Selenium Dynamics in Cereal Biofortification

Optimising Fertiliser Strategies and Assessing Residual Fate

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Abstract

Selenium (Se) is an essential micronutrient for humans and animals and hence, a low intake of Se in the diet can lead to health problems. The application of Se fertilisers to staple crops, a process called agronomic biofortification, can effectively improve humans' Se intake levels. The overarching aim of this study was to develop improved strategies for Se biofortification through an enhanced understanding of Se dynamics in arable systems.

A pot trial was set up to investigate whether the application of 3.33 μg kg^{-1} of Se (equivalent to 10 g ha^{-1}) to wheat can be made more efficient by its co-application with macronutrient carriers, either to the soil or to the leaves. In the soil, Se was applied either on its own (selenate only) or as a granular, Se-enriched macronutrient fertiliser supplying nitrogen, phosphorus, potassium or sulphur. Selenium was also applied to leaves at head emergence with, or without, 2% w/v N fertilisers. With grain Se concentrations varying from 0.13–0.84 mg kg^{-1}, soil application of selenate-only was 2–15 times more effective than granular Se-enriched macronutrient fertilisers in raising grain Se concentrations. Foliar Se application was superior to soil-applied Se treatments in increasing grain Se levels, especially when foliar Se was co-applied with an N carrier. Under foliar Se+N treatments, grains accumulated twice as much Se as those fertilised with foliar Se only, the majority of which was highly bioavailable (selenomethionine). This study was perhaps the first to show the efficiency of co-applying foliar Se with N in improving Se uptake and recovery in wheat. Such findings support the hypothesis that the efficacy of existing agronomic practices for Se biofortification can be improved through the use of macronutrient carriers, which could potentially reduce costs associated with fertiliser application and management.

The second experiment shed light on the residual fate of Se in different soils over a 300-day period, using both chemical and biological assays to estimate Se availability. Eight soils varying in physicochemical properties were spiked with 0.5 mg kg^{-1} Se in the form of sodium selenate and incubated at 25°C for different periods (1, 30, 60, 90 and 300 d). At the end of the ageing period, soil Se was fractionated by sequential extraction procedures into soluble, adsorbed and organically-bound Se fractions. Simultaneously, a pot trial was set up where wheat was grown in the Se-aged soils for six weeks. A rapid decline in Se solubility (> 50% within 24 h) was observed in the Oxisol, probably due to its high mineral oxides and clay contents. Over time, calcareous soils showed more pronounced Se ageing than non-calcareous soils as solubility reached 0 at 300 d, probably due to the fixation of Se onto calcite surfaces. In highly calcareous soils, plant Se concentrations decreased from 37 mg kg^{-1} to < 5 mg kg^{-1} within 30 days. Comparable Se concentrations were only observed > 100 days in plants grown in non-calcareous soils. The soluble Se fraction at specific ageing times was best represented by a reversible first order model, and was primarily influenced by soil pH. Understanding how added Se behaves in soils over time could be used to make more informed decisions about the rate and frequency of Se fertiliser application in agronomic biofortification programs.

The third experiment was undertaken to investigate time-dependent changes in the uptake and partitioning of Se in wheat. It also investigated whether the uptake efficiency of Se in wheat was influenced by timing of fertiliser application. In a pot trial, 3.33 µg kg^{-1} Se was as ^{77}Se-enriched sodium selenate (Se_{fert}) to wheat at two growth stages – stem elongation (GS1) and heading stage (GS2), by two methods – soil and foliar (foliar Se on its own and foliar Se + 2% urea-N). Wheat was harvested 3, 10 and 17 d and 3, 10, and 34 d after Se application at GS1 and GS2, respectively. Only foliar treatments were effective in raising grain Se concentrations (> 0.25 mg kg^{-1}) above the

target level of 0.1 mg kg^{-1} for biofortification. However, the poor efficiency of the soil-applied Se fertiliser was speculated to be predominantly caused by accidental leaching of the applied Se from the free-draining pots. This study showed that, when applied at an early growth stage, foliar Se with N improved the uptake of Se into wheat, compared to foliar application of Se on its own. At the later growth stage, N inclusion to foliar Se fertilisers significantly increased grain Se concentration in the grain (0.32 mg kg^{-1}) compared to foliar Se on its own (0.26 mg kg^{-1}), the majority of which was highly bioavailable. Speciation analysis data of the foliar-treated leaves suggested that the presence of N in foliar solutions improved the assimilation and translocation of organic Se compounds. Practical knowledge gained about the optimisation of Se fertiliser formulation, method and timing of application will be of importance in refining biofortification programs across different soil and climatic regimes.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Chandnee Ramkissoon

Preface

This project was undertaken to obtain a fuller understanding of Se dynamics in agronomic systems. It addressed, more specifically, the potential improving current Se fertiliser strategies associated with biofortification and the transformation of applied Se within arable systems over time. The project was a collaborative venture between the University of Adelaide and the University of Nottingham.

