



RESEARCH PAPER

# Respiratory potential and Se compounds in pea (*Pisum sativum* L.) plants grown from Se-enriched seeds

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## Abstract

Selenium (Se) has been proved to be an essential element for humans and animals. However, less is known about its effects on plants. Pea plants were treated foliarly once (OT) and twice (TT) with Se solution during their flowering period. Seeds obtained from these plants contained 383 and 743 ng Se g<sup>-1</sup>, respectively, and, together with control seeds from untreated plants (UT) containing 21 ng Se g<sup>-1</sup>, were sown in soil in a greenhouse. Se content and its chemical form in young plants were studied, and its impact on plant respiratory potential, measured as terminal electron transport system (ETS) activity, determined. ETS activity was highest in young pea leaves with the highest Se content. Higher ETS activity possibly reflected increased glutathione peroxidase (GSH-Px) activity in mitochondria. The Se content of leaves and stems of plants grown from control seeds was similar to that in the seed, being around 40 ng Se g<sup>-1</sup>. Se concentration in leaves of young plants grown from OT and TT seeds was 605%, and 1340% higher, respectively, than the control, and in their stems 355%, and 680% higher, respectively. The ratio of Se concentrations in OT and TT seeds was the same as in the leaves and stems in the young plants grown from them. SeMet was the major Se compound in Se-rich pea seeds and leaves, comprising 49% and 67% of the total Se content in OT and TT seeds, respectively, and 85% and 79% in the corresponding leaves.

Key words: Electron transport system activity, pea, selenium, selenium compounds.

## Introduction

Selenium (Se) is a trace element that can function as an essential nutrient for humans and animals or as an environmental toxicant; the boundary between the two is narrow and depends on its chemical form, concentration, and other environmentally regulating variables (Fan *et al.*, 2002; Shardendu *et al.*, 2003). Slovenia is a country with a low content of Se in the soil (Pirc and Šajn, 1997). Cultivation of plants enriched with Se could be an effective way of producing Se-rich foodstuffs and thereby increase health benefits (Ip and Lisk, 1994; Finley *et al.*, 2001; Lyons *et al.*, 2005). Se plays a role in the prevention of atherosclerosis, specific cancers, arthritis, and altered immunological functions. The beneficial effects of Se are dependent on the chemical form, and SeMet is known to be the most readily assimilated form of Se (Patrick, 2004). Duffield-Lillico *et al.* (2003) reported that supplementation of the human diet with selenium yeast, containing SeMet as the main chemical form, significantly reduced the occurrence of prostate cancer.

Se has not been classified as an essential element for plants, although its role has been considered to be beneficial in plants capable of accumulating large amounts of the element (Terry *et al.*, 2000). Uptake and accumulation of Se by plants is determined by the chemical form and concentration, soil factors such as pH, salinity, and CaCO<sub>3</sub> content, the identity and concentration of competing ions, and the ability of the plant to absorb and metabolize Se (Kabata Pendias, 2001). Kahakachchi *et al.* (2004) stated that actively growing tissues usually contain the largest amounts of Se. The majority of plants accumulate more Se in shoot and leaf than in root tissues, but there are

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Abbreviations: ETS, terminal electron transport system activity; GSH-Px, glutathione peroxidase; OT, once-treated maternal plants; TT, twice-treated maternal plants; UT, untreated maternal plants.

exceptions (Zayed *et al.*, 1998). Hartikainen *et al.* (2000) reported a growth-promoting effect of Se in ryegrass. Senescence stress is partly counteracted by enhanced antioxidation which is associated with an increase glutathione peroxidase (GSH-Px) activity. These enzymes are particularly active in mitochondria. Data on terminal electron transport system (ETS) activity in mitochondria enable the general metabolic activity of individual organisms to be assessed. When organisms are stressed and demand more energy, ATP production and O<sub>2</sub> consumption are increased in mitochondria (Packard, 1985; Bartoli *et al.*, 2005). However, no direct studies have been published on a relationship between respiratory potential and Se content in plants. Few studies have looked at the feasibility of biofortifying Se by growing high-Se cereals and soybean, by soil or foliar application of Se (Sima and Gissel-Nielsen, 1985; Gupta and MacLeod, 1999).

