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REGULAR ARTICLE

Agronomic biofortification of *Brassica* with selenium—enrichment of SeMet and its identification in *Brassica* seeds and meal

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Abstract Selenium (Se) is an essential micronutrient and is circulated to the food chain through crops. *Brassica* species are efficient in Se accumulation and thus, good species for Se biofortification purposes. The residual fraction obtained after oil processing of *Brassica* seeds, the meal, is an important protein source in animal diets and used in feed concentrates. The accumulation of soil or foliar applied Se in the seeds and meal of *Brassica napus* and *B. rapa* as well as its effects on growth and yield formation was studied in two field experiments. Also, a HPLC-ICP-MS based method for the identification and quantification of Se species in *Brassica* seeds and meal was developed. Selenium application did not affect the yield or oil content. High accumulation of Se in the seeds and meal (1.92–1.96 μg Se g^{-1}) was detected. Biotransformation of inorganic Se was evaluated by using HPLC-ICP-MS previous enzymatic hydrolysis for species extraction. The Se speciation studies showed that up to 85% of the total Se was SeMet whereas other Se-species were not detected. We conclude that the agronomic biofortification of *Brassica* species can improve the nutritive quality of the protein rich meal fraction as it contains significant amount of SeMet.

Keywords Biofortification · *Brassica napus* · *Brassica rapa* · Fertilization · Oil seed rape · Selenium · SeMet · Speciation · Turnip rape

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Introduction

Selenium (Se) is recognized as essential microelement for humans and animals, mainly due to its antioxidative properties and role in hormone balance (Schwartz and Foltz 1957; Flohe et al. 1973; Rotruck et al. 1973). Moreover, dietary Se concentrations that are either too low or too high can be lethal to humans and animals (Combs 2001). In plants, evidence of the beneficial effects of Se on plant growth and stress tolerance is increasing (Hartikainen 2005), but it has not been

recognized as an essential micronutrient. Selenium is mainly circulated to the food chain via crop plants and feed, the Se concentrations of which are dependent on the Se level of the soil. A sufficient content of Se in feed for livestock is 0.05–0.1 mgkg^{-1} DM whereas a daily intake of 1 mgkg^{-1} is toxic (Gissel-Nielsen 1998). In areas where the agricultural soils are low in Se, the daily intake of Se by humans is low, approximately 0.025 mgday^{-1} (Hartikainen 2005). Agronomic biofortification has successfully increased the daily Se intake to 0.08 mgday^{-1} in 2001 and elevated the blood serum Se level of Finns from the lowest in Europe (0.63–0.76 μmol L^{-1}) to an adequate level (1.2–1.4 μmol L^{-1}) (Hartikainen 2005).

The additional health benefits of Se, such as improved immune system and reduced cancer risk, require higher than the currently recommended daily intakes (Rayman 2008). To reduce the incidences of lung, prostate and colo-rectal cancers in humans, a high Se concentration (200 μgday^{-1}) in diet is required (Combs 2001). Selenium can be supplied to the food chain in either inorganic or organic forms. The organic forms of Se are safer, their LD_{50} values to rats being approximately 40 mgkg^{-1}, whereas that of inorganic sodium selenate is close to 15 mgkg^{-1} (Rayman 2004). The retention of organic Se species in tissue is also better, and it is thought that stored tissue Se can act as a reservoir, especially for production animals (Rayman 2008). Selenium enriched yeast (SY) is a feed additive that contains approximately 70% of organic Se in the form of selenomethionine (SeMet) (Rayman 2004). Selenium enriched yeast has been shown to increase the total Se concentration and blood Se status of production animals more efficiently than sodium selenite and, consequently to elevate the Se content of meat, milk and cheese (Juniper et al. 2008, 2009; Phipps et al. 2008). Applied SY does not improve animal productivity, *e.g.* growth (Haug et al. 2008) or milk production (Phipps et al. 2008), but it exerts a beneficial effect on the food chain since meat and dairy products are the main source of Se in human diets (Hartikainen 2005).

Selenium circulated to the food chain via biofortification of crops is mainly in the form of organic SeMet. The ability of plants to accumulate Se and metabolize it into organic Se compounds is dependent on soil available Se and sulphur (S) and varies among plant species. Plants can be divided to Se non-accumulators, Se-indicators or Se-accumulators (White et al. 2004 and references therein). Typically plants contain less than 25 μg Se g^{-1} DM, but the Se-indicators can accumulate up to 1,000 μg Se g^{-1} DM and the Se-accumulators (*e.g.* few species of *Astragalus*) up to 20–40 mg Se g^{-1} DM. According to current knowledge, the same transporters and enzymes active in sulphur uptake and metabolism are also responsible for Se metabolism (Pilon-Smits et al. 2009) The Se hyperaccumulating plants may however have specific transporters for Se uptake (Pilon-Smits et al. 2009). *Brassica* species favour sulphur, and the ability of *B. napus* to accumulate Se as well has been utilized for the phytoremediation of Se-rich soils. The harvested leaf material containing up to 3.5 mg Se kg^{-1} DM is then used as a dietary Se supplement for sheep and cattle (Bañuelos and Mayland 2000). The Se accumulation in the seeds of *Brassica* species has been studied less. Lyons et al. (2009) reported that although the aerial parts of *B. rapa* contained 250 μg Se kg^{-1} DW, the concentration in seeds was significantly lower, 150 μg Se kg^{-1} DW.