The thesis consists of five chapters and an Appendix. The experimental chapters (Chapter 2, 3 and 4) have been written in manuscript style for publication in scientific journals. Hence, some unavoidable overlap in the information presented, especially between the introductory chapter (Chapter 1) and in the introduction sections of the experimental chapters.

Chapter 1 covers extensive literature about the research topic. Special emphasis was put on presenting up-to-date research findings about fertilisation strategies and current understanding of Se behaviour in the soil in a clear and concise manner. In the light of such literature, the aim and objectives of the project were set.

Chapter 2 describes an experiment carried out at the University of Adelaide, investigating ways of improving the efficacy of Se fertilisers for wheat biofortification. This chapter was published in Scientific Reports (see Appendix). Minor changes, predominantly stylistic in nature, were made to keep the presentation of the chapter consistent with subsequent ones.

Chapter 3 describes an experiment undertaken at the University of Adelaide, which aimed to assess the effect of soil properties and contact time on the availability of Se in soils varying in physicochemical properties.

Chapter 4 describes an experiment carried out at the University of Nottingham, which assessed the effect of Se fertiliser formulation, method and timing of application on its transfer within wheat.

Chapter 5 is a summary and critical analysis of the findings contained within the study. It also gives recommendations for future work.

The Appendix consists of a copy of the published research article and some additional information to support observations reported in the text.

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Over these last four years, I have crossed paths with many individuals, who have, in one way or another, helped shape this experience. I wish to sincerely thank the following people for making a positive impact on my journey.

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List of publications and conference presentations

Publications

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Conference presentations

Ramkissoon C., Degryse, F., McLaughlin, M. J., da Silva, R. C, Baird, R., Young, S. D. Wheat biofortification using selenium-enriched macronutrient fertilisers. 2017. 5th International Conference on Selenium in the Environment and Human Health. Stockholm, Sweden.

Ramkissoon C., Degryse F., McLaughlin M. J., Young, S. D., Bailey, E. H. Assessing the effect of soil properties and contact time on the ageing of selenium. 2019. 6th International Conference on Selenium in the Environment and Human Health. Yangling, China.

Ramkissoon C., Young, S. D, Bailey E. H., Degryse F., McLaughlin, M. J. 2019. Optimising fertiliser strategies for maximal wheat nutritional quality. 2019. 22nd Annual Conference of the International Fertiliser Society. Cambridge, United Kingdom.

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1 Introduction and Literature Review

1.1 Introduction

Selenium (Se) is an essential micronutrient for humans, animals and certain lower plants. Its biological importance was first flagged by Schwarz and Foltz in 1957, as they demonstrated the role of Se in preventing muscle dystrophy and liver necrosis in rats (Hartikainen, 2005). Later on, the discovery of Se in enzymes such as glutathione peroxidase (GPx) highlighted its importance in preventing oxidative cell damage and maintaining a healthy immune system in humans (Garousi, 2017). A suboptimal intake of Se in the diet can lower the functionality of the immune system and increase disease susceptibility, while a more overt deficiency can lead to physiological disorders such as dilated cardiomyopathy and skeletal muscle myopathy (Rayman, 2000). The worst cases of Se deficiency have been observed in low-Se areas in China and Eastern Siberia, leading to two endemic conditions, namely Keshan and Kashin-Beck diseases. Keshan disease is characterised by myocarditis, affecting mostly children (2 – 10 years old) and women of child-bearing age while Kashin-Beck is a rheumatoid condition causing enlarged joints, shortened legs and fingers, and in extreme cases, dwarfism (Hartikainen, 2005). However, an excess of Se, albeit less frequent than deficiency, is also detrimental to humans. The narrow range between Se deficiency and toxicity is the reason why Se is called the “double-edged sword” element (Brozmanova´ et al., 2010). Almost two decades ago, Combs (2001) determined that about 0.5 - 1 billion people worldwide were not consuming enough Se in their diet, and hence were at risk of health diseases. With the world population having reached 7.8 billion people in 2019, a quarter of whom is suffering from the ‘hidden hunger’ of micronutrient deficiency (United Nations, 2019), there is an urgent need to move towards nutritionally-sensitive and sustainable agricultural practices. The agronomic biofortification of staple crops could be an effective way of sustainably increasing dietary levels of Se (Lyons, 2018). One success story of Se biofortification is Finland. In response to a decline in dietary Se intake in its population, the Finnish Government

made it compulsory to enrich compound fertilisers with sodium selenate, which over the years, has increased Se concentrations in animal feeds, primary food groups and human blood serum (Eurola et al., 2000).

However, the success of biofortification programs is dependent on several factors, such as the specific properties of and the climatic conditions at the site at which the program is being set up as well as the staple crops of the population targeted, amongst others. