and oligo (dT)^{18} primers. RNA that was complementary to the cDNA was removed by treatment with 1 µl (2 units) of *Escherichia coli* (Migula) Castellani & Chalmers RNase H and incubation at 37 °C for 20 min.

The primers were designed for S metabolism genes in *B. napus*. For the internal control, β-actin primers (F946-atcgtcctcagtggtggttc, R1049-gcgaccaccttgatcttcat) were designed according to GenBank sequence AF111812. APS1 (F373-AACATGTCGGTGCCTATCGT, R482-AGGATAGCGACGGGATTACC), APS2 (F236-TCTCTTGCTTCACCCTTTGG, R341-CGGGTCAAGAACTCCATCTT) and APS3 (F188-TCCGGAAAGAGCTTGAGAAA, R289-GGCGAGTGTCAGTCATGAGA) primers were designed according to GenBank sequences U68218, EE463359 and EV120399, respectively, and the SMT (F279-ATCTCCAGCGCTTCTTACCA, R394-TGCGAGCTTCACATGCTATT) primers to the EST JCVI_9670 (http://brassica.bbsrc.ac.uk/). A new set of primers (F385 5'ACCAGGCCACGATTCAGGGGT-3', R1216 5'-ACGTGAGTGAAGGTGGAGGAGGA-3') was designed to examine the potential Ala-184 to Thr replacement in the putative SMT protein sequence of *B. napus*.

AGRICULTURAL AND FOOD SCIENCE

M.M. Seppänen et al. (2018) 27: 38–49

A 648-nucleotide-long sequence was obtained, translated into an amino-acid sequence (http://web.expasy.org/translate/) and compared with its homologues in wild cabbage *Brassica oleracea* L. (AY817737.1) and *A. bisulcatus* (AJ131433.1), using Clustal W (http://www.genome.jp/tools/clustalw/).

Primer testing was done, using a 50-µl polymerase chain reaction (PCR) that contained 0.5 U of Taq polymerase (DyNAzyme II; Finnzymes OY, Espoo, Finland), 1 x buffer (DyNAzyme™), 0.2 µmol of each primer, 200 µmol of the deoxynucleotide phosphates (dNTPs) and 100 ng of genomic DNA. The PCR was performed (Eppendorf Netheler-Hinz, Hamburg, Germany) under the following conditions: 5 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at 57 °C and 90 s at 72 °C. The PCR fragments were excised from the 1% Tris-acetate-EDTA (TAE) agarose gel, purified using the QIAquick® gel extraction kit (Qiagen N.V., Hilden, Germany) and cloned into the pCR® 2.1-TOPO cloning vector (Invitrogen) according to the manufacturer's manual. The cloned fragments were sequenced at the Institute of Biotechnology (University of Helsinki) for verification of the amplification product.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was carried out in a Light Cycler® 480 Real-Time PCR System (Roche Applied Science, Penzberg, Germany). A 20-µl reaction contained 10 µl of DyNAmoTM (Finnzymes) SYBR® Green master mix, 0.5 µM of each primer, 100 ng of cDNA and 0.4 µl of passive ROX dye. All primers were tested in a cDNA dilution series (1, 5, 10, 25, 50 and 100%), using the following program: 95 °C 15 min, 40 x (94 °C for 10 s, 59 °C for 30 s and 72 °C for 30 s), with a melting curve at 95 °C for 15 s, 60 °C for 20 s and 95 °C for 30 s. The efficiency (E) values for the APS1, APS2 APS3, SMT and β-actin primers were 2.07, 1.92, 2.15, 2.11 and 2.03. The expression levels of the target genes were normalized, using β-actin as the reference gene, and the relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Selenium analysis

The plant samples were dried overnight at 70 °C to determine the total concentration of Se. The soil samples were subjected to an acid mixture digestion (HNO_3, $HClO_4$ and H_2SO_4), and all the Se forms were reduced to SeIV by HCl, which was subsequently chelated with ammonium pyrrolidine dithiocarbamate. The reduced Se was then extracted withy methyl isobutyl ketone. The analyses were executed with the electrothermal atomic absorption spectrometric (AAS) method (Kumpulainen et al. 1983), using a Zeeman spectrometer (model 5100; PerkinElmer Inc., Waltham, MA, USA) connected to a graphite furnace (model HGA 600; PerkinElmer) and equipped with an AS-60 autosampler (PerkinElmer). The accuracy of the measurement was confirmed by including an in-house reference sample for each set of samples.

Se speciation in the various samples was analysed with high-performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) after extraction of the Se species, using ultrasonic probe-assisted enzymatic hydrolysis. About 0.05 g of fresh plant material and 3 ml of Milli-Q water (Millipore Corp., Burlington, MA, USA) were placed into a tube with 20 mg of Protease XIV (Sigma-Aldrich Corp., Steinheim, Germany) and sonicated at 40% ultrasound amplitude for 2 min in a Sonoplus ultrasonic homogenizer (Bandelin Electronic GmbH & Co. KG, Berlin, Germany) that was equipped with a Titanium 3-mm-diameter microtip and fitted with a high-frequency generator of 2200 W at 20 kHz. The samples were filtered through a 0.22-µm filter, and the extracts were analysed with HPLC-ICP-MS. Two chromatographic columns were used for the analysis to correctly identify the organic Se species. The first chromatographic column was a PRP X-100 anion-exchange column and the second a Shodex Asahipak column (Showa Denko K.K., Tokyo, Japan), both using combined size-exclusion and ion-exchange separation mechanisms. Standard stock solutions of 1000 mg l^{-1} of SeMet, SeMetSeCys and selenocystine (SeCys$_2$) (Sigma) were prepared in ultrapure water (18.2 MΩ cm^{-1}) obtained from a Milli-Q water purification system (Millipore), and 3% HCl was added to enhance the dissolution of SeCys$_2$ and SeMetSeCys. Inorganic Se solutions (1000 mg l^{-1}) were prepared by dissolving Na_2SeO_3 and Na_2SeO_4 (Merck KGaA, Darmstadt, Germany) in Milli-Q water. The stock solutions were stored at 4 °C.