In the last decade, pea has been studied as a nutritional source of non- or slowly digestible carbohydrates (Skrabanja *et al.*, 1999). In Asian cuisine young pea shoots or leaves are used as a vegetable food. The aim of this work was to study the distribution and chemical form of Se in plant tissue and the influence of Se on the respiratory potential of pea plants. Further, there was the possibility that, in areas where the soil is low in Se, plants could be enriched in Se to produce foodstuffs containing adequate amounts of Se.

## Materials and methods

### Plant material

Pea (*Pisum sativum* L., cv. Petit Provençal) plants were grown outdoors in Ljubljana, Slovenia (320 m above sea level, 46°35' N, 14°55' E), on a Se-poor soil. When flowering began, plants were foliarly sprayed once (OT) or twice (TT) (second treatment 2 weeks after the first) with an aqueous solution containing 10 mg Se l<sup>-1</sup> in the form of sodium selenate. A small sprayer was used and about 0.9 ml of solution per plant was used for each spraying. 20 plants were treated in each group, and 20 untreated plants served as controls (UT). Seeds obtained from the three groups of plants were sown in soil in a greenhouse. The soil contained less than 0.1 mg Se kg<sup>-1</sup> and the plants were watered with water containing no detectable Se (i.e. less than 0.5 µg l<sup>-1</sup>). The temperature was 9±2 °C during the night (12 h dark) and 20±2 °C during the day (12 h light), 80 plants in each group were cultivated under 100 µmol m<sup>-2</sup> s<sup>-1</sup> photosynthetically active radiation (PAR). The scheme of the study and the analysis is shown in Fig. 1.

### Determination of the Se content and its species

Se was determined in seeds from the three groups of plants, and in leaves and stems of the resulting groups of young pea plants (36-d-old, 24 plants from each group), using hydride generation atomic fluorescence spectrometry (HG-AFS) (Smrkoj and Stibilj, 2004).

To determine the Se species, a modified method of Bodo *et al.* (2003) was used. To 900 mg sample was added 12 g 25 mM phosphate buffer (KH<sub>2</sub>PO<sub>4</sub>) (pH 7.5) or 150 mg of the non-specific protease *Streptomyces griseus* (Protease XIV, Merck) dissolved in 12 g of the same buffer. In both cases, the samples were stirred at

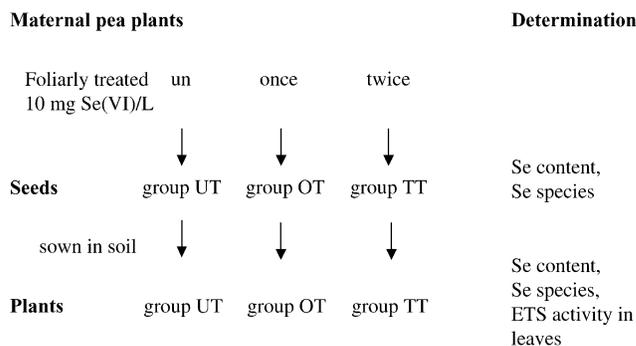


Fig. 1. The scheme of the study.

200 rpm for 24 h at 37 °C (SW 22, Julabo). Subsequently, samples were centrifuged at 14 000 g for 45 min at 4 °C (5804R, Eppendorf). The supernatants were filtered through a 0.25 µm filter (Millipore).

To determine soluble Se in the supernatants, the digestion with HNO<sub>3</sub> was used (Stibilj *et al.*, 2003). To 0.5 g of supernatant 0.5 ml of concentrated HNO<sub>3</sub> was added and heated for 10 min on an electric hot plate at about 100 °C in a capped Teflon vial. Then 0.5 ml H<sub>2</sub>O<sub>2</sub> was added three times and evaporated each time to one-quarter volume. Addition of 1 ml of 6 mol l<sup>-1</sup> HCl was added for the reduction of Se<sup>VI</sup>, samples were diluted and then Se was measured by HG-AFS.