To establish Se metabolism in plants, it is necessary to carry out speciation studies. Species extraction is the first stage of the process and the methods should prevent species interconversion. In the case of Se, enzymatic hydrolysis by using non-specific proteases such as Pronase E is the method of choice, because it offers the advantages of mild conditions and selectivity (Moreno et al. 2001). However, the methodology is extremely time-consuming and an alternative method for reducing sample treatment time is the combination of ultrasonic energy combined with enzymatic hydrolysis, the so-called enzymatic probe sonication (EPS) (Capelo et al. 2004). The use of this combination allows quantitative extraction of Se species in a short time. The resulting extract is further analyzed for species separation, detection and quantification, mainly by using the coupling HPLC-ICP-MS (Pederero and Madrid 2009). One of the main challenges related to Se speciation and the identification of individual Se-species is the lack of available Se compounds as standards. The loosely retained Se compounds can also be easily co-eluted from chromatographic column, leading to erroneous identification (Pederero and Madrid 2009). Thus, the establishment of a the protocol to circumvent these challenges is of a great importance for correct and unambiguous Se species identification in plants.

The aim of this study was to investigate the potential of *B. rapa and B. napus* to assimilate and biotransform Se and to accumulate organic Se species in their seeds. Special focus was on the protein fraction of seeds, the oil-free meal which is an important protein source in ruminant diets. A further aim was to identify the main Se species accumulating in *Brassica* seeds and meal and the nutritional value of Se enriched *Brassica* meal as a source of organic Se compounds in ruminant diet is discussed.

Materials and methods

The field experiments were carried out at Viikki Experimental Farm, University of Helsinki, Finland (EXP1) and at Kotkaniemi Experimental Farm, Yara-Suomi Ltd., Finland (EXP2). *B. rapa* (Hilight) and *B. napus* (4021B) were sown in separate experiments in a completely randomized block design with four replications. All 8 m^2 plots were fertilized at the time of sowing with 100 $kgNha^{-1}$ and three application levels of Se, 0, 5.6 and 20 g Se ha^{-1} as Na_2SeO_4. The composition of fertilizers were; N27-P0-K0-Mg4-Se0, N27-P0-K0-Mg1-S4-B0.02-Se0.0015 and N26-P0-K1-S3-Mg1-B0.02-Se0.005. A foliar spray of Na_2SeO_4 or Na_2SeO_3 of 0 and 30 g Se ha^{-1} was applied a month after the sowing when the plants were at rosette stage. Before sowing, the field was treated with the pre-emergent herbicide Super Treflan (Dow AgroSciences) and pesticide spray (Decis 25EC, Bayer CropScience) was applied as required. The soil was characterized as silty clay soil with pH 6.2 (EXP1) and as medium clay soil with pH 6.3–6.4 and Se <0.01 mg L^{-1} (EXP2).

During the growing period the following observations were made; density of seedlings (BBCH 12), vitality of plants (BBCH 60) and the length of siliques (BBCH 63). At the end of the growing season, ten plants from each plot were collected for the analysis of plant height and major yield components describing the canopy structure: number of side branches and the number of siliques in main branch and side branches. Plots were harvested at maturity by threshing machine (Massey Ferguson), and dried at 35°C to 9% moisture content. The 1,000 seed weight (g) was determined and the oil content was measured using NMS110 minispec NMR analyzer (Bruker) using 20 g of seeds. Five grams of seeds were used for the extraction of oil and separation of the meal fraction. The seeds were shaken and ground for 40 min in metal tubes containing 30 mL of petroleum ether (Sigma-Aldrich) and three metal balls. The liquid fraction containing oil was separated by filtration and the meal fraction was dried at $^{+}$80°C for 20 min.

Analysis of total selenium concentration

The Se concentration of the seed meal (Table 3, EXP1 and EXP2) was determined by the electrothermal atomic absorption spectrophotometric method (Kumpulainen et al. 1983). An in-house reference sample was included for every analytical round. For Se determination of seeds and meal (Table 5), ICP-MS was used. Samples of approximately 0.2 g were digested in a microwave oven by using 2.5 mL of HNO_3 (65%) and 0.5 mL H_2O_2 (35%). The solutions obtained were diluted with ultrapure water up to 25 mL. The operating conditions for ICP-MS are given in Table 1.

Separation, identification and quantification of Se species

Selenium speciation in different samples was analysed by HPLC-ICP-MS after extraction of Se species with enzymatic hydrolysis. Enzymatic hydrolysis was performed by using two methods: incubation in a controlled temperature incubator and ultrasonic probe. In controlled temperature incubation, about 0.05 g of sample were weighed into 5 mL tubes with 20 mg of enzyme (Protease type XIV, Sigma-Aldrich, Steinheim, Germany) and 3 mL of 30 mM Tris solution (pH 7.5) (Fluka). The tubes were incubated in a gravity convection oven (Heraeus D-6450 Hanau, USA) for 24 h at 37°C. In the ultrasonic probe method a 0.05 g portion of sample and 3 mL Milli-Q water were placed in a tube with 20 mg of Protease XIV. The samples were sonicated for 2 min at 40% ultrasound amplitude in a Sonoplus ultrasonic homogenizer (Bandenlin, Germany) equipped with a titanium 3 mm diameter microtip and fitted with a high-frequency generator of 2,200 W at frequency of 20 KHz. After proteolysis, the samples were centrifuged at 11,000 rpm for 15 min (Eppendorf Centrifuge 5804 R F34-6-38, Hamburg, Germany) using 10 KDa cut-off filters (Millipore, USA). The aqueous extracts were filtered through a 0.22 μm filter.

Table 1 Instrumental operating conditions for LC and ICP-MS systems

ICP-MS parameters	
Forward power	1,250 W
Plasma gas flow rate	15 Lmin^{-1}
Auxiliary gas flow rate	0.73 Lmin^{-1}
Nebulizer gas flow rate	0.83 Lmin^{-1}
Collision gas	93% He, 7%H
Collision gas flow rate	8.8 mLmin^{-1}
Nebulizer	Meinhard
Spray chamber	Impact Bead Quartz Spray
Isotope monitored	^{80}Se, ^{78}Se, ^{76}Se and ^{74}Se
Dwell time per point	200 ms
Replicates	3
LC parameters	
Anion exchange	
Column	Hamilton PRP-X100 (250×4.1 mm, 10 μm)
Mobile phase	0.2% citric acid, 2% methanol (pH 5)
Injection volume	100 μL
Flow rate	1 mLmin^{-1}
Column temperature	25°C
Elution mode	Isocratic
Size exclusion anion exchange	
Column	Shodex Asahipak GS-220HQ (300×7.6 mm, 2 μm)
Mobile phase	25 mM ammonium acetate (pH 6.7)
Injection volume	100 μL
Flow rate	0.7 mLmin^{-1}
Column temperature	25°C
Elution mode	Isocratic

The resulting extracts were analysed by HPLC-ICP-MS. The HPLC consisted of a PU-2089 HPLC pump (JASCO, Tokio, Japan) fitted with a six-port injection valve (model 7725i, Rheodyne, Rohner Park, CA, USA) with a 100-μL injection loop. In order to get unambiguous identification of the species, the extracts from enzymatic hydrolysis were run through two different chromatographic columns, a Hamilton PRP-X100 (250×4.1 mm, 10 μm) column and a Shodex Asahipak GS-220HQ (300×7.6 mm, 2 μm) column, with different retention mechanisms: anion exchange and a combination of size exclusion and anion exchange, Analyses were performed by ICP-MS in time resolved analysis mode and the operating conditions of LC-ICP-MS are summarized in Table 1.

Standard stock solutions of 1,000 mg L^{-1} of selenomethionine (SeMet), selenomethylselenocysteine (SeMetSeCys) and selenocystine ($SeCys_2$) (Sigma) were prepared in ultra-pure water (18.2 MΩ cm^{-1}) from a Milli-Q water purification system (Millipore, MA, USA), and 3% hydrochloric acid was added for better dissolution of $SeCys_2$ and SeMetSeCys. Inorganic selenium solutions were prepared by dissolving sodium selenite (Na_2SeO_3) and selenate (Na_2SeO_4) (Merck) in Milli-Q water. Stock solutions were stored at 4°C, whereas working standard solutions were prepared daily by dilution.

Data analysis

The data was tested using analysis of variance (ANOVA) in the GLM procedure of SAS version 9.1 (SAS Institute Inc., Cary, NC). Significantly different means between treatments were separated with Tukey HSD tests.

Results

Yield parameters and selenium content of *Brassica* meal

Both of the field experiments (EXP1 and EXP2) were affected by the spring drought, but the densities of the plant canopies were dissimilar at the two locations. EXP1 suffered from spring drought more severely and the canopy was thin (73 to 86 plants m^{-2}), whereas in EXP2 the canopy was dense (113 to 201 plants m^{-2}) (Table 2). The plants showed plasticity in the formation of yield components; at low density the plants had more side branches and siliques per side branch and yield reduction was not observed (Table 3). In the dense canopy of *B. rapa* in EXP2, the Se supplementation to soil (6 g Se ha^{-1}) followed with foliar application of Na_2SeO_4 was associated with a statistically significant increase in the number of siliques in the main branch (Table 2). A similar trend, although not significant, was seen also in EXP1. In *B. napus* no effect on yield components was observed. The increased number of siliques in main branch in the Se-treated *B. rapa* was not associated with a decrease in the seed size (Table 3). As the number of seeds per silique was not calculated, the influence of Se application on seed formation could not be assessed.

Table 2 Canopy density (number of plants m^{-2} $^{-1}$), plant height (cm), number of siliques in main and side branches, and number of side branches in *B. rapa* and *B. napus* plants supplemented with Na_2SeO_4 in soil Se0, Se6 and Se20 (0. 6 or 20 g Se ha^{-1}) and leaves as Na_2SeO_4 (Se^{6+}) or Na_2SeO_3 (Se^{4+}) (30 g Se ha^{-1}) in two experimental locations (EXP1, EXP2)