Statistical analyses

The data were classified, based on the harvesting time, and each time was analysed separately. First, one-way analysis of variance (ANOVA) was carried out to reveal the differences between all the treatments and on which the tables presented here are formulated. In addition, to show the main effects and interactions between the soil and leaf applications of Se, a two-way ANOVA analysis was carried out. All statistical analyses were carried out, using SPSS software version 24 (IBM Corp., Armonk, NY, USA). Significantly different means between the treatments were analysed, using Tukey´s test with $p \leq 0.05$.

AGRICULTURAL AND FOOD SCIENCE

M.M. Seppänen et al. (2018) 27: 38–49

Results

Plant growth and development

Even very high Se accumulations in the leaves (33 µg Se g^{-1}, Table 1) did not delay development of the plants, and no significant alterations in the (SPAD) values for leaf senescence were observed (data not shown). Fourteen days after application, the Se applied (to soil or leaves) lowered the DM content, but this reduction was transient, and the Se-induced impact on the seed yield was insignificant (data not shown). Furthermore, the Se addition did not alter the plant architecture, since the development of the yield components was similar in all treatments (data not shown).

Table 1. The concentrations of total Se in shoot and different Se species (µg g^{-1} DM) in *Brassica napus* plants at 6 h, 7 days or 14 days after treatment with 0 or 130 µg Se $plant^{-1}$ as Na_2SeO_4 (Soil0 and SoilVI,), in soil and 0 or 30 µg Se $plant^{-1}$, foliar spray of Na_2SeO_4 or Na_2SeO_3, (Leaf0, LeafVI and LeafIV respectively). Selenomethionine (SeMet), selenomethyl selenocysteine (SeMeSeCys) and inorganic SeVI and SeIV were detected. The recovery (R %) was calculated by ratio of the sum of Se species to the total Se obtained by the acid digestion method. The number inside brackets is the percentage of different Se species from total Se.

Treatments	Total Se in shoot µg g^{-1}	SeMet	SeMeSeCys	SeVI	SeIV	R %
6 h						
$Soil_0/Leaf_0$	0.23^f	0.39^d (54)	0.00^c (0)	0.07^d (37)	0.04^{cd} (9)	78^c
$Soil_0/Leaf_{VI}$	1.40^e	0.11^d (12)	0.00^c (0)	0.84^d (87)	0.01^d (1)	70^d
$Soil_0/Leaf_{IV}$	2.40^d	0.36^d (71)	0.00^c (0)	0.24^d (2.5)	0.09^c (18)	21^f
$Soil_{VI}/Leaf_0$	18.72^c	2.83^b (15)	0.8^a (4)	14.84^a (80)	0.00^d (0)	99^a
$Soil_{VI}/Leaf_{VI}$	20.85^b	2.05^c (16)	0.26^b (2)	10.06^c (80)	0.26^b (2)	61^e
$Soil_{VI}/Leaf_{IV}$	23.04^a	5.76^a (29)	0.79^a (4)	12.80^b (64)	0.60^a (3)	87^b
±SE	0.13	0.20	0.02	0.27	0.02	1.31
Soil application	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$
Leaf application	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$
Soil*Leaf application	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$
7d						
$Soil_0/Leaf_0$	0.15^d	0.08^d (49)	0.00^d (0)	0.04^d (24)	0.04^d (27)	100^b
$Soil_0/Leaf_{VI}$	0.30^d	0.07^d (37)	0.00^d (0)	0.12^d (63)	0.00^e (0)	65^d
$Soil_0/Leaf_{IV}$	0.30^d	0.14^d (41)	0.00^d (0)	0.03^d (9)	0.17^b (49)	115^a
$Soil_{VI}/Leaf_0$	22.15^a	7.03^a (33)	0.23^a (1)	14.53^a (66)	0.11^c (1)	99^{bc}
$Soil_{VI}/Leaf_{VI}$	15.60^b	2.04^c (33)	0.12^b (2)	4.00^c (64)	0.06^d (1)	40^e
$Soil_{VI}/Leaf_{IV}$	13.03^c	5.54^b (46)	0.06^c (1)	6.15^b (51)	0.37^a (3)	93^c
±SE	0.16	0.13	0.00	0.19	0.01	1.94
Soil application	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$
Leaf application	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$
Soil*Leaf application	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$
14 d						
$Soil_0/Leaf_0$	0.14^d	0.06^d (46)	0.00^b (0)	0.05^d (36)	0.02^d (17)	98^{ab}
$Soil_0/Leaf_{VI}$	0.27^d	0.16^d (59)	0.00^b (0)	0.08^d (30)	0.03^d (11)	100^{ab}
$Soil_0/Leaf_{IV}$	0.63^d	0.13^d (20)	0.01^b (2)	0.05^d (8)	0.46^c (70)	103^a
$Soil_{VI}/Leaf_0$	33.00^a	7.70^a (29)	0.00^b (0)	17.37^a (64)	1.93^a (7)	82^c
$Soil_{VI}/Leaf_{VI}$	12.90^c	4.02^c (32)	0.25^a (2)	8.00^c (65)	0.13^d (7)	96^b
$Soil_{VI}/Leaf_{IV}$	22.03^b	5.81^b (32)	0.00^b (0)	11.22^b (61)	1.28^b (7)	83^c
±SE	0.15	0.15	0.00	0.27	0.04	2.21
Soil application	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$
Leaf application	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$
Soil*Leaf application	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$

Numbers followed by different letters in the same column differ significantly at the $p \leq 5\%$ by Tukey's test; n=3. *P* value of main effects and interaction between soil and leaf application has been reported.