Se compound analysis in supernatants was made by HPLC-UV-HG-AFS for which the operating conditions are described by Mazej *et al.* (2006). The separation system consisted of a high pressure pump (Varian Pro Star 210), a Hamilton PRP X-100 anion exchange column (4.1 mm×250 mm×10 µm) and a Chrompack IonoSpher C column (4.6 mm×250 mm×10 µm). The mobile phase for the anion exchange column was 40 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> (pH 6) with a flow rate of 0.5 ml min<sup>-1</sup> and for the cation exchange column 7 mM pyridine solution (pH 2.7) with a flow rate of 0.5 ml min<sup>-1</sup>. These conditions enabled the separation of five Se species (Se<sup>IV</sup>, Se<sup>VI</sup>, SeMet, SeMeSeCys, SeCys<sub>2</sub>) (Fig. 2). Standards were prepared at concentrations of approximately 100 ng Se g<sup>-1</sup> for each species, except for SeMet (approximately 400 ng Se g<sup>-1</sup>).

### Terminal electron transport system activity

Terminal electron transport system (ETS) activity was measured four times; twice in young plants: 13 December 2004 (13-d-old) and 21 December 2004 (21-d-old), and twice in flowering plants: 18 January 2005 (49-d-old) and 10 February 2005 (72-d-old). All measurements were made on UT, OT, and TT groups. The respiratory potential of mitochondria was measured via ETS activity, as described by Packard (1971). Leaves of known fresh weight were crushed in a mortar in chilled 0.1 M sodium phosphate buffer (pH=8.4) containing 0.15% (w/v) polyvinyl pyrrolidone, 75 µM MgSO<sub>4</sub>, and 0.2% (v/v) Triton-X-100, and then homogenized by ultrasound. The extract was centrifuged in a refrigerated centrifuge (2K15, Sigma, Osterode, Germany) at 8500 g for 4 min at 0 °C. Then (i) 1.5 ml substrate solution (0.1 M sodium phosphate buffer (pH=8.4), 1.7 mM NADH, 0.25 mM NADPH, 0.2% (v/v) Triton-X-100), and (ii) 0.5 ml of INT (20 mg 2-*p*-iodo-phenyl 3-*p*-nitrophenyl 5-phenyl tetrazolium chloride in 10 ml of bidistilled water) were added to 0.5 ml of the supernatant. The mixture was incubated at 20 °C for 40 min. After stopping the reaction with stopping solution (formaldehyde and phosphoric acid, 1:1 v:v), the formazan absorbance at 490 nm was determined. ETS activity was calculated as the rate of INT reduction, which was converted to the amount of oxygen utilised per mg of leaf dry mass (DM) per unit time (Kenner and Ahmed, 1975).

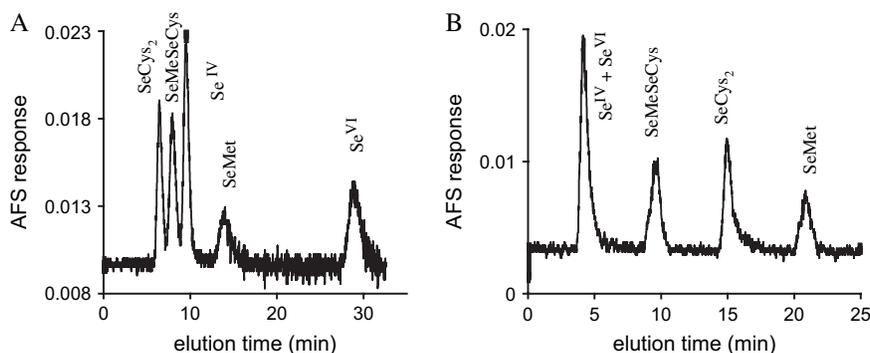


Fig. 2. Separation of Se standards on anion (A) and cation (B) columns.

### Statistical analysis

ANOVA was used to test the effect of Se on ETS activity of plants. Young and flowering plants were tested separately.

## Results and discussion

### Agronomic data

The average fresh weight of 24 plants from each group (36-d-old) was 1.3 g above-ground and 0.3 g below-ground; plants had, on average, five leaves. No significant differences were observed between plants grown from seeds with different Se contents. The average mass of the lyophilized seeds was 0.212 g; of leaves on progeny plants 0.089 g, stems 0.015 g, and roots 0.050 g. There were again no significant differences between groups.

### Se content

The Se content of pea seeds obtained from the untreated (UT group), once (OT) and twice (TT) foliarly treated plants, was, in each case, directly proportional to the number of spraying applications (Table 1). Seeds are usually a moderate source of Se, but several studies dealing with cereal and legume seeds showed, as in this study, that they are able to accumulate high amounts of Se (Stadlober *et al.*, 2001; Smrkoj *et al.*, 2005, 2006). Higher Se contents were found in the leaves than in the stems of plants grown from both OT and TT seeds, and these contents were, as for the seeds, directly proportional to the number of original foliar treatments (Table 1). The proportion of Se translocated from seeds to leaves and stems of progeny plants was calculated on the basis of Se contents of the individual parts and their lyophilized mass. In the OT and TT groups, 35%, on average, of the Se in the seed was translocated to the leaves and stems, irrespective of the initial Se content in the seeds. By contrast, in the UT group, 95% of the Se in the seed was transferred to leaves and stems. This suggests that Se has a specific role in the plant.

Table 1. Selenium mass fraction in pea seeds, and in leaves and stems of the progeny (young plants)

Group <sup>b</sup>	Se mass fraction (ng g <sup>-1</sup> ) <sup>a</sup>		
	Seeds	Plants	
		Leaves <sup>c</sup>	Stems <sup>c</sup>
UT	21±2	41±4	40±4
OT (9 µg Se per parent plant)	383±19	289±14	182±9
TT (18 µg Se per parent plant)	743±37	591±59	312±16

<sup>a</sup> Results are given as the average ±SD (standard deviation, *n*=3).

<sup>b</sup> UT, untreated; OT, once-treated; TT, twice-treated plants, see explanation in Fig 1.

<sup>c</sup> In lyophilized samples.

### The chemical form of the Se in Se-enriched seeds and leaves

The distribution of the different compounds of Se was studied in Se-enriched seeds and leaves; stems were omitted due to the insufficient mass. In Se-enriched seeds 32% of the Se was water soluble, but, following hydrolysis with non-specific protease, the proportion of water-soluble Se increased to 92% (Table 2).

After hydrolysis, selenomethionine was the only Se compound found in supernatants by anion and cation exchange chromatography (Fig. 3), comprising 49% and 67% of the total Se content in the OT and TT groups, respectively. Thus, 43% in OT and 26% in TT groups of the soluble Se were not detected by the ion exchange HPLC-UV-HG-AFS system. The reason for that could be that Se is present in the non-seleno-amino acid compounds that were not digested with the UV digestion unit. The presence of SeMet was confirmed by the standard addition method, because the elution time of Se species from samples was shorter than those for standard mixtures prepared in water, due to the matrix effect.

SeMet has been found to be the major Se species in other plant seeds enriched with Se in different ways. In buckwheat and pumpkin seeds from plants that were foliarly treated with selenate solution, SeMet was the

main Se species, comprising 93% and 81% of the total Se content, respectively (Smrkolj *et al.*, 2005, 2006). Stadlober *et al.* (2001) cultivated different cereals in soil to which selenate was added and in wheat, barley, and rye between 70% and 83% of the Se was found in the form of SeMet.

The results of this study show that selenium-enriched pea seeds are a potential source of dietary selenium, on account of their ability to accumulate Se, and that this Se is present mainly as SeMet, known to be favourable for human consumption.

In pea leaves, 91%, on average, of the Se was found to be water soluble after enzymatic hydrolysis. SeMet was, as in seeds, the only Se species obtained on both anion and cation exchange columns, comprising 82% of the total

Se content (Table 2; Fig. 4). In leaves, a higher proportion of SeMet (85% and 79% in OT and TT groups, respectively) was found than in seeds (49% and 67% in OT and TT groups, respectively). By calculating the part of Se as SeMet in leaves and in seeds, 38% of the Se was present in the form of SeMet in leaves of plants in both OT and TT groups.