	B. rapa					*B. napus*				
	Density	Height	Siliques in main branch	Side branch	Siliques in side branch	Density	Height	Siliques in main branch	Side branch	Siliques in side branch
EXP 1										
Se0	82[a]	128[a]	34.5[a]	4.6[a]	81.4[a]	83[a]	114[a]	23.2[a]	3.7[a]	59.4[a]
Se0+Se^{6+}	85[a]	129[a]	36.0[a]	4.9[a]	93.1[a]	73[a]	108[a]	23.1[a]	3.7[a]	49.2[a]
Se0+Se^{4+}	75[a]	133[a]	36.7[a]	5.4[a]	98.4[a]	75[a]	109[a]	22.4[a]	3.8[a]	61.7[a]
Se6	86[a]	125[a]	33.3[a]	5.4[a]	86.3[a]	80[a]	109[a]	22.9[a]	3.2[a]	39.0[a]
Se6+Se^{6+}	73[a]	132[a]	36.2[a]	5.3[a]	111.5[a]	76[a]	105[a]	22.4[a]	2.7[a]	32.1[a]
Se6+Se^{4+}	78[a]	127[a]	32.4[a]	4.5[a]	76.4[a]	77[a]	103[a]	24.6[a]	3.5[a]	52.1[a]
Se20	78[a]	130[a]	34.6[a]	4.8[a]	87.4[a]	76[a]	107[a]	23.2[a]	3.9[a]	40.4[a]
Se20+Se^{6+}	82[a]	133[a]	38.1[a]	5.0[a]	102.3[a]	75[a]	109[a]	24.7[a]	3.7[a]	44.8[a]
Se20+Se^{4+}	75[a]	127[a]	33.7[a]	4.8[a]	93.9[a]	82[a]	108[a]	26.2[a]	3.4[a]	46.4[a]
EXP 2										
Se0	146[a]	114[a]	18.9[b]	2.7[a]	37.3[a]	161[a]	99[a]	21.5[a]	2.3[a]	32.9[a]
Se0+Se^{6+}	160[a]	115[a]	22.7[ab]	2.7[a]	33.5[a]	201[a]	101[a]	22.3[a]	2.2[a]	34.8[a]
Se0+Se^{4+}	168[a]	108[a]	23.8[ab]	2.7[a]	46.8[a]	190[a]	100[a]	22.8	2.2[a]	33.8[a]
Se6	137[a]	107[a]	26.9[ab]	3.3[a]	63.2[a]	175[a]	105[a]	19.2[a]	2.1[a]	26.9[a]
Se6+Se^{6+}	113[a]	112[a]	27.7[a]	3.0[a]	47.5[a]	179[a]	108[a]	21.6[a]	2.1[a]	26.9[a]
Se6+Se^{4+}	145[a]	115[a]	24.8[ab]	2.9[a]	45.3[a]	190[a]	103[a]	20.3[a]	2.1[a]	22.6[a]
Se20	157[a]	113[a]	23.3[ab]	2.6[a]	37.5[a]	185[a]	101[a]	20.8[a]	2.1[a]	27.2[a]
Se20+Se^{6+}	148[a]	116[a]	26.3[ab]	3.0[a]	47.2[a]	181[a]	102[a]	20.4[a]	2.3[a]	31.0[a]
Se20+Se^{4+}	123[a]	113[a]	24.5[ab]	3.1[a]	47.4[a]	175[a]	102[a]	22.2[a]	2.3[a]	32.5[a]

Means (n=4) within a column followed by a different letter differ significantly ($p<0.05$, Tukey HSD). Experiments and columns are tested separately

The Se application did not affect seed yield or oil content of the seeds (Table 3). The level of Na_2SeO_4 applied to soil was reflected in the Se concentration in the protein rich meal fraction. The foliar Se spray of Na_2SeO_4 solution alone was sufficient to increase the Se concentration of the meal from 0.05 μg g^{-1} to 0.54–1.00 μg g^{-1}, and the low and the high Se soil application increased it further to 0.88–1.15 μg g^{-1} and 1.48–1.84 μg g^{-1}, respectively. Foliar Na_2SeO_3 solution spray had a minor effect on Se content in the meal. At any given dosage, the Se concentration of the meal was slightly higher in *B. rapa* than in *B.napus*, especially in EXP1. At the time of foliar spray, the *B. rapa* plants had reached rosette stage at developmental stage BBCH 33, whereas *B.napus* was still at BBCH 30. As the target Se concentration of *Brassica* meal for feed industry can be set to 1.0 μg Se g^{-1}, the level can be obtained in both locations and species by supplementing the soil Na_2SeO_4 application (6 g Se ha^{-1}) with foliar spray of Na_2SeO_4 at the rosette stage. The total recovery (grain and straw) of applied Se was measured for the plots supplemented Se in soil. The *Brassica* seed yield in these experiments was 2,000 to 3,200 kg ha^{-1} and the recovery of Se 5.5–6.0% (6 or 20 g Se ha^{-1} in soil).

Selenium speciation studies

Similar Se concentration values were obtained from both proteolytic and acid digestion, suggesting that enzymatic hydrolysis was capable of extracting the Se compounds from the samples using either the ultrasonic probe or incubation bath (Table 4). Enzymatic hydrolysis with ultrasonic probe sonication was selected for further studies since it drasti-

Table 3 Yield (kg ha^{-1}), 1,000 seed weight (sw, g), oil content (%) and Se concentration of *Brassica* meal (μg g^{-1} DW) of plants supplemented with Na_2SeO_4 in soil Se0, Se6 and Se20 (0.6 or 20 g Se ha^{-1}) and leaves as Na_2SeO_4 (Se^{6+}) or Na_2SeO_3 (Se^{4+}) (30 g Se ha^{-1}) in two experimental locations (EXP1. EXP2)