AGRICULTURAL AND FOOD SCIENCE

M.M. Seppänen et al. (2018) 27: 38–49

Activation of gene expression

Of the four S metabolism genes monitored, *APS1* showed the greatest response in transcript accumulation (Fig. 1a). However, the levels of transcript accumulation of all the studied genes did not vary significantly between the treatments (Figs. 1a–d). The results indicate, however, that the soil Se application (Soil$_{IV}$/Leaf$_0$, Soil$_{IV}$/Leaf$_{VI}$, Soil$_{IV}$/Leaf$_{IV}$) tended to result in higher transcript accumulation of *APS1* and *APS2* than did the foliar Se application solely after 14 d. Moreover, the foliar application of SeIV (Leaf$_{IV}$) seemed to result in more rapid transcript accumulation of all the studied genes.
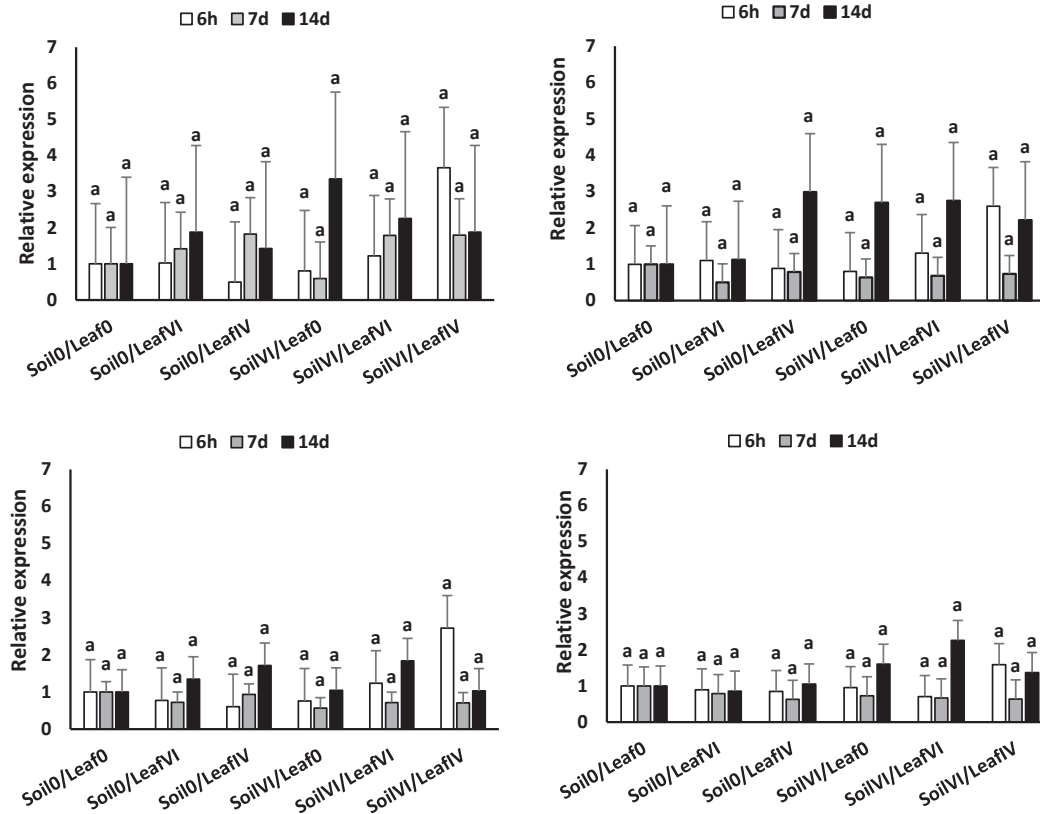

Fig. 1. Relative expression of a) APS1, b) APS2, c) APS3 and d) SMT genes at 6 h (white), 7 d (grey) and 14 d (black) after treatment with 0 or 130 µg Se plant^{-1} as Na$_2$SeO$_4$ in soil (Soil0 and SoilVI,) and 0 or 30 µg Se plant^{-1}, foliar spray of Na$_2$SeO$_4$ or Na$_2$SeO$_3$, (Leaf0, LeafVI and LeafIV respectively); mean ± SE; n=2

