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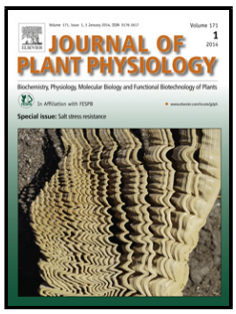

Title: Plant selenium toxicity: Proteome in the crosshairs

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Title: PLANT SELENIUM TOXICITY: PROTEOME IN THE CROSSHAIRS

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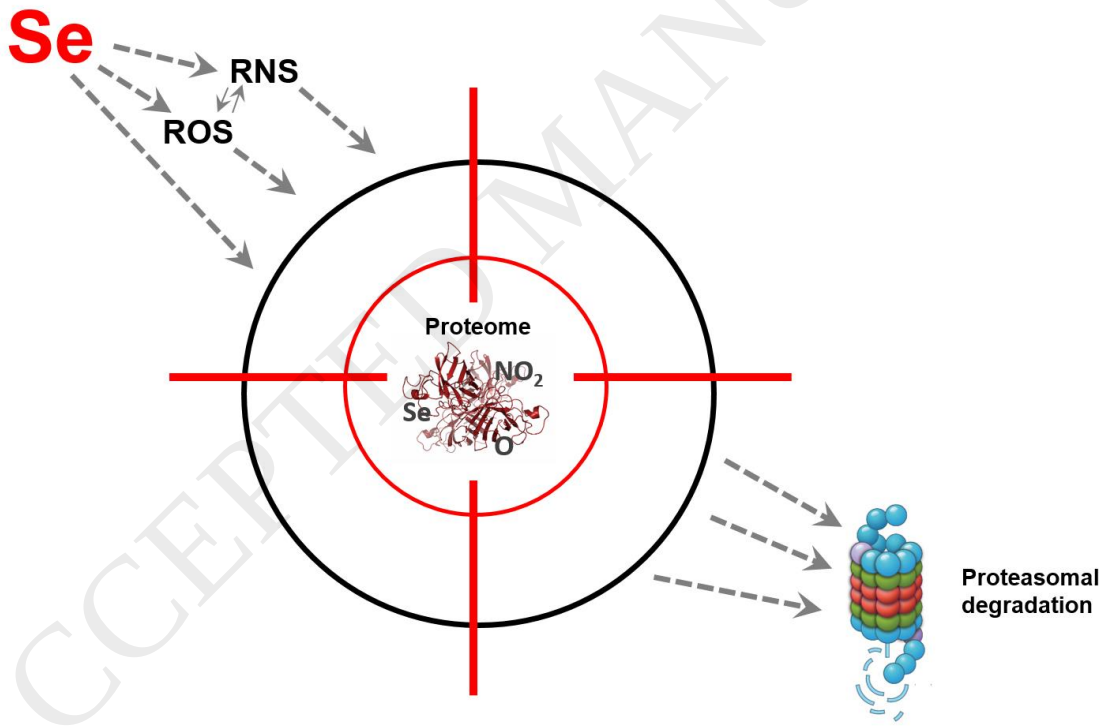
Graphical Abstract:



Summary

The metalloid element, selenium (Se) is in many ways special and perhaps because of this its research in human and plant systems is of great interest. Despite its non-essentiality, higher plants take it up and metabolize it *via* sulfur pathways, but higher amounts of Se cause toxic symptoms in plants. However, the molecular mechanisms of selenium phytotoxicity have been

only partly revealed; the data obtained so far point out that Se toxicity targets the plant proteome. Besides seleno- and oxyproteins, nitroproteins are also formed due to Se stress. In order to minimize proteomic damages induced by Se, certain plants are able to redirect selenocysteine away from protein synthesis thus preventing Se-protein formation. Additionally, the damaged or malformed selenoproteins, oxyproteins and nitroproteins may be removed by proteasomes. Based on the literature this review sets Se toxicity mechanisms into a new concept and it draws attention to the importance of Se-induced protein-level changes.

Key words: selenium, phytotoxicity, oxyprotein, nitroprotein, selenoprotein, proteome

Introduction: overview of Se uptake and metabolism in plants

Selenium (Se) is a naturally occurring non-metal element that exists in the (+6), (+4), (0), and (-2) oxidation states. The most oxidized forms - selenate (SeO_4^{2-}) and selenite (SeO_3^{2-})- are water soluble and therefore both have a high degree of bioavailability and bioaccumulation potential in the environment (Saha et al., 2017). As sessile organisms, plants take up selenium from the soils due to the chemical similarities with the essential macroelement sulfur (S). Principally, land plants take up Se in the form of selenate or selenite using active transport processes. Due to several mechanisms for selenite removal from soil solution (e.g. absorption of selenite by organic and inorganic soil particles, assimilation of selenite by soil microbes) selenate is the major soluble form in soil solution (Barrow and Whelan, 1989). Selenate is primarily taken up by plants via sulfate carrier proteins (Shinmachi et al., 2010; Sors et al., 2005; Terry et al., 2000; White et al., 2004, 2007), while selenite has been shown to enter the root cells through phosphate transporters (Hopper and Parker, 1999; Li et al., 2008; Zhang et al., 2014). Furthermore, a silicon (Si) influx transporter OsNIP2;1 (Lsi1) belonging to the nodulin 26-like intrinsic membrane protein subfamily of aquaporins, has been shown to be permeable to selenite (Zhang et al., 2006). Land plants also absorb organic Se forms such as selenocysteine (SeCys) and selenomethionine (SeMet) with the help of the activities of amino acid permeases (White and Broadley, 2009), and the rate of their uptake can be higher compared to oxidized selenium forms in species like durum wheat and spring canola (Kikkert and Berkelaar, 2013; Zayed and Terry, 1992). In other species, like *Brassica juncea*, the uptake of selenate is dominant over SeMet and selenite (Montes-Bayón et al., 2002). Root-to-shoot translocation of Se primarily depends on the selenium speciation. The more toxic selenite is converted to less mobile and non-toxic organic forms such as SeMet, therefore Se translocation from root to shoot is considerably lower in plants supplied with selenite than those treated with selenate (Arvy, 1993; de Souza et al., 1998; Hopper and Parker, 1999; Li et al., 2008). In case of selenate; however, most of it is translocated into the above-ground plant parts *via* xylem (Zayed et al., 1998). The assimilation of selenium occurs through S assimilation enzymes and pathway in chloroplasts. In the first steps, selenate is activated by ATP sulphurilase (APS) and reduced by APS reductase resulting in the formation of selenite which is further reduced to selenide (Se^{2-}) either by sulfite reductase or by a non-enzymatic reduction with glutathione (Van Hoewyk, 2013). In the subsequent reaction between selenide and O-acetylserine (OAS), SeCys is formed which is detoxified by various alternative ways. It can be converted to the less toxic SeMet and volatile dimethyl-selenide (DMSe) or it can be converted to elemental Se (Se^0)

and alanine *via* the enzyme NifS (Van Hoewyk et al., 2005). The formations of methylselenocysteine (MeSeCys) and the volatile dimethyl-diselenide (DMDSe) are further possible SeCys detoxification mechanisms and are characteristic of hyperaccumulator plant species (Pilon-Smits and Quinn, 2010). The uptake and assimilation mechanisms of inorganic Se forms and the participating enzymes are depicted in Fig 1.

Selenium speciation within the plant tissues partly depends on the form of selenium which is supplemented to the plant. A nice example is the work of de Souza et al. (1998), where selenate-treated Indian mustard accumulated selenate while selenite-treated mustard principally accumulated SeMet and selenomethionine-Se-oxide. In carrot, however, SeMet and γ-glutamyl-selenomethyl-selenocysteine was detected in roots and only SeMet was present in the leaves regardless of the Se forms applied (Kápolna et al., 2009). Se speciation also depends on the accumulation capability of the certain plant species. Hyperaccumulators, like *Stanleya pinnata* and *Astragalus bisulcatus* contains high amounts of organic Se species like MeSeCys, gamma-glutamyl-SeCys, selenocystathionine. Based on these, we can assume that the accumulated Se forms in hyperaccumulator plants are mainly organic MeSeCys (Freeman et al., 2006), while in non-accumulators and accumulators, the majority of Se remains as inorganic selenate (Van Hoewyk et al., 2005). The species of the accumulated selenium determine the rate of Se phytotoxicity, since the inorganic forms are thought to be more toxic compared to organic Se species (Garousi, 2015).

Se toxicity in plants

The concentration of Se in the world's soils is typically within the range 0.01-2.0 mg kg^{-1} (Johnson et al., 2010). There are areas with high Se concentrations (parts of North America, China, India), and these naturally Se-rich soils may contain vegetation the consumption of which can be toxic for grazers. Moreover, due to anthropogenic activities such as mining, agriculture, household or oil production (Dhillon and Dhillon, 2003), Se can accumulate in soils and therefore cause environmental problems and public health concerns. Toxic levels of Se in plant tissues are generally above 5 mg kg^{-1} (Reilly, 1996), but the Se tolerance of crop plants actually greatly differ. For rice 2 mg kg^{-1} dry weight (DW) selenium concentration proved to be toxic, wheat showed toxic symptoms at 4.9 mg kg^{-1} DW Se (Tripathi and Misra, 1974), while Dutch clover tolerated 330 mg kg^{-1} DW selenium in their tissues (Mikkelsen et al., 1989).

At the whole-plant level, excess Se causes general symptoms including chlorosis, withering, and stunted shoot and root growth (Hawrylak-Nowak et al., 2015; Lehotai et al., 2016; Molnár et al., 2018a;b) as shown in Fig 2. Se toxicity impairs both non-proteomic processes (*e.g.*, lipid peroxidation and altered redox status) and the proteome; proteomic damages include stress specific to selenium (*i.e.*, malformed selenoproteins) as well as protein damage associated with general abiotic stress (*i.e.*, oxidized or nitrated proteins).

Protein modifications unique to Se stress: selenoprotein formation

Organisms that have a requirement for Se are equipped with machinery- including $tRNA^{sec}$- to incorporate SeCys into gene encoded selenoproteins (Carlson et al., 2018; Tobe and Mihara, 2018). In contrast to many prokaryotes, protists, and animals, higher plants do not require Se but can still accumulate and assimilate it into SeCys (White, 2016). The synthesis of selenocysteine in plants is problematic due to its structural similarity with cysteine, and therefore $tRNA^{cys}$ can compete with selenocysteine during polypeptide synthesis (Stadtman, 1990). Therefore, a non-specific route for the formation of selenoproteins exists in plants, which is a result of a cysteine to selenocysteine substitution.

Additionally, an alternative route to selenocysteine formation may also exist. Recently it was discovered in yeast that the trans-sulfuration pathway can covert SeMet into SeCys *via* the intermediate seleno-adenosylmethionine (Lazard et al., 2015; Plateau et al., 2017). This conclusion was based on the presence of SeCys in yeast treated with SeMet. Because photoautotrophic organisms also possess homologous enzymes involved in methionine catabolism, it is very likely that the trans-sulfuration pathway in plants represents a second route for the formation of selenocysteine. However, even though SeMet can also result in the formation of selenoproteins, this amino acid in proteins is more inert compared to SeCys and is generally considered harmless in plant proteins (Stadlober et al., 2001); thus, the following discussion on cytotoxic selenoproteins focus on those containing SeCys.

Non-specific selenoproteins containing SeCys have been associated with Se toxicity in non-accumulator plants for almost forty years (Brown and Shrift, 1980; 1981). Toxicity of nonspecific selenoproteins can be explained on several accounts, and because this topic was recently reviewed (Van Hoewyk, 2013), it will be briefly discussed herein. (*i*) The replacement of cysteine with the bigger SeCys is predicted to alter protein structure. For example, a diselenide bond is 0.2 Å longer than a disulfide bond and will also have a lower redox potential (Hondal et al., 2013). (*ii*) Because SeCys is more reactive than cysteine- which often occupies

the active site in enzymes- a SeCys substitution can also alter enzyme kinetics (Kim and Gladyshev, 2005; Hazebrouck et al., 2000). (*iii*) Furthermore, a cysteine to selenocysteine substitution at a protein residue that binds cofactors or ions can also compromise protein function (Aldag et al., 2009). (*iv*) Lastly, many chloroplastic and mitochondrial proteins have Fe-S clusters that participate in electron transport, and there is evidence that the replacement of an Fe-Se clusters can dramatically alter protein activity (Hallenbeck et al., 2009). In summary, the formation of selenoproteins in plants is viewed as toxic, and minimizing their formation is associated with improved selenium tolerance.

Reactive oxygen species (ROS)-induced protein modifications

Most terrestrial plants will transport selenium from the soil across the root cell membranes as selenate, which is readily reduced enzymatically into selenite (Terry, 2000). However, selenite is rapidly reduced into selenide non-enzymatically *via* glutathione, which generates superoxide (Chen et al., 2007) and stimulates an anti-oxidative response at the transcriptional (Van Hoewyk et al., 2008) and metabolomic level (Dimkovikj and Van Hoewyk, 2014). It is now very well established that treating plants with both forms of inorganic selenium generate ROS (including hydrogen peroxide and superoxide) as summarized in Table 1. If anti-oxidative metabolism is overwhelmed, Se treatment will ultimately depletes the glutationine pool and alter cellular redox state (Hugouvieux et al., 2009; Grant et al., 2011). Downstream targets of ROS accumulation include proteins, and protein oxidation occurs by direct oxidative attack of amino acids like Met, Cys, Tyr, Trp in proteins. As a consequence, the enzyme activity is altered and the protein becomes more susceptible toward proteolysis (Berlett and Stadtman, 1997; Dunlop et al., 2002). Recently it has been shown that selenium can increase levels of oxidized proteins (Sabbagh and Van Hoewyk, 2012; Vallentine et al., 2014). Because selenium is a pro-oxidant, the long-term consequences of the toxic effects of selenium are not too different compared to other abiotic stressors that are also known to cause oxidative stress, *e.g.* decreased plant growth, necrosis, etc. In fact, the inhibitory effects of selenium-induced oxidative stress on plant physiology- particularly photosynthesis- have been well documented (Geoffery et al., 2007; Freeman et al., 2010, Grant et al., 2011).

More recent research has indicated that selenium generates mitochondrial superoxide (Vallentine et al., 2014), which interferes with mitochondrial processes governing primary metabolism. Hydroponically grown *Brassica napus* treated with selenite accumulated mitochondrial superoxide within 90 minutes, which was associated with an increase in amino

acids, but a decrease in most tricarboxylic acid (TCA) cycle metabolites in root tissue (Dimkovikj and Van Howeyk, 2014). After 24 h, selenite decreased the activity of aconitase- a mitochondrial enzyme that participates in the initial steps of the TCA cycle; these data provide evidence that the decrease in metabolites was caused by TCA cycle inhibition rather than decreased demand. Aconitase contains an iron-sulfur cluster, and its cofactor is known to be impaired by superoxide (Verniquet et al., 1991); this supports the conclusion that selenite-induced oxidative stress can alter mitochondrial processes. It is possible that decreased aconitase activity and impaired mitochondrial processes likely contribute to decreased starch utilization that have been observed in selenium-treated *Arabidopsis* plants (Ribeiro et al., 2016).

The photosynthetic electron transport chain also contains several proteins that require an iron-sulfur cluster for activity, and thus it is also likely that selenium- as well as other stressors that generate superoxide- decreases photosynthesis by targeting such proteins. Support for this possibility comes from recent biochemical experiment using the iron-sulfur protein ferredoxin purified from spinach (Fisher et al., 2016). Superoxide generated from the *in vitro* reduction of selenite rapidly altered the integrity of ferredonxin's iron-sulfur cluster, which was concomitant with a decrease in its activity and the formation of apo-ferredoxin, *i.e.*, non-native protein without its cluster. Although *in planta* experiments are lacking at this point, these biochemical data point to a possible mechanistic understanding of how selenium impairs photosynthesis.

Lastly, a recent study has shed light on the possibility that Se can also target cytosolic enzymes. Selenium-treated rice plants exhibited oxidative stress as judged from lipid peroxidation, which might have stemmed from an accumulation of methylglyoxal (Mostofa et al., 2017); this metabolite is an unwanted byproduct of glycolytic metabolism, that can be quenched by glyoxylases. Intriguingly, the authors link methylglyoxal toxicity to Se-induced inhibition of activities in both glyoxylase I and II. Whether or not Se directly or indirectly inhibits glyoxylases warrants further study.

Reactive nitrogen species (RNS)-induced protein modifications

In biological systems, nitric oxide (NO) is able to react with different types of molecules like reactive oxygen species, the tripeptide glutathione or molecular oxygen, which can respectively yield peroxynitrite ($ONOO^-$), nitrous acid (HNO_2), S-nitrosoglutathione (GSNO), nitrogen dioxide radical ($NO_2^{\cdot}$) or dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4) (del

Río, 2015). The perception of NO and its reaction products (RNS) and the transfer of their bioactivity is realized through protein-centric signaling during which NO and their reaction products target specific groups of proteins resulting in redox-based, post-translational modifications (PTMs) (Nathan, 2003; Umbreen et al., 2018). These RNS-dependent PTMs include S-nitrosylation, metal nitrosylation and nitration. In case of the reversible S-nitrosylation, thiol groups in specific cysteine amino acids are targeted by NO resulting in structural and activity changes in the protein through the formation of S-nitrosothiol groups (Astier et al., 2012). Also, protein-protein interactions (Hara et al., 2005) or protein localization (Tada et al., 2008) have been revealed to be modulated by S-nitrosylation. Nitric oxide rapidly reacts with metal centers (Zn^{2+}, Cu^{2+}, Fe^{2+} or Fe^{3+}) of certain proteins (e.g. plant haemoglobins, Seregélyes et al., 2004) resulting in the formation of metal-nitrosyl complexes and consequently causing activity changes of the target protein (Astier and Lindermayr, 2012). Moreover, RNS (primarily peroxynitrite) catalyze the irreversible nitration of certain aromatic amino acids (primarily tyrosine, tryptophan). In case of tyrosine, a nitro group is added to the *ortho* carbon atom of the aromatic ring resulting in the formation of 3-nitrotyrosine and causing conformational changes in proteins that can inactivate enzymatic activity (Mata-Pérez et al., 2016). Therefore, the accumulation of 3-nitrotyrosine can be considered a marker for nitrosative stress (Valderrama et al., 2007). Because of the commonly affected amino acid residues we can suspect interactions between oxidation, nitration and S-nitrosylation. For instance, in case of Cys, S-nitrosylation resulting in lower oxidation state may prevent these residues from irreversible sulfonic acid formation (Lindermayr 2018). The link between amino acid nitration and oxidation is evidenced by the fact that the first step during nitration is the one electron oxidation of Tyr yielding tyrosyl radical which can be susceptible to further nitration modification (Souza et al., 2008). Although there is no direct evidence for it, but it can be assumed that the selenized Cys residues are not targets of further oxidation or nitration.

Previous studies evidenced that excess selenium disturbs the homeostasis of RNS in plant cells (Table 1). During early seedling development in *Arabidopsis* roots, selenite decreased NO levels in a concentration-dependent manner, while in the longer term NO levels increased as selenite toxicity increased (Lehotai et al., 2012). The NO and GSNO overproducer *gsnor1-3* mutant showed improved selenite tolerance compared to the wild-type, but nitrate reductase (NR) deficient *nia1nia2* plants with low endogenous NO proved to be Se sensitive suggesting that NO/GSNO contributes to Se tolerance (Lehotai et al., 2012). In another experiment, the roots of selenite-treated *Arabidopsis* showed decreased endogenous NO levels and this NO diminution proved to be NR-independent (Lehotai et al., 2016a). Selenite

intensified cytokinin signaling in the root system which contributes to the Se-induced NO depletion (Lehotai et al., 2016a).

Further studies associate nitrosative stress with Se phytotoxicity. For instance, in selenite-treated *Brassica rapa* roots, NO levels showed concentration- and time-dependent accumulation, which was responsible for NADPH-oxidase dependent ROS production and consequently for Se phytotoxicity (Chen et al., 2014). In this case, biochemical evidence suggested that Se-induced NO production is associated with NR and nitric oxide synthase (NOS)-like activities (Chen et al., 2014). Similarly, pea plants supplemented with selenite increased endogenous NO contents both in their leaves and roots and showed toxic symptoms (Lehotai et al., 2016b). Also peroxynitrite levels and protein nitration increased in their shoot, reflecting for the first time that nitrosative damages coincide with Se phytotoxicity (Lehotai et al., 2016b). The Se-induced alterations in the physiological nitro-proteome of pea can be considered as organ specific and were observed to be dependent on the concentration of the applied Se (Lehotai et al., 2016b).

The involvement of RNS-induced nitrosative stress in Se toxicity has been further supported recently. In hydroponically grown *Brassica juncea*, the severe phytotoxicity of selenite was accompanied by intense protein tyrosine nitration as well as alterations in nitration pattern. This means that a protein band with approximately 60 kDa molecular weight showed selenite-dependent nitration. At the same time, selenate had a less toxic effect compared to selenite both on the physiological performance and on protein nitration suggesting a correlation between the degree of Se forms-induced toxicities and nitrosative stress in *Brassica* organs (Molnár et al., 2018a).

In contrast to *B. juncea*, the selenium sensitive *Arabidopsis thaliana* exhibited pronounced oxidative stress and was accompanied by slight modifications in protein nitration; *Brassica juncea* exhibited moderate oxidative and intense nitrosative stress. The results of this comparative study suggest that selenite tolerance or sensitivity is more associated with oxidative processes than secondary nitrosative modifications in the above plant species (Molnár et al., 2018b). Recently, nitrosative and oxidative processes were examined in Se hyperaccumulator and non-accumulator *Astragalus* species in order to evaluate the possible role of RNS metabolism and signaling in Se (hyper)tolerance (Kolbert et al., 2018). Selenate intensified the production or disturbed the metabolism of RNS (NO, $ONOO^-$, GSNO) consequently resulting in increased protein tyrosine nitration in sensitive *Astragalus membranaceus*. Interestingly, in the (hyper)tolerant and hyperaccumulator *A. bisulcatus*, Se-induced RNS accumulation and tyrosine nitration proved to be negligible suggesting that this

species is able to prevent Se-induced nitrosative stress. These results support the correlation of protein nitration with Se phytotoxicity/tolerance (Kolbert et al., 2018).

According to a biochemical study, exogenous NO in the form of sodium nitroprusside (SNP) induced the expression of phosphate and sulfate transporters (OsPT2, OsSultr1;2 and OsSultr4;1), resulting in enhanced SeCys and MeSeCys concentration in rice roots. Furthermore, NO supplementation stimulated glutathione biosynthesis by up- regulating γ-glutamylcysteine synthetase (γ- ECS) and glutathione synthetase (GS), and increased the expression of cysteine synthase (CS) resulting in triggered Se metabolism. These data imply the possibility that not only selenium affects endogenous NO/RNS metabolism and signaling, but also that NO has an effect on Se uptake and metabolism (Xiao et al., 2017).

The recently accumulated data show that the effect of Se on NO/RNS metabolism is diverse and depends, *inter alia*, on the plant species, on the applied selenium form and concentration. Furthermore, as a consequence of disturbed NO/RNS metabolism, intense protein nitration occurs in selenium-stressed plants which together with protein oxidation potentially contributes to Se phytotoxicity. Similar to the oxidative protein modifications, the intensification of protein nitration leads to the increase of inactive nitroproteins that likely have to be cleared from the cell to maintain protein quality control.

Strategies to minimize proteomic damages induced by selenium

Redirecting selenocysteine away from protein synthesis

The malformed selenoprotein hypothesis posits that minimizing selenocysteine misincorporation in protein prevents Se toxicity in plants. This can be achieved by either decreasing the synthesis of selenoproteins or increasing their degradation (Van Hoewyk, 2013). Several pieces of evidence demonstrated that preventing the synthesis of selenoproteins is coupled to increased Se tolerance, and are summarized in Fig. 2. (*i*) Selenium-tolerant selenium hyperaccumulating plants such as *Astragalus bisulcatus* possess a selenocysteine methyltransferase; this enzyme prevents methylated selenocysteine from being incorporated into protein and is part of a pathway that leads to the volatization of Se as DMDSe (Neuhierl and Böck, 1996). Over-expression of this enzyme in *Arabidopsis* and *Brassica juncea* similarly lead to MeSeCys and increased Se tolerance (LeDuc et al., 2004). Collectively, these data indicate that the production of MeSeCys prevents Se toxicity. In fact, in a comparative study between two plants with different tolerance to Se, the Se hyperaccumulating plant *Stanleya*

pinnata predominantly accumulated MeSeCys, whereas the Se-sensitive *Stanleya albescens* did not (Freeman et al., 2010). (*ii*) In another approach, overexpression of an endogenous cystathionine gamma-synthase in *Brassica juncea* also resulted in a two-fold increase in volatilized Se; this enzyme catalyzes the reaction of selenocysteine into Se-cystathionine, and the increased selenite tolerance in transgenic plants was explained by diverting selenocytseine away to protein (Van Huysen et al., 2003). (*iii*) Lastly, over expression of CpNifS in *Arabidopsis* results in increased selenate tolerance; this chloroplastic enzyme possess selenocysteine lyase activity whose products are alanine and elemental Se. Although these plants accumulated more Se, the observed phenotypes of the transgenics was explained by the nearly two-fold decrease of selenium in protein compared to wild type plants (Van Hoewyk et al., 2005).

Proteasomal removal of non-specific selenoproteins, oxyproteins and nitroproteins

As discussed above, nonspecific selenoproteins containing a cysteine to selenocysteine substitution have long suspected to be misfolded. This assumption is based on the well-established effects of canavanine and azetidine-2-carboxylic acid; these two non-proteinaceous amino acids can respectively replace arginine and proline, resulting in protein misfolding (Kurepa et al., 2008). Misfolded proteins are cytotoxic if they are not removed by proteolytic pathways because they can lead to protein aggregates that impede cellular processes, including trafficking (Liu and Howell, 2010). Although autophagy is involved in bulk degradation of macromolecules, the ubiquitin-proteasome pathway (UPP) is the predominant proteolytic system that maintains specificity in eukaryotes (Smalle and Vierstra, 2004). In the past several years, it has become evident that this pathway is implicated in Se tolerance by removing selenoproteins once they are created.

The proteasome is a multi-subunit complex in eurkaryotic cells that can selectively remove regulatory and misfolded proteins. Substrate specificity of the 26S proteasome requires the concerted action of E1, E2, and E3 enzymes that covalently attach the small protein ubiquitin onto targeted proteins. Upon delivery to the proteasome, ubiquitinated proteins enter the catalytic chamber of the proteasome where they are degraded by three different proteases (Smalle and Vierstra, 2004).

The effect of selenium on the UPP was first studied in the selenium hyperaccumulating plant *Stanleya pinnata* (Sabbagh and Van Hoewyk, 2012). Nontoxic levels of selenate increased the accumulation of ubiquitinated proteins in a dose-dependent manner. Inhibition of the proteasome did not affect protein levels of copper, sulfur, or iron in plants treated with selenate; however, proteasome inhibition increased the amount of Se in protein nearly two-fold, indicating that proteasomes acts to remove misfolded selenoproteins. Additionally, an experiment was designed to investigate the elemental composition of an enriched fraction of ubiquitinated proteins isolated from plants treated with selenate. In this approach, elemental analysis revealed that ubiquitinated proteins accounted for 25% of the total Se found in protein; this value was almost two-fold greater than the amount of S found in the ubiquitinated protein fraction. These data indicate that selenoproteins are preferentially ubiquitinated and targeted for proteasomal removal. To further assess if the proteasome alleviates Se toxicity, *Arabidopsis* plants with three different mutations in the 26S proteasome were treated with selenate. All three mutants exhibited elevated selenate sensitivity compared to wildtype plants.

However, the ability of the proteasome to remove malformed selenoproteins is dependent upon the level of stress, speciation of selenium, and possibly plant species. A follow-up study examined the effect of mild and severe selenite stress on the UPP in the free alga *Chlamydomonas reinhardti* (Vallentine et al., 2014). Proteasome inhibition exacerbated selenite sensitivity at 50 and 200 µM. Algae treated with 50 µM selenate had increased proteasome activity and levels of ubiquitinated proteins; chemically inhibiting the proteasome with MG132 at this concentration of selenite increased the amount of selenium in protein, providing strong evidence that the proteasome removes nonspecific proteins in lower plants. However, a different story emerged when *Chlamydomonas* was challenged with 200 µM selenate. At this concentration, there was a 2-fold increase in Se in protein compared to 50 µM, but the amount of Se in protein was not affected by proteasome inhibition. This result was explained by the selenite-induced oxidative stress that had already inhibited the proteasome at this high concentration. In line with this conclusion, there was a decrease in proteasome activity and levels of ubiquitinated proteins at 200 µM selenite. Proteasome inhibition during severe stress was attributed to the accumulation of ROS, including mitochondrial superoxide, which can directly impair the UPP in non-plant model systems (Huang et al., 2013).

The same effect of severe Se stress on the UPP was also observed in *Brassica napus*. In this study, selenite-induced superoxide coincided with decreased proteasome activity and levels of ubiquitinated proteins (Dimkovikj and Van Hoewyk, 2014). However, treating the plants with selenocysteine had the opposite effect, as this increased proteasome activity and

ubiquitinated proteins (Dimkovikj et al., 2015). Moreover, proteasome inhibition increased the amount of Se in protein in SeCys-treated plants. Taken together, even though Se-induced oxidative stress might decrease proteasome activity, these data suggest that the proteasome can remove misfolded selenoproteins **(Fig 3**).

Analysis of selenium-treated *Arabidopsis* plants with mutations in proteins involved in the endoplasmic reticulum-associated degradation (ERAD) of misfolded proteins also supports the malformed selenoprotein hypothesis. The chaperone binding protein 2 (Bip2) resides in the ER lumen where it binds to unfolded proteins prior to their retrograde transport and proteasomal degradation in the cytosol. *bip2-1* seedlings were severely affected by selenocysteine and failed to germinate (Sabbagh and Van Hoewyk, 2012). Likewise, mutations in the HRD complex, which localizes to the ER membrane and facilitates retrograde transport of misfolded proteins, also decrease selenium tolerance (Van Hoewyk, 2016).

It is also worthwhile to discuss a recent study in yeast that also supports the involvement of proteasomes in the removal of nonspecific selenoproteins. In this study, a cysteine to selenocysteine in protein resulted in protein aggregation (Lazard et al., 2017). A mutant yeast strain unable to synthesize SeCys suppressed protein aggregation, which importantly demonstrates the link between SeCys and protein misfolding. This study also observed an enrichment of genes involved in the assembly of the proteasome in yeast sensitive to Se.

An end target of Se-induced oxidative stress are oxidized proteins; therefore, measuring oxidized proteins may serve as a better biomarker for oxidative stress compared to activities of enzymes (*e.g*. SOD and catalase) or levels of metabolites (*e.g*. GSH:GSSG). *Chlamydomonas* challenged with selenite for 3, 8, and 48 h increased levels of oxidized proteins at all time points, but was not time-dependent (Vallentine et al., 2014). A similar story emerged in selenate-treated *Stanleya pinnata;* in this study, selenate increased levels of oxidized proteins. Importantly, inhibition of the proteasome resulted in a severe accumulation of oxidized proteins, demonstrating that proteasomes can remove oxidized proteins caused by selenium stress (Sabbagh and Van Hoewyk, 2012) similar to other stressors that generate ROS.

Additionally, it is also possible that Se-induced nitroproteins may be removed by proteasomes. Pioneering work by Souza et al. (2000) demonstrated that nitroproteins in mammalian cells can be degraded by 20S proteasomes, and this has now been extended to plants (Tanou et al., 2012). Recently, nitrated abscisic acid receptor PYR/PYL/RCAR was shown to become polyubiquitylated and consequently it underwent proteasome-regulated degradation (Castillo et al., 2015), thus providing experimental evidence for proteasomal

degradation of plant nitroproteins. Deciphering if selenium-induced nitroproteins are also proteasomally degraded is the task for future research.