	B.rapa				*B.napus*			
	yield	1,000 sw	oil	Se	yield	1,000 sw	oil	Se
EXP 1								
Se0	3,188^{a}	n.d.	39.1^{a}	0.05^{f}	2,473^{a}	n.d.	40.3^{a}	0.05^{e}
Se0+Se^{6+}	3,033^{a}	n.d.	39.0^{a}	1.00^{bc}	2,441^{a}	n.d.	40.2^{a}	0.94^{b}
Se0+Se^{4+}	3,009^{a}	n.d.	39.2^{a}	0.23^{ef}	2,508^{a}	n.d.	39.6^{a}	0.18^{e}
Se6	3,184^{a}	n.d.	39.0^{a}	0.23^{ef}	2,499^{a}	n.d.	40.1^{a}	0.24^{de}
Se6+Se^{6+}	3,087^{a}	n.d.	39.0^{a}	1.15^{b}	2,486^{a}	n.d.	40.3^{a}	1.07^{ab}
Se6+Se^{4+}	3,170^{a}	n.d.	39.0^{a}	0.43^{def}	2,487	n.d.	40.2^{a}	0.38^{cde}
Se20	3,109^{a}	n.d.	39.0^{a}	0.60^{cde}	2,475^{a}	n.d.	40.3^{a}	0.68^{bcd}
Se20+Se^{6+}	3,103^{a}	n.d.	39.0^{a}	1.84^{a}	2,289^{a}	n.d.	40.0^{a}	1.48^{a}
Se20+Se^{4+}	3,206^{a}	n.d.	39.0^{a}	0.79^{bcd}	2,418^{a}	n.d.	40.4^{a}	0.75^{bc}
EXP2								
Se0	2,179^{a}	2.4^{a}	45.0^{a}	0.05^{e}	2,222^{a}	2.9^{a}	43.9^{a}	0.05^{e}
Se0+Se^{6+}	2,061^{a}	2.5^{a}	44.5^{a}	0.95^{b}	2,173^{a}	2.9^{a}	43.6^{a}	0.54^{c}
Se0+Se^{4+}	2,060^{a}	2.4^{a}	44.7^{a}	0.31^{de}	2,306^{a}	2.9^{a}	43.0^{a}	0.12^{e}
Se6	1,862^{a}	2.4^{a}	44.5^{a}	0.25^{de}	2,330^{a}	3.0^{a}	43.7^{a}	0.23^{de}
Se6+Se^{6+}	2,075^{a}	2.5^{a}	44.8^{a}	0.88^{b}	2,233^{a}	3.0^{a}	44.2^{a}	0.89^{b}
Se6+Se^{4+}	2,018^{a}	2.4^{a}	44.6^{a}	0.50^{cd}	2,351^{a}	2.8^{a}	44.1^{a}	0.35^{cd}
Se20	2,173^{a}	2.4^{a}	44.6^{a}	0.76^{bc}	2,336^{a}	2.9^{a}	43.3^{a}	0.80^{b}
Se20+Se^{6+}	2,040^{a}	2.4^{a}	44.3^{a}	1.59^{a}	2,293^{a}	3.0^{a}	44.2^{a}	1.55^{a}
Se20+Se^{4+}	2,145^{a}	2.4^{a}	44.7^{a}	1.05^{b}	2,151^{a}	2.9^{a}	44.2^{a}	0.85^{b}

Means (n=4) within a column followed by a different letter differ significantly ($p<0.05$, Tukey HSD)

Experiments and columns are tested separately. N.d. not determined

cally decreased the sample treatment time from 12 h to 2 min.

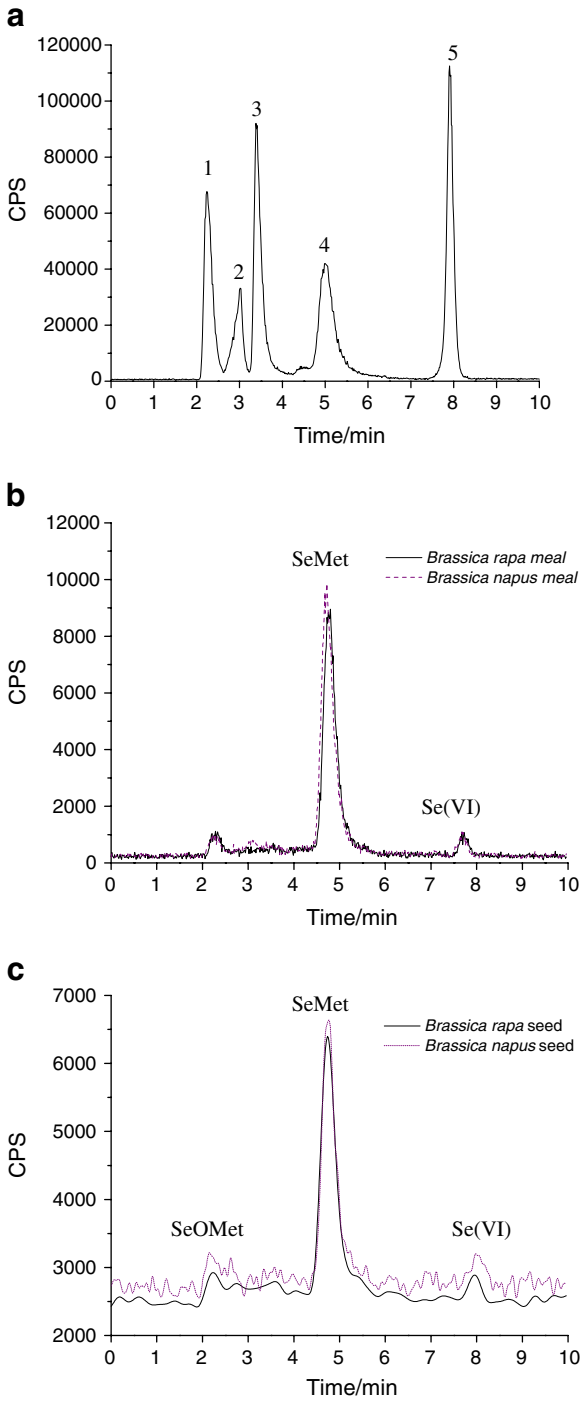
Figure 1a and b show the chromatographic profiles of Se standards and the enzymatically hydrolyzed meal samples by anion exchange LC-ICP-MS, respectively.

Table 4 Total selenium concentration following different sample preparation methods in *B. rapa* and *B. napus* seed meal from plants supplemented with 6 g Se ha^{-1} as Na_2SeO_4 in soil and 30 g Se ha^{-1} as foliar spray Na_2SeO_4. Means (n=3) ± Stdev

Meal samples	Acid digestion (μg Se g^{-1})	Enzymatic hydrolysis (μg Se g^{-1})	
		Ultrasonic probe	Gravity convection oven
B. rapa	2.24±0.20	2.40±0.07	2.24±0.06
B. napus	2.40±0.08	2.32±0.30	2.15±0.12

SeMet was identified by comparing the retention times of standards and by spiking experiments. Other minor species were found in *Brassica* meal by anion exchange LC-ICP-MS, such as Se (VI) (Fig. 1b). A second chromatographic column separation combining anion exchange and size exclusion mechanisms detected a peak corresponding to SeMet (Fig. 2a) in the enzymatically hydrolyzed meal samples (Fig. 2b). These results corroborate the data from the anion-exchange column. The main selenocompound in all meal samples tested was SeMet, regardless of Se treatment applied to the plants. Similarly in the seeds, the main accumulating Se compound was SeMet for all treatments (Figs. 1c and 2c).

The amounts of SeMet and SeO_4^{2-} found after each treatment along with mass balance are presented in Table 5. Up to 80% of the total Se was assimilated to SeMet and only traces of inorganic SeO_4^{2-} were



◀ **Fig. 1** Cromatographic profile obtained by anion exchange LC-ICP-MS for ^{80}Se corresponding to **a** Mixture of Se standards containing 50 μg L^{-1} of each Se species standards 1. $SeCys_2$, 2. SeMeSeCys, 3. Se (IV), 4. SeMet, 5. Se (VI). **b** Enzymatic extraction of *B. napus* and *B. rapa* meal in plants supplemented with 6 g Se ha^{-1}as Na_2SeO_4 in soil and 30 g Se ha^{-1} as foliar spray **c** Enzymatic extraction of *B. napus and B rapa* seeds in plants supplemented with 6 g Se ha^{-1} as Na_2SeO_4 in soil and 30 g Se ha^{-1} as foliar spray

detected. The Se application method and the Se species added in the foliar spray did not affect the percentage of SeMet in the total Se. Thus, the high total Se concentration resulted also in a high SeMet concentration in both the seeds and the meal. In the plants given additional foliar Na_2SeO_4, the accumulation of SeMet ranged from 0.861 to 1.365 μg g^{-1}. The foliar application of Na_2SeO_4 efficiently increased SeMet concentration in the meal, whereas that of Na_2SeO_3 had only a small effect. When the total Se fertilization increased from 6 to 20 g Se ha^{-1}, the SeMet content rose from 0.199 to 0.567 μg g^{-1}. Thus, the foliar application of Na_2SeO_4 alone was more efficient resulting in 0.861 μg SeMet g^{-1}. The two *Brassica* species did not differ in their efficiency in assimilating the applied Se to SeMet.

The variation in the recovery of Se (%) was larger while entire seeds (55–102%) than in the meal (66–82%) samples, which challenges the interpretation of the localization of Se within the seed. On average, the seeds consist of 40% oil, and the remainder is mainly in the forms of protein, carbohydrates and seed coat fraction which is called the meal. The results show that most of the Se accumulated in the seed was in the meal components.

Discussion

There is increasing evidence that Se can have beneficial effects on the growth, yield formation and stress tolerance of plants (Hartikainen 2005). The physiological, biochemical or molecular mechanisms behind the stimulated growth and improved tolerance have not yet been determined. Nevertheless, enhanced antioxidant capacity (reviewed in Hartikainen 2005) and more efficient accumulation of carbohydrates (Turakainen et al. 2004) are thought to be contributing factors in the better performance of the plants. In greenhouse experiments (Lyons et al. 2009), where the cross-contamination of control plants by volatile Se compounds was carefully restricted, Se increased the seed production of *Brassica rapa* and there were positive effects of Se on the germination of *Brassica* seeds and pollen viability of the flowers. In the present study, no yield improvement or changes in the

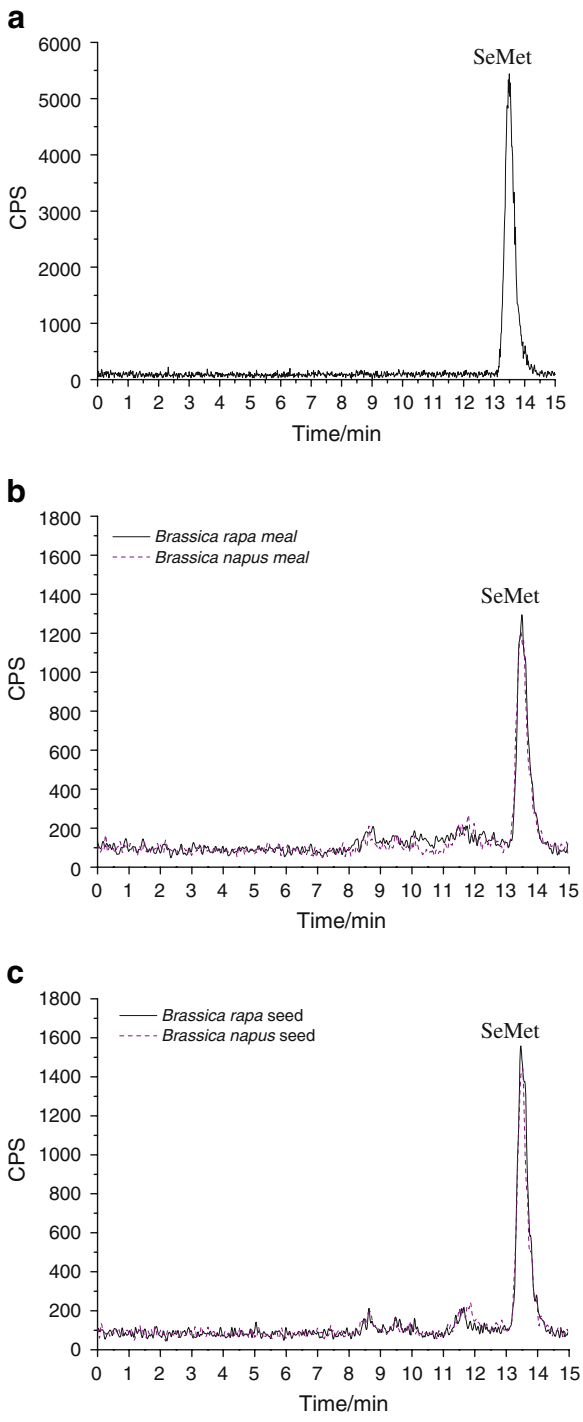


Fig. 2 Chromatographic profile obtained by size exclusion anion exchange LC-ICP-MS for ^{80}Se corresponding to **a** SeMet standard, 50 μg g^{-1}; **b** Enzymatic extraction of *B. napus* and *B. rapa* meal in plants supplemented with 6 g Se ha^{-1} as Na_2SeO_4 in soil and 30 g Se ha^{-1} as foliar spray **c** Enzymatic extraction of *B. napus and B rapa* seeds in plants supplemented with 6 g Se ha^{-1} as Na_2SeO_4 in soil and 30 g Se ha^{-1} as foliar spray

oil concentration were attributed to the Se treatment of either *B. rapa* or *B. napus* plants. However, the plant morphology was affected. In the Se–treated *B. rapa*, more siliques tended to be formed in the main branch rather than in side branches. This indicates that the availability or distribution of carbohydrates within the plant could be altered. Supporting observations were obtained in a greenhouse experiment in *B. rapa* (data not shown). Lyons et al. (2009) reported on positive responses of seed yield to Se in greenhouse experiments but not in the field. The discrepancy may have been due to trace amounts of Se in the field soil. In our study, the field experiment was conducted in soils that have been fertilized with Se in previous years, and trace amounts of Se (0.05 μg Se g^{-1}) accumulated in seeds and meal of control plants. As compared to the greenhouse experiment, where yield benefits were detected, the Se concentration of the seeds was only 0.004 μg Se g^{-1} in the control plants (Lyons et al. 2009). Thus, the trace amounts of Se in soil may have been sufficient for the yield formation also in the present field experiment.

Both *Brassica* species accumulated high amounts of Se in the protein fraction (meal) of the seed. The total recovery of applied Se to seeds and straw was approx. 6% which is lower than the 20–35% of Se recovery reported for wheat by Broadley et al. (2010). The substantially lower seed yield of *Brassica* (2 to 3 tha^{-1}) compared to winter wheat (6 to 10 tha^{-1}) most probably explains these differences in Se recovery. Sprayed Na_2SeO_4 and Na_2SeO_3 were both substantially metabolised into SeMet, and up to 85% of Se was in the form of SeMet. The dominance of SeMet as the main Se species in both meal and seeds implies that processing of *Brassica* seed to defatted meal does not alter the SeMet originally present in seeds. The percentage of total Se as SeMet was the same as that in the selenised yeast used in feed additives (see *e.g.* Rayman 2004). The SeMet content in meal increased to 0.86 μg g^{-1} when Na_2SeO_4 was applied as foliar spray and the soil application of 6 or 20 mg Se ha^{-1} elevated the level further to 0.98 and 1.37 μg g^{-1}, respectively. Without Se application, only traces of SeMet were detected in the meal. In feeding experiments with SY, where positive effects on blood GSH-Px activity have been reported, the Se content of the concentrate blends were 0.98–1.98 mg Se kg^{-1} DM, resulting 0.30–0.51 mg kg^{-1} in the mixed feed (Juniper et al.

Table 5 Concentration of SeMet (μg g^{-1}), inorganic selenate (μg g^{-1}), total Se (μg g^{-1}) and ratio of SeMet to total Se (%) in the meal and seeds of plants supplemented with Na_2SeO_4 in soil Se0, Se6 and Se20 (0. 6 or 20 g Se ha^{-1}) and leaves as Na_2SeO_4 (Se^{6+}) or Na_2SeO_3 (Se^{4+}) (30 g Se ha^{-1}) in EXP1