When the partial *SMT* homologue of *B. napus* was aligned with the corresponding sequences of the Se accumulator *A. bisulcatus* and the nonaccumulator *B. oleracea* (Fig. 2), the amino-acid alteration of Ala-184 to Thr, specific for Se nonaccumulators, was found in the *B. napus* sequence. The SMT protein sequence of *B. napus* showed closer homology with that of *B. oleracea* than with that of *A. bisulcatus*.
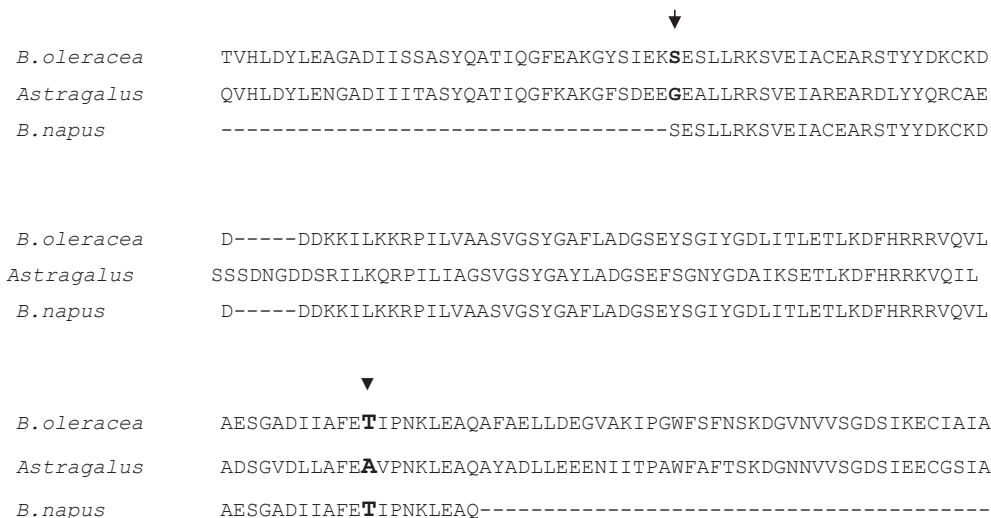


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                                                                        ↓
 B.oleracea    TVHLDYLEAGADIISSASYQATIQGFEAKGYSIEKSESLLRKSVEIACEARSTYYDKCKD
 Astragalus    QVHLDYLENGADIIITASYQATIQGFKAKGFSDEEGEALLRRSVEIAREARDLYYQRCAE
 B.napus       -------------------------------SESLLRKSVEIACEARSTYYDKCKD


 B.oleracea    D-----DDKKILKKRPILVAASVGSYGAFLADGSEYSGIYGDLITLETLKDFHRRRVQVL
 Astragalus    SSSDNGDDSRILKQRPILIAGSVGSYGAYLADGSEFSGNYGDAIKSETLKDFHRRKVQIL
 B.napus       D-----DDKKILKKRPILVAASVGSYGAFLADGSEYSGIYGDLITLETLKDFHRRRVQVL


                             ▼
 B.oleracea    AESGADIIAFETIPNKLEAQAFAELLDEGVAKIPGWFSFNSKDGVNVVSGDSIKECIAIA
 Astragalus    ADSGVDLLAFEAVPNKLEAQAYADLLEEENIITPAWFAFTSKDGNNVVSGDSIEECGSIA
 B.napus       AESGADIIAFETIPNKLEAQ----------------------------------------
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Fig. 2. Partial protein sequence alignment of selenomethyltransferase (SMT) of *Brassica oleracea* (GenBank: AY817737.1). *Astragalus bisulcatus* (AJ131433.1) and putative SMT sequence of *Brassica napus*. The Ala-184 residue unique to the Se accumulator and replacement by Thr residue in non-accumulators is marked in bold and indicated with a black arrowhead.

AGRICULTURAL AND FOOD SCIENCE

M.M. Seppänen et al. (2018) 27: 38–49

Identification of selenium species in leaves and seeds

Analyses of the Se species revealed the dynamics of Se assimilation in the leaves of *B. napus* (Fig. 3). Six hours after SeVI application to the soil, 81% of the Se in the leaves was in the original inorganic form of SeVI, and this proportion decreased to 60% by 14 d. Inorganic SeIV, in turn, was detected in the leaves after 7 d, and the accumulation was substantial after 14 d. This response was probably attributable to the intensive activation of the *APS* genes (Fig. 1) and accumulation of SeIV as an intermediate product of Se assimilation. The concomitant increase in the total Se concentration of the leaves from 18.7 to 33.0 $\mu g\ g^{-1}$ indicated that the soil still contained Se available for plant uptake (Table 1). The proportion of SeMet of 15% only 6 h after application increased to 33% at 14 d, which indicated that the assimilation of Se into organic compounds is efficient in *B. napus*. The accumulation of SeMetSeCys detected as early as 6 h coincided with a two-fold increase in *SMT* expression (Fig. 1d). Although the *SMT* expression remained elevated, the SeMetSeCys content decreased to zero within 14 d (Fig. 3c, Table 1).
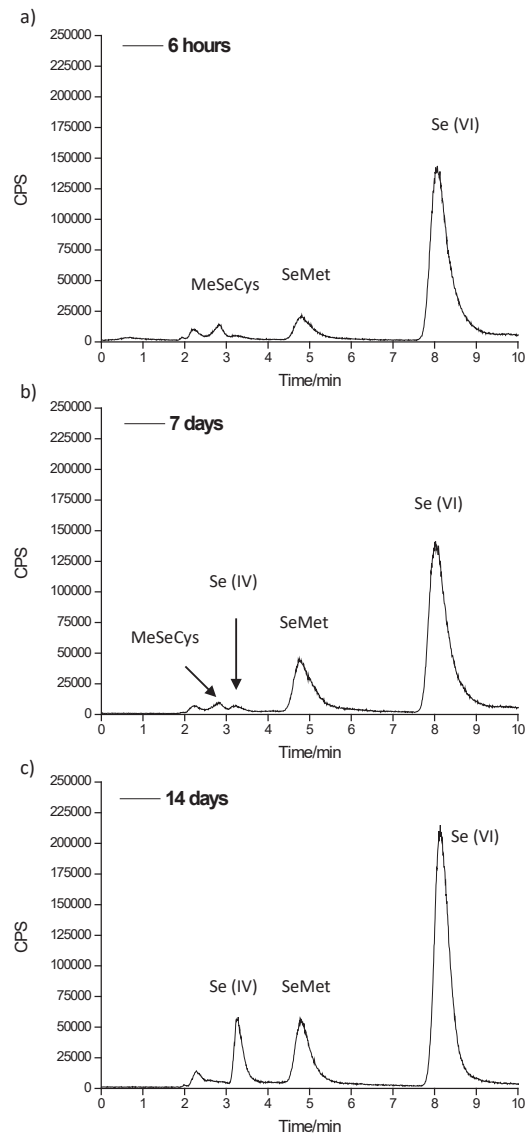