Yoshida *et al.* (2005) determined selenium species in pea leaves and flowers, and in immature beans and pods. The pea plants were cultivated on a soil fertilized with Se granules composed of sodium selenate and barium selenate. The average Se mass fraction was  $66 \mu\text{g Se g}^{-1}$  in immature bean,  $68 \mu\text{g Se g}^{-1}$  in immature pods,  $46 \mu\text{g Se g}^{-1}$  in leaves, and  $69 \mu\text{g Se g}^{-1}$  in flowers, all on a dry matter

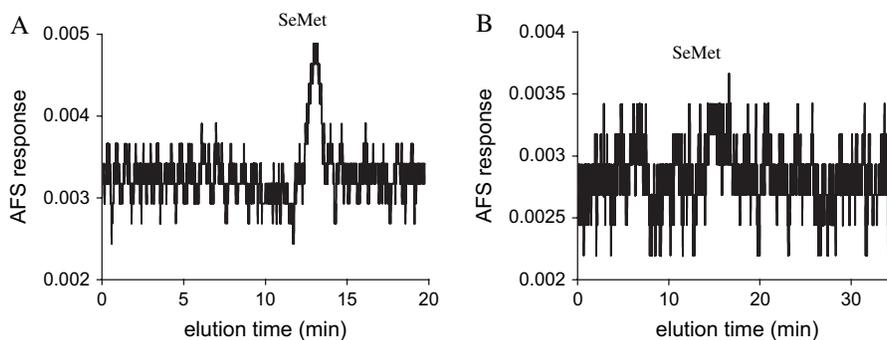
**Table 2.** Mass fractions of water-soluble Se and SeMet in pea seeds from Se-treated plants and in the leaves of their progeny

Group <sup>a</sup>	Seeds			Leaves		
	Water-soluble Se after proteolytic hydrolysis	Se in the form of SeMet		Water-soluble Se after proteolytic hydrolysis	Se in the form of SeMet	
	(%) <sup>b</sup>	(ng g <sup>-1</sup> ) <sup>c</sup>	(%) <sup>b</sup>	(%) <sup>b</sup>	(ng g <sup>-1</sup> ) <sup>c</sup>	(%) <sup>b</sup>
OT	92±1	188±8	49±2	94±4	167±11	85±4
TT	93±2	499±12	67±2	88±4	465±46	79±8

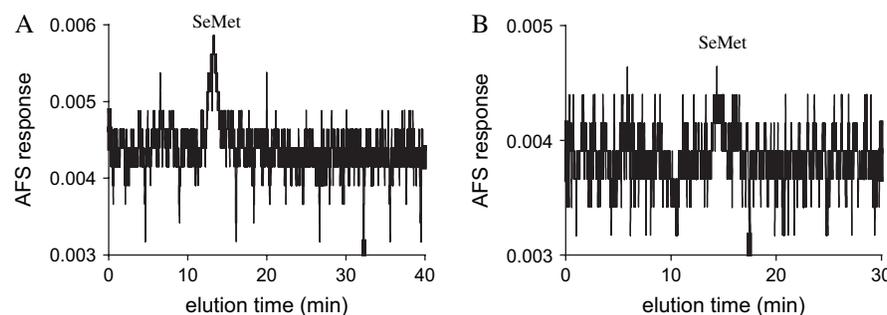
<sup>a</sup> OT, once-treated; TT, twice-treated plants, see explanation in Fig. 1.

<sup>b</sup> Based on the total Se mass fraction.

<sup>c</sup> Samples were analysed in triplicate at least, Se mass fraction in leaves was based on lyophilized matter.