	B.rapa					*B.napus*				
	SeMet	Se^{6+}	Total Se	SeMet/ Se	R (%)	SeMet	Se^{6+}	Total Se	SeMet/ Se	R (%)
Meal										
Se0	0.033±0.002	0.003±0.000	0.046±0.005	72	79±5	0.047±0.003	0.003±0.000	0.060±0.010	78	84±5
Se0+Se^{6+}	0.861±0.052	0.055±0.003	1.137±0.050	76	81±5	0.793±0.048	0.026±0.002	1.235±0.075	64	66±4
Se0+Se^{4+}	0.237±0.015	–	0.327±0.025	72	72±5	0.192±0.008	–	0.250±0.020	77	77±3
Se6	0.199±0.011	–	0.268±0.019	74	74±4	0.199±0.018	–	0.245±0.034	81	81±7
Se6+Se^{6+}	0.988±0.014	–	1.445±0.080	68	68±1	0.951±0.023	–	1.345±0.095	71	71±2
Se6+Se^{4+}	0.388±0.016	0.057±0.002	0.571±0.020	68	78±3	0.301±0.019	0.028±0.002	0.428±0.040	70	77±5
Se20	0.567±0.028	0.038±0.002	0.779±0.050	73	78±4	0.581±0.023	0.032±0.001	0.817±0.070	71	75±3
Se20+Se^{6+}	1.365±0.038	–	1.958±0.090	70	70±2	1.198±0.024	–	1.454±0.090	82	82±2
Se20+Se^{4+}	0.749±0.021	–	0.932±0.070	80	80±2	0.783±0.047	0.004±0.000	1.042±0.085	75	75±5
Seed										
Se0	0.141±0.020	–	0.170±0.024	83	83±12	0.042±0.003	–	0.076±0.006	55	55±4
Se0+Se^{6+}	1.005±0.023	–	1.521±0.035	66	66±1	0.427±0.017	–	0.659±0.021	65	65±3
Se0+Se^{4+}	0.226±0.018	–	0.410±0.032	55	55±4	0.118±0.008	–	0.171±0.012	69	69±5
Se6	0.380±0.008	0.042±0.002	0.433±0.009	88	97±2	0.120±0.004	–	0.169±0.005	71	71±2
Se6+Se^{6+}	1.612±0.096	0.057±0.005	1.586±0.095	100	105±7	0.525±0.026	–	0.657±0.033	80	80±4
Se6+Se^{4+}	0.565±0.032	–	0.739±0.042	76	76±4	0.254±0.013	–	0.391±0.018	65	65±3
Se20	0.864±0.026	–	1.149±0.036	75	75±2	0.314±0.018	–	0.474±0.030	66	66±4
Se20+Se^{6+}	1.885±0.050	–	1.921±0.052	98	98±3	1.067±0.056	–	1.095±0.060	97	97±5
Se20+Se^{4+}	0.814±0.047	–	0.890±0.050	91	91±5	0.459±0.027	–	0.452±0.024	100	102±6

[a] Average value ± standard deviation (n=3)

[b] Recoveries calculated by comparing the sum of species with values of total Se obtained by acid digestion

2008). The Se-enriched *Brassica* meal can be used up to 50% as protein source in feed concentrates and it can significantly increase the amount of SeMet in ruminant diet.

Se accumulation was greater when Na_2SeO_4 rather than Na_2SeO_3 was used in foliar application. This finding agrees with previous studies with Indian mustard (*B. juncea*) where Se-enrichment with Na_2SeO_4 proved to be more efficient than that with Na_2SeO_3 (Montes-Bayón et al. 2002). In general, *Brassica* species can accumulate high concentrations of Se, and canola (*B. napus*) leaf material can contain as much as 3.5 mg Se kg^{-1} (Bañuelos and Mayland 2000). The Se tolerance in plants is thought to be related to the synthesis of non-structural selenoamino acids, allowing them to accumulate high amounts of Se without symptoms of toxicity (Ximenéz-Embúm et al. 2004). The accumulated SeMet can be incorporated into proteins, associated to biomolecules (Rúiz Encinar et al. 2003), or trapped in the cell walls (Polatajko et al. 2004), which are also the main components of the *Brassica* meal.

In the present study, methodology based on the use of HPLC-ICP-MS was optimized for seed and meal samples. The use of enzymatic probe sonication allowed quantitative extraction of Se species in a short period of time (2 min). No differences were observed in the chromatographic profiles when comparing the enzymatic hydrolysis using either control temperature incubator or ultrasonic probe (data not shown) showing that the use of the ultrasonic probe not only allowed quantitative extraction of species but also prevented species transformation. The action of ultrasound enhances the cleavage of proteins and complex structures (Capelo et al. 2004) and it has been successfully applied for samples

as diverse as vegetables (Pedrero et al. 2008), meat (Cabañero et al. 2005) and selenized yeast (Capelo et al. 2004). Despite the good results obtained previously, the methodology should be validated for each particular sample. Previous studies have shown that the main organic Se compound in the leaves, flower structures and roots of Se-enriched broccoli (*B. oleracea*) was selenomethylselenocysteine (SeMeSeCys) and not SeMet (Pedrero et al. 2008). Similarly, SeMeSeCys was the main Se compound also in Se-enriched garlic, onion and leek, whereas SeMet and selenate were the predominant Se species in cereal grains (Zhu et al. 2009) and potato tubers (Cuderman et al. 2008). In *Brassica*, the reported Se specification studies have been done with fresh leaf material and there are no other reports on Se species composition in seeds. The lack of other Se species in the seeds and meal may be due to instability of these compounds during seed drying and oil extraction. Results on rice indicate that the endosperm can accumulate SeMeSeCys if the seed Se concentration is high, up to 8 mg Se kg^{-1} DW (Williams et al. 2009), so the retranslocation to seeds may not be the limiting factor for the accumulation of SeMeSeCys in *Brassica*.

The field experiments conducted in this study show that while Se application may not affect the quantity of *Brassica* seed or oil yield, it has a significant positive effect on the yield quality, as the valuable SeMet in the seed protein fraction increased. The Se species were successfully validated by the use of two chromatographic separation mechanisms that allowed a proper identification of SeMet and the performance of mass balance. The presence of other Se species was not detected in either seeds or meal, which may have been due to the degradation of unstable Se species during drying and processing of the seeds. Our results show that the agronomic biofortification with Se can improve the nutritional quality of *Brassica* meal and the capacity of *Brassica* species to accumulate Se gives an attractive option for increasing the SeMet concentration of animal diets.

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