Fig. 3. Chromatographic profiles obtained by anion exchange with HPLC-ICP-MS for 80Se following enzymatic extraction of *Brassica napus* leaves at (a) 6 h, (b) 7 d and (c) 14 d after treatment with 130 μg Se plant^{-1} as Na_2SeO_4 in soil alone; n=3

Foliar spraying of SeVI or SeIV resulted in the rapid accumulation of Se in the leaves. As expected, no changes in the main Se species were detected (Figs. 4 a and b). The concentration of inorganic Se thereafter gradually became reduced as a result of the biotransformation of inorganic Se into organic forms of Se, especially into SeMet and its oxidation product selenomethionine Se oxide (SeOMet). The concentration of SeIV in the respective foliar spraying treatment was highest at 14 d after Se treatment (Fig. 4b). The main Se species in seeds and meal, however, was SeMet, and only traces of SeOMet were detected (Figs. 5a and b).

Accumulation of selenium species

Se uptake was rapid. The Se concentration in the leaves for the $Soil_{VI}/Leaf_0$ treatment only 6 h after the soil application was 18.7 $\mu g\ g^{-1}$ (Table 1). The total Se concentration in the shoots found for the $Soil_{VI}/Leaf_{IV}$ treatment

AGRICULTURAL AND FOOD SCIENCE

M.M. Seppänen et al. (2018) 27: 38–49

increased more rapidly than for the Soil$_{VI}$/Leaf$_{VI}$ treatment. Furthermore, the SeIV resulted in a more rapid bio-transformation of inorganic Se species into SeMet than did SeVI, and this occurred at a level that was higher in the Leaf$_{IV}$ treatments than for their Leaf$_{VI}$ counterparts, irrespective of the Se application rate to the soil. Further-more, the biotransformation of SeMet in the SeIV-treated plants was more rapid than in the SeVI-treated plants. The concentration of SeMet was higher in the Leaf$_{IV}$ treatment plants than in the Leaf$_{VI}$ plants, irrespective of the application rate. The SeMet concentration decreased in the plants supplied only with foliar Se additions. This re-sponse was probably caused by a dilution effect resulting from the increase in biomass, which lowered the con-centrations of Se available for SeMet biosynthesis.
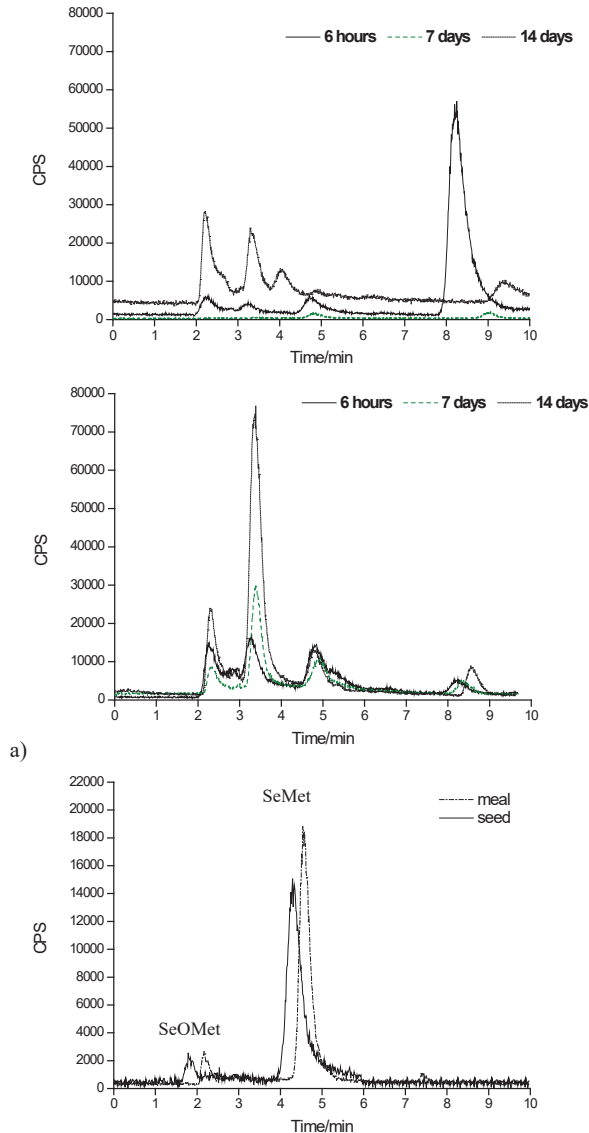