**Fig. 3.** Selenium species in Se-enriched pea seeds, results by anion (A) and cation (B) exchange columns.



**Fig. 4.** Selenium species in Se-enriched leaves of pea plants obtained by anion (A) and cation (B) exchange columns.

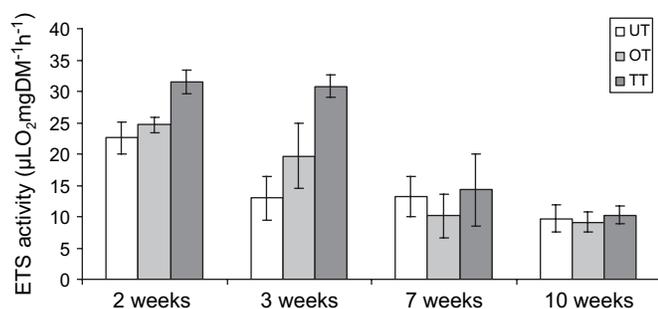
basis, and 71.4–92.7% of Se was water soluble. They found  $\text{Se}^{\text{VI}}$ , SeMeSeCys, SeMet, and an unknown Se compound in the water extract of parts of pea plants. The major part of water-soluble Se was in the form of selenate, 61.9% and 77.7% in immature pods and in leaves, respectively. In the water extract and after protease hydrolysis of immature bean, SeMeSeCys was the main compound (around 35%). SeMet was present in a minor proportion in all parts of the pea plant except in pods. On the basis of these results and the results obtained in this study, it is concluded that the composition of Se species varies with plant parts, plant maturity and Se-enrichment of the pea plants.

Sugihara *et al.* (2004) germinated soybean and kidney bean, both leguminous plants, in selenite solution and harvested the resulting sprouts. The Se mass fraction was 8–10  $\mu\text{g g}^{-1}$  of wet weight, and the major part of Se was in the form of SeMeSeCys, with only a minor proportion in the form of SeMet and one unidentified Se species.

### ETS activity

Respiratory potential, measured as ETS activity, was approximately twice as high in leaves of young pea plants as in those of flowering plants (Fig. 5). Plants demand more energy during intensive growth and development, in order to build structural components. Smillie (1962) reported that the respiratory rates of pea leaves decrease continuously during leaf maturation and that this may result from the decreasing ability of the leaf cells to use their potential enzymic capacity fully, or it may be the direct consequence of a decrease in the respiratory enzymic capacity of the leaf. The highest ETS activity at the beginning of growth was reported for an aquatic plant *Potamogeton crispus* (Mazej and Gaberšič, 1999) and for *Fagopyrum esculentum* and *F. tataricum* (Breznik *et al.*, 2005).

ETS activity was highest in the leaves of young plants with the highest concentration of Se ( $591 \pm 59 \text{ ng g}^{-1}$ ) ( $P < 0.0001$ ,  $n=5$ ) (Fig. 5). The possible explanations are (i) higher ETS activity reflected increased GSH-Px activity in mitochondria. It was shown (Xue and Hartikainen, 2000; Hartikainen *et al.*, 2000; Xue *et al.*, 2001) that Se



**Fig. 5.** Terminal electron transport system (ETS) activity in pea plants grown from seeds with different Se contents [UT, untreated; OT, once-treated; TT, twice-treated maternal plants (in weeks after germination)], see explanation in Fig. 1.

exposure increased GSH-Px activity in ryegrass and lettuce. (ii) Plants need energy to repair damage caused by Se. The latter is consistent with the fact that Se can mimic sulphur, forming Se analogues of S compounds, for example replacing S in amino acids (methionine and cysteine). The conformation of proteins containing seleno-amino acids could be perturbed, and their catalytic activity thereby disturbed (Brown and Shrift, 1982). However, nutrient solutions containing up to 5  $\mu\text{g ml}^{-1}$  Se as selenite or selenate showed no adverse effect on plant growth in the report of Kahakachchi *et al.* (2004).

ETS activity was the highest in plants with the highest content of SeMet in soluble proteins in the leaves, so it is possible that SeMet enhances respiratory activity over that with Met. It is not known whether other Se species would induce the same response.

In flowering plants, there was no difference between control and Se groups (Fig. 5). Similarly, in the studies of Germ *et al.* (2005) and Breznik *et al.* (2005), no difference in terminal ETS activity was observed in Se-treated plants.

### Conclusions

The transfer of Se from Se-enriched seeds to young plants has been established. Following hydrolysis with non-specific protease from *Streptomyces griseus*, SeMet was found to be the major Se species in Se-enriched seeds and leaves. Therefore, Se-enriched pea is a potential source of dietary Se, given the accumulation of Se and the fact that it is present mainly as SeMet, known to be a very favourable nutrient for humans. However, further work is needed to characterize the remaining soluble Se species resulting from enzymatic hydrolysis. ETS activity was highest in young plants. Plants need a lot of energy in times of intensive growth and development. The Se that was transferred from seeds to leaves clearly intensified respiratory potential in young plants. Higher ETS activity might be partly due to increased GSH-Px activity in mitochondria.

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