Additional (non-proteomic) processes contributing Se phytotoxicity

As the result of oxidative stress, selenium as a stressor is often responsible for the development of lipid peroxidation and cell death in plants like wheat, *Arabidopsis*, bean, turnip or rice (Łabanowska et al., 2012; Lehotai et al., 2012; Mroczek-Zdyrska and Wójcik, 2012; Chen et al., 2014; Mostofa et al., 2017). Moreover, in *Coffea* cell suspensions both 0.05 and 0.5 mM selenite enhanced the amount of lipid peroxidation product after 24 hours of treatments (Gomes-Junior et al., 2007). These results indicate that besides proteins also lipids are oxidized by selenium-induced ROS.

Recent evidences show that the hormonal system is highly affected which is partly responsible for growth alterations in selenium-stressed plants. According to Lehotai et al. (2012, 2016a) growth regulators like auxins, cytokinins and ethylene (ET) are involved in Se-induced root growth responses. Furthermore, transcriptomic analyses revealed that selenium upregulates ET- and jasmonic acid (JA)-associated genes in *Arabidopsis* and mutants defective in ET or JA signaling exhibited Se sensitivity relative to the wild type (Van Hoewyk et al., 2008). Also, in tea plants, selenite treatment resulted in the upregulation of ET and JA biosynthetic genes and genes encoding the ethylene-response factor (EFR) (Cao et al., 2018). These suggest a relevant role for especially ET and JA as stress hormones in Se tolerance.

Selenium uptake interferes with other micro- and macroelements resulting in disturbed element homeostasis in organs of selenium-supplemented plants. As an example selenium at a low concentration (2 mg kg^{-1}) results in enhanced nitrate reductase activity, nitrogen content and sulfur content in wheat (Iqbal et al., 2015). Regarding Se-S interactions, some fundamental observations were made by White et al. (2004) who found that external sulfate inhibits selenate uptake of *Arabidopsis*, but rhizosphere selenate induces sulfate uptake due to the preventing effect of Se on the inhibition of S transporters in the presence of excess S. It was also stated that the reason for Se toxicity is partly manifested by interfering with sulfur metabolism due to competition between Se and S for biochemical processes (White et al., 2004). As for other micro- and macroelements, Se supplementation has various effects on them in different plant species (e.g. Filek et al., 2010; Zembala et al., 2010; Hawrylak-Nowak et al., 2015) resulting in disturbance of element homeostasis in plant organs which in turn may contribute to Se phytotoxicity.

Conclusion and future perspectives

The processes of Se toxicity observed so far are only partly specific for selenium. The incorporation of Se into amino acids and proteins gives rise the formation of malformed selenoproteins considered to be toxic. At the same time, the proteome is targeted not only by direct Se incorporation but also by Se-induced reactive species, like ROS and RNS resulting in protein oxidation and nitration **(Fig 4)**. Excess selenium-induced oxidative and nitrosative protein modifications can be considered as secondary, non-Se-specific toxicity processes. While Se-specific modifications primarily affect the proteome, the effects of oxidative and nitrosative stresses are widespread, because lipids and nucleic acids can also be affected. Future research has to answer the following question: to what extent are ROS- and RNS-induced or Se-specific modifications of the proteome responsible for Se phytotoxicity? The identification of nitroproteins and examination of other RNS-induced PTMs (e.g. S-nitrosylation) in selenium-exposed plants can also provide insights into nitrosative processes responsible for Se phytotoxicity.

Another interesting idea which needs further testing is whether or not Se-tolerant plants possess enhanced proteasome activity compared to Se-sensitive plants. Under this scenario, increased proteasome activity in Se-hyperaccumulating plants may allow these unique plants to be more fit in seleniferous soils. Already it has been demonstrated that the Se hyperaccumulator *Stanleya pinnata* can preferentially remove selenoproteins, while proteasome activity decreases in more sensitive plants treated with selenium (Dimkovij and Van Hoewyk, 2014; Vallentine et al., 2014). Furthermore, within the past year, two different groups have performed transcriptomic studies aimed at identifying genes that are differentially regulated in Se-treated hyperaccumulators (*Stanleya pinnata* and *Cardamine hupingshanensis*). Intriguingly, both studies identified increased expression of genes encoding components of the ubiquitin proteasome pathway in the Se hyperaccumators compared to the non-hyperaccumulators (Wang et al., 2018; Zhou et al., 2018). However, it still has not been experimental determined if Se hyperaccumulators have elevated proteasome activity compared to non-hyperaccumulators.

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AUTHOR STATEMENT

ZsK wrote the manuscript, prepared figures and organized the preparation of the manuscript. DVH participated in writing and figure preparation. Also GF and ÁM participated in writing.

Figure legends

Fig 1 Uptake and assimilation of selenate and selenite by higher plants. Abbreviations: SeO_4^{2-} selenate; SeO_3^{2-} selenite; HAST high affinity sulfate transporter; PHT high affinity phosphate transporter; SeMet selenomethionine; APSe adenosine phospho selenate; Se^{2-} selenide; OAS *O*-acetylserine; SeCys selenocysteine; SeMeCys methyl-selenocysteine; DMDSe dimethyldiselenide; Ala alanine; Se^0 elemental selenium; OPH *O*-phosphohomoserine; Secystatione selenocystatione; SehomoCys seleno-homocysteine; methyl-SeMet methyl-selenomethionine; DMSeP dimethylselenoproprionate; DMSe dimethylselenide. Numbers indicate enzymes as follows: (1) ATP sulphurylase; (2) adenosine phosphosulphate reductase; (3) sulfite reductase; (4) O-acetylserine thiol lyase; (5) selenocysteine methyltransferase; (6) selenocysteine lyase; (7) cysthathionine-γ-synthase; (8) cysthathionine-β-lyase; (9) methionine synthase; (10) methionine methyltransferase; (11) dimethylselenoproprionate lyase; (12) γ-glutamyl-cysteine synthetase (modified after Pilon-Smits and Quinn 2010).

Fig 2 The negative effect of excess selenium on plants. Control (A) and 100 µM selenite-treated pea (adapted from Lehotai et al., 2016, bar=5 cm), shoot of control (C,E) and 100 µM selenite (D) or selenate (F)-treated Brassica juncea (adapted from Molnár et al., 2018a,b, bars=3 cm).

Fig 3 Schematic overview of plant metabolism that generates and removes or prevents nonspecific selenoproteins containing selenocysteine. Enzymatic reactions are in red, and the nonenzymatic step mediated by glutathione is in blue. Abbreviations: SeCys- selenocysteine; GSH- glutathione; HSe- selenide; Se^0- elemental selenium; CS- cysteine synthase; SiR- sulfite reductase; CpNifS- chloroplastic selenocysteine lyase; SMT- selenocysteine methyltransferase; CgS- cystathionine gamma-synthase.