Fig. 4. Chromatographic profiles obtained by anion exchange HPLC-ICP-MS for 80Se following enzymatic extraction of the *Brassica napus* leaves at 6 h, 7 d and 14 d after foliar spray with a) 30 µg Se plant^{-1} as Na2SeO4 and b) 30 µg Se plant^{-1} as Na$_2$SeO$_3$ respectively; n = 3
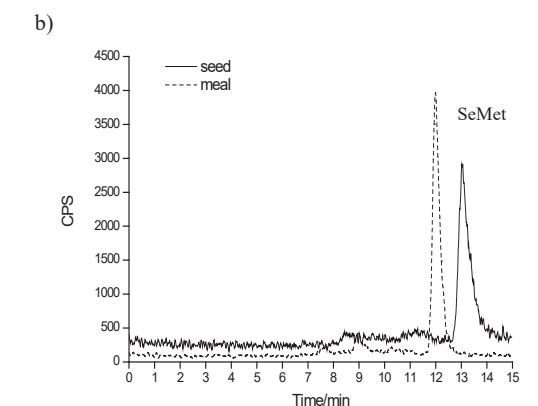
Fig. 5. Chromatographic profiles obtained by (a) anion exchange HPLC-ICP-MS and (b) size exclusion anion exchange HPLC-ICP-MS for 80Se following enzymatic extraction of *Brassica napus* meal and seeds from plants after treatment with 130 µg Se plant^{-1} as Na$_2$SeO$_4$ in soil and 30 µg Se plant^{-1}, foliar spray of Na$_2$SeO$_4$; n=3

AGRICULTURAL AND FOOD SCIENCE

M.M. Seppänen et al. (2018) 27: 38–49

The Se accumulation was generally lower in the plants that were only supplied with foliar Se, compared with those plants in which it was applied to the soil (Table 1). Foliar spraying seemed to interfere both with the uptake of inorganic Se and its conversion into SeMet. The inorganic Se and SeMet concentrations were lower in the plants that received the combined foliar and soil application than in those in which Se was added only to the soil ($Soil_{VI}$/$Leaf_0$). This was clearly seen in the SeMet concentration 7 d after the Se application (Table 1). The expression of the *APS1* homologue gene in the plants treated with foliar Se peaked earlier at 7 d than at 14 d for those *B. napa* supplied with Se to the soil (Fig. 1a). The *APS1* expression in the $Soil_{VI}$/$Leaf_{IV}$ treatment decreased radically, which may partly explain their lower SeMet concentrations (Table 1). The accumulation of SeIV in these treatments was also slower than that of SeVI and its peak concentration in the $Soil_0$/$Leaf_{IV}$ plants, which was measured after 14 d.

The chromatograms show that SeMet was the main organic Se compound (Figs. 3 and 4) and that its proportion of all Se species varied significantly between treatments and sampling times, the range being 12−71% (Table 1). The accumulation of SeMetSeCys (2−4% of the total Se) was detected in the leaves 6 h after the Se application to the soil, but its concentration diminished at the two subsequent time points. The expression of *SMT* homologue 6 h after the Se was applied to the soil was already up-regulated in the $Soil_0$/$Leaf_{VI}$ and $Soil_{VI}$ treatments (Fig. 1d), but the expression levels clearly did not correlate with the (detected) SeMetSeCys concentrations (Table 1).

Se application to the soil resulted in high accumulations of total Se and SeMet in the seeds and the dried meal (Table 2), with up to 94% of the Se in the meal being in the form of SeMet. Although the concentrations of inorganic Se species were high in the leaves, inorganic SeVI and SeIV were not detected in the seeds.

Table 2. The concentrations of total Se and different Se species ($\mu g\ g^{-1}$ DM) in *Brassica napus* seeds and meal in mature plants after treatment with 0 or 130 μg Se plant^{-1} as Na_2SeO_4 (Soil0 and SoilVI) in soil and 0 or 30 μg Se plant^{-1}, foliar spray of Na_2SeO_4 or Na_2SeO_3, (Leaf0, LeafVI and LeafIV respectively). Selenomethionine (SeMet) and inorganic SeVI were detected. The recovery (R %) was calculated by ratio of the sum of Se species to the total Se obtained by the acid digestion method. The number inside brackets is the percentage of different Se species from total Se.

Treatment	Total Se in seed/meal	SeMet	SeVI	R %
Seeds				
$Soil_0$/$Leaf_0$	0.46 [d]	0 [e] (0)	0.0 [a] (0)	0 [e]
$Soil_0$/$Leaf_{VI}$	0.29 [d]	0.17 [d] (59)	0.0 [a] (0)	59 [bc]
$Soil_0$/$Leaf_{IV}$	0.34 [d]	0.2 1[d] (62)	0.0 [a] (0)	62 [b]
$Soil_{VI}$/$Leaf_0$	5.56 [a]	2.92 [b] (53)	0.0 [a] (0)	53 [d]
$Soil_{VI}$/$Leaf_{VI}$	5.05 [b]	3.38 [a] (67)	0.0 [a] (0)	67 [a]
$Soil_{VI}$/$Leaf_{IV}$	4.41 [c]	2.40 [c] (55)	0.0 [a] (0)	55 [cd]
±SE	0.09	0.05	n.s.	0.02
Soil application	$p \le 0.00$	$p \le 0.00$	n.s.	$p \le 0.00$
Leaf application	$p \le 0.00$	$p \le 0.00$	n.s.	$p \le 0.00$
Soil*Leaf application	$p \le 0.00$	$p \le 0.00$	n.s.	$p \le 0.00$
Meal				
$Soil_0$/$Leaf_0$	0.07 [c]	0.04 [c] (66)	0.006 [a] (9)	79 [bc]
$Soil_0$/$Leaf_{VI}$	0.27 [c]	0.20 [c] (71)	0.000 [b] (0)	70 [c]
$Soil_0$/$Leaf_{IV}$	0.17 [c]	0.16 [c] (93)	0.000 [b] (0)	93 [a]
$Soil_{VI}$/$Leaf_0$	4.40 [b]	3.72 [b] (85)	0.003 [ab] (1)	84 [ab]
$Soil_{VI}$/$Leaf_{VI}$	5.34 [a]	4.63 [a] (87)	0.000 [b] (0)	86 [ab]
$Soil_{VI}$/$Leaf_{IV}$	5.23 [a]	4.74 [a] (91)	0.000 [b] (0)	90 [ab]
±SE	0.08	0.09	0.00	0.04
Soil application	$p \le 0.00$	$p \le 0.00$	$p \le 0.24$	$p \le 0.01$
Leaf application	$p \le 0.00$	$p \le 0.00$	$p \le 0.00$	$p \le 0.00$
Soil*Leaf application	$p \le 0.00$	$p \le 0.00$	$p \le 0.25$	$p \le 0.01$

Numbers followed by different letters in the same column differ significantly at the $p \le 5\%$ by Tukey's test; n=3; p value of main effects and interaction between soil and leaf application has been reported; n.s. = non-significant

AGRICULTURAL AND FOOD SCIENCE

M.M. Seppänen et al. (2018) 27: 38–49

The two-way ANOVA results showed that apart from significant interaction between the soil and leaf applications, both the main effects on total Se accumulation and the concentration of Se species in the leaves were also significant ($p \leq 0.00$) (Table 1). In the case of meal, only the main effect of the soil application on the SeVI concentration was not significant ($p \leq 0.24$), and no significant interaction between the soil and leaf applications was shown ($p \leq 0.25$) (Table 2).

Discussion

Reports on the effects of Se on plant growth and yield are somewhat contradictory. The Se added in the present experiment improved the seed set in accordance with previous reports on *B. napus* (Lyons et al. 2009, Ebrahimi et al. 2015). In field experiments, however, no increase in *Brassica* yields as a response to Se application has been reported (Lyons et al. 2009, Seppänen et al. 2010). However, some studies showed that low concentrations of Se in other plant species enhance plant growth and DM accumulation (Hartikainen and Xue 1999, Xue et al. 2001, Ebrahimi et al. 2015) or do not affect either parameter (Hartikainen et al. 1997, Djanaguiraman et al. 2010). Recently Li et al. (2018) reported that significant increase in turnip (*B. rapa*) DM was obtained with 0.5 Se mg kg^{-1} soil as SeVI or SeIV, which is higher than our soil application as SeVI. They also showed that the foliar application of SeVI and SeIV had no significant effect on plants biomass.

Se concentrations above a threshold level can reduce growth without any symptoms of toxicity (Terry et al. 2000, Lyons et al. 2005). In our experiment, transient reductions in the range of 3–28% in leaf DM accumulation were detected 14 d after the Se application coincided with a rapid Se accumulation in plant tissue and activation of its biotransformation into organic compounds. Thus, the growth retardation may have been attributable to metabolic disturbance caused by accumulated inorganic Se and its conversion into organic compounds, such as SeMet. In this experiment, SeIV was more rapidly biotransformed into selenoamino acids than reported earlier (Sors et al. 2005a) for SeVI, and it also caused a more severe reduction in the growth of *B. napus* than did SeVI.

The main form of Se that accumulated in the secondary accumulator white princesplume *Stanleya albescens* M.E. Jones was Se-cystathione (Se-Cyst), a metabolic intermediate between SeCys and SeMet (Freeman et al. 2010). The results of our *SMT* gene sequencing and Se speciation analysis showed that the Se metabolism of *B. napus* resembles that of the secondary accumulators. In our experiments, up to 4% of the Se was found as SeMetSeCys in *B. napus*, 6 h to 7 d after the Se application, similar to that in milk-vetch *Astragalus* L. species (Sors et al. 2005b). The moderate accumulation of SeMetSeCys in *B. napus* indicates that the incorporation of excess Se into nonprotein amino acids is not efficient, so the transient growth retardation indicated that the tissue Se concentration had exceeded a Se toxicity threshold value.

The predicted partial SMT amino-acid sequence of protein *B. napus* revealed homology with nonaccumulating species, including: Drummond's milk-vetch *Astragalus drummondii* Douglas ex Hook.*,* groundplum milk-vetch *Astragalus crassicarpus* Nutt. and freckled milk-vetch *Astragalus leptocarpus* Torr. & A. Gray in having a tyrosine (Tyr) residue at position 148 instead of phenylalanine (Phe) (Sors et al. 2009). Moreover, the Ala-184-Thr substitution indicates that the SMT enzyme in *B. napus* has no substrate specificity for SeCys, but does have a dual SMT/HMT (homocysteine S-methyltransferase HMT EC 2.1.1.10) function with the ability to methylate both SeCys and HoCys (homocysteine). *Astragalus bisulcatus* SMT (AbSMT) shows a high level of homology with the coding region of the HMT enzyme, but due to the presence of Ala at position 184, it appears to be specific for the methylation of SeCys and to lack the ability to methylate HoCys (Sors et al. 2009). Flowering plants have an S-methylmethionine (SMM) cycle in which SMM is synthesized from Met, converted to S-adenosylmethionine (AdoMet) and back again to Met, with the last conversion step being catalysed by HMT (Mudd and Datko 1990, Ranocha et al. 2001). The dual SMT/HMT function in the nonaccumulator species may provide an alternative pathway for the methylation of SeCys and HoCys and play a unique role in plant Se and S metabolism (Lyi et al. 2007). The dual SMT/HMT function in *B. napus* may have contributed to the low level of SeMetSeCys and the high accumulation of SeMet that was observed in this study and also in our previous study of SeMet (Seppänen et al. 2010). It should also be noted that SeCys is a substrate for SeMetSeCys synthesis and was not detected after the Se application when significant amounts of SeMet were synthesized in the leaf tissue.