Fig 4 Summarizing model of selenium-induced alterations in ROS (superoxide radical, hydrogen peroxide) and RNS (nitric oxide, S-nitrosoglutathione, peroxynitrite) metabolism and protein-level consequences (selenoprotein formation, protein oxidation, protein nitration). High concentrations of selenium result in non-specific selenoprotein formation which is related to Se toxicity. Excess selenium induces NADPH-oxidase (NOX) activity which produces superoxide radical ($O_2^{\cdot-}$) (Kolbert et al., 2018). At the same time, selenium depletes glutathione (GSH) pool which can be explained by that glutathione reacts with Se yielding selenoglutathione and superoxide radical (Wallenberg et al., 2010). Superoxide dismutases (SODs) convert

superoxide to hydrogen peroxide (H_2O_2) which in turn oxidizes proteins. Selenium induces nitric oxide (NO) production through nitrate reductase (NR) and/or nitric oxide synthase (NOS) activities (Chen et al., 2014) and NO influences ROS production through the regulation of NOX (Yun et al., 2011). NO reacts with GSH and S-nitrosoglutathione (GSNO) is formed which can be reduced by S-nitrosoglutathione reductase (GSNOR) enzyme (Wink et al., 1994). Another molecule which reacts with NO is superoxide yielding peroxynitrite ($ONOO^-$) which is primarily responsible for protein tyrosine nitration (Sawa et al., 2000). Protein level changes (selenization, oxidation, nitration) lead to the inactivation of certain proteins which in turn are removed from the active proteome by proteasomal degradation. The affected amino acids are indicated. Selenium (hyper)accumulator plants possessing selenocysteine methyltransferase (SMT) enzyme are able to redirect selenoamino acids from protein synthesis (Neuhierl and Böck, 1996).

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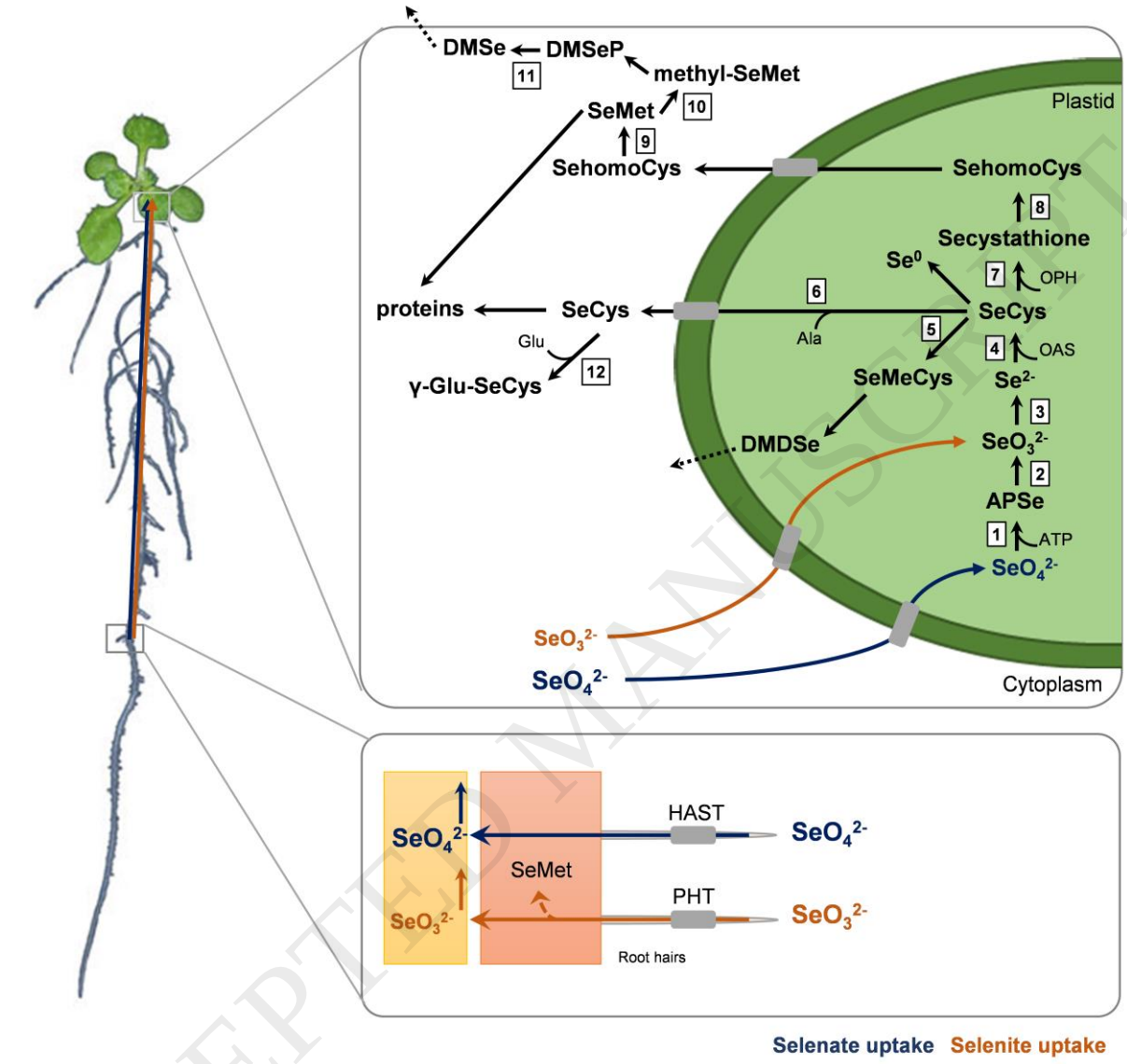
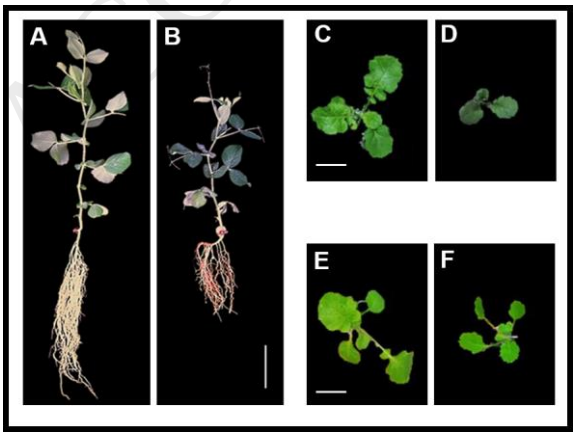
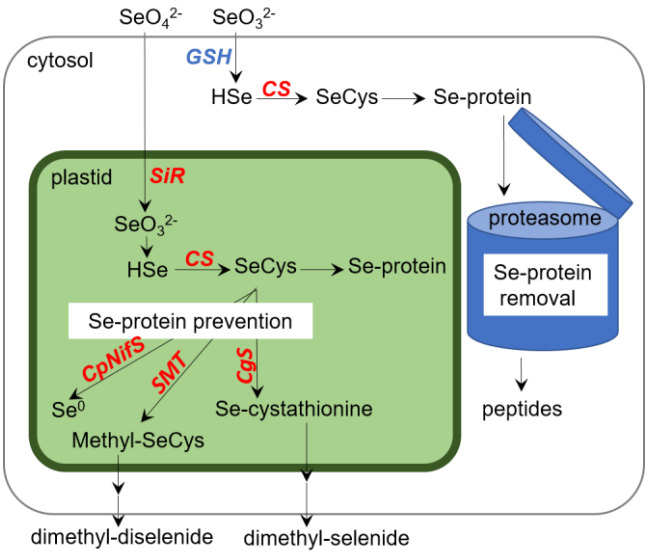
Figures







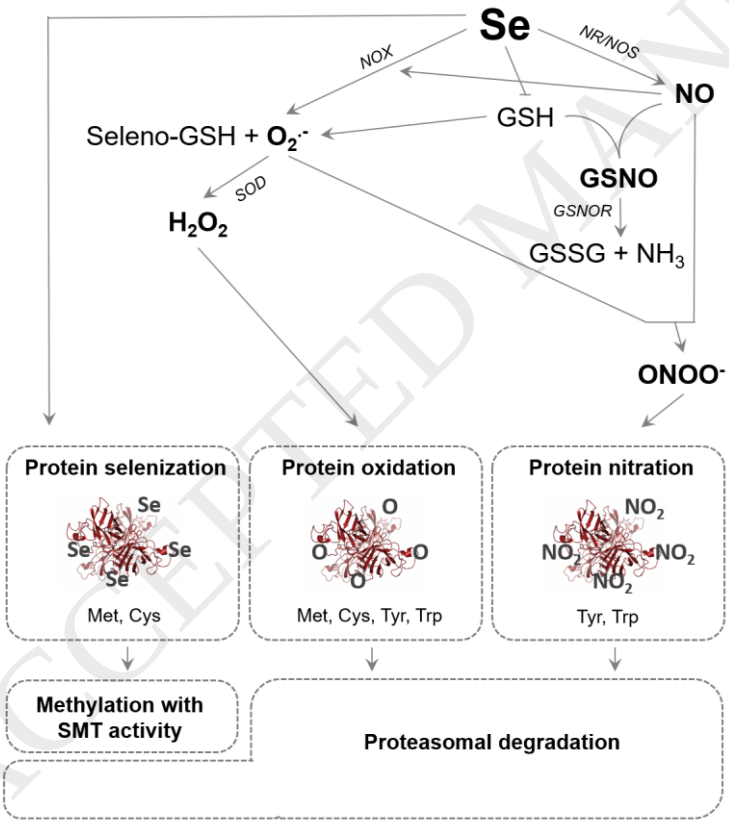


Table:

Table 1 Examples for reactive oxygen- and nitrogen species modified by different selenium forms and concentrations in different plant species.

Reactive species	Se form and concentration	Plant species/organ	Effect of Se application	Reference
Superoxide radical ($O_2^{\cdot-}$)	15 µM selenite	A. thaliana/cotyledon	increase	Tamaoki et al., 2008
	20 µM selenate	B. juncea/root	decrease	Molnár et al., 2018a
	100 µM selenite	B. juncea/root	increase	Molnár et al., 2018a
	30, 60, 120, 230, 460 µM selenite	B. rapa/root	increase	Chen et al., 2014
	20, 50, 100 µM selenite	A. thaliana/root, B. juncea/root	increase	Molnár et al., 2018b
	50 µM selenite	B. napus/root	increase	Dimkovikj and Van Hoewyk, 2014
	50 µM selenate	A. membranaceus/root cotyledon	increase	Kolbert et al., 2018
	250, 500, 750, 1500 µM selenate	O. sativa/leaf	increase	Mostofa et al., 2017
	20, 40 µM selenite or selenate	B. oleracea/leaf	increase	Tian et al., 2017
	40 µM selenate	A. thaliana/shoot	increase	Grant et al., 2011
	20 µM selenate	S. pinnata/leaf S. albescens/leaf	increase	Freeman et al., 2010
	6 µM selenite	V. faba/root	increase	Mroczek-Zdyrska and Wójcik, 2012
Hydrogen	5, 10, 20, 40, 60, 80, 120 µM selenate or selenite	L. sativa	increase	Ríos et al., 2009

peroxide (H_2O_2)	15 µM selenite	A. thaliana/cotyledon	increase	Tamaoki et al., 2008
	300 mg/L selenate	O. europea/pollen	increase	Tedeschini et al., 2015
	4 and 6 ppm selenate	V. faba seedlings	increase	Aggarwal et al., 2011
	50, 100 µM selenite	P. sativum/leaf, root	increase	Lehotai et al., 2016b
	50, 100 µM selenite	A. thaliana/root B. juncea /root	increase	Molnár et al., 2018b
	100, 250, 500, 750, 1500 µM selenate	O. sativa/leaf	increase	Mostofa et al., 2017
	10,20,40 µM selenite	A. thaliana/root	increase	Lehotai et al., 2012
total ROS	30, 60, 120, 230, 460 µM selenite	B. rapa/root	increase	Chen et al., 2014
Nitric oxide (NO)	30, 60, 120, 230, 460 µM selenite	B. rapa/root	increase	Chen et al., 2014
	10, 20, 40 µM selenite	A. thaliana/root	decrease	Lehotai et al., 2012
	10, 50, 100 µM selenite	P. sativum/leaf	increase	Lehotai et al., 2016b
	20, 50 µM selenite	A. thaliana/root	decrease	Molnár et al., 2018b
	50 uM selenate	A. membranaceus/ root, cotyledon	increase	Kolbert et al., 2018
Peroxy-nitrite (ONOO-)	50, 100 µM selenate	B. juncea/root	decrease	Molnár et al., 2018a
	50, 100 µM selenite	B. juncea/root	increase	Molnár et al., 2018a
	20, 50, 100 µM selenite	A. thaliana/root B. juncea/root	increase	Molnár et al., 2018b
	50, 100 µM selenate	A. membranaceus/ root, cotyledon	increase	Kolbert et al., 2018

S-nitroso-glutathione (GSNO)	50, 100 µM selenate	A. membranaceus /root A. bisulcatus/root, cotyledon	decrease	Kolbert et al., 2018
	50, 100 µM selenate	A. membranaceus/ cotyledon	increase	Kolbert et al., 2018