A 1.5–2-fold increase in the accumulation *SMT* transcription was detected after the application of SeVI to soil in the present study. Lyi et al. (2007) showed that the expression of *Brassica oleracea SMT* (*BoSMT*) in *Brassica oleracea* responded to the applied, SeVI whereas the highly homologous *Brassica oleracea HMT* (*BoHMT*) was up-regulated by the Se status and to a lesser extent by Met, SMM, HoCys or cadmium. In rockcresses *Arabidopsis* (DC.)

AGRICULTURAL AND FOOD SCIENCE

M.M. Seppänen et al. (2018) 27: 38–49

Heynh., the *HMT2* gene was activated by SeVI (Zhang et al. 2006), which suggests a role in the detoxification of SeCys via methylation to prevent its incorporation into proteins (LeDuc et al. 2004). Methylated SeCys, however, was not detected in these plants. The accumulated organic Se species in our experiment (SeMet, SeOMet and Se-MetSeCys), were the same compounds that had accumulated in wheat *Triticum* L. during a short 24-h feeding regime with SeVI or SeIV (Li et al. 2008). No SeCys or methylated SeCys was detected in SeVI-treated *B. napus* plants, which suggests that neither the synthesis nor the methylation of SeCys is the main detoxification mechanism, nor is it plausible that these molecules are so labile that they are lost during extraction and detection by HPLC-ICP-MS.

ATPS is a key enzyme of the SO_4 assimilation pathway in plants (Leustek and Saito 1999) and can also be activated by SeVI (Renosto et al. 1993). The importance of ATPS and SMT activities for Se metabolism has been verified, using transgenic approaches (Pilon-Smits et al. 1999, LeDuc et al. 2004, LeDuc et al. 2006). Our experiment showed that the accumulation of 33 µg Se g^{-1} DM by *B. napus* in only 1 week was comparable to that of the 15 µg Se g^{-1} dry weight (DW) by *S. albescens* in the same time interval (Zhang et al. 2006). *APS1* responded most to the Se applied in our experiments. The amount and chemical form of the Se applied and the feeding time varied in these expression studies, which can in turn explain why different isoforms of the *APS* genes were activated in the various experiments (Zhang et al. 2006, Hoewyk et al. 2008, Freeman et al. 2010).

The soil SeVI was rapidly taken up and assimilated as SeMet throughout our experiment, and the high proportion of Se in SeMet increased, whereas the quantities of inorganic SeVI decreased. When either SeVI or SeIV was applied to the leaves, substantially less SeMet accumulated, while SeOMet accumulated only in the plants undergoing the foliar Se treatment. The study by Li and colleagues (Li et al. 2008) showed that a short 24-h feeding period for wheat with SeVI and SeIV resulted in low accumulation of SeMet, SeOMet and SeMetSeCys in the roots. The foliar application of SeVI and SeIV in the present study seemed to interfere with the root SeVI uptake to a similar extent as that found for the SeIV applied to the soil, which suppressed SeVI uptake by wheat roots (Zhang et al. 2006).

Up to 67% and 94% of the total Se was found as SeMet in the seeds and meal of *B. napus*, respectively, similar to our findings in a previous field experiment (Seppänen et al. 2010). In the current experiment, the Se concentrations of the seeds were 5- to 10-fold higher than those we obtained in the previous field experiment, yet the proportion of SeMet in the total Se remained high. This indicates that the synthesis of SeMet in *B. napus* is efficient and that seeds are a strong sink for SeMet. Whether SeMet in seeds is incorporated in storage proteins or exists as a free amino acid is not known.

In the Leaf$_{IV}$ and Leaf$_{VI}$ treatments, traces of SeOMet were detected in the seeds and meal. SeOMet is an oxidation product of SeMet and can be formed during sampling, storage and sample treatment. One of the main problems related to Se speciation is the lack of available compound standards, and coelution is also a problem. The proper identification of peaks requires the use of at least two chromatographic columns with different separation mechanisms, and two different chromatographic columns were indeed used in the present experiment. By these combined means, the presence of both SeOMet and SeMet was unambiguously confirmed.

We conclude that all SeVI applications to the soil activate the S assimilation pathway in *B. napus*, which rapidly takes up Se within 6 h. The intensive Se uptake causes a transient growth retardation, activation of Se detoxification and modest synthesis of nonprotein SeMetSeCys. The SMT enzyme in *B. napus* carries an Ala-184-Thr mutation, which causes dual SMT/HMT functioning of the enzyme, and this may explain the low accumulation of SeMetSeCys. The main organic Se compound that accumulated in the leaves and seeds was SeMet, whereas SeCys, the substrate for SeMetSeCys synthesis, was not detected. A transient accumulation of methylated Se compounds indicates that the volatilization of Se compounds may be activated and function as a detoxification mechanism in *B. napus*.

Acknowledgements

AGRICULTURAL AND FOOD SCIENCE

M.M. Seppänen et al. (2018) 27: 38–49

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AGRICULTURAL AND FOOD SCIENCE

M.M. Seppänen et al. (2018) 27: 38–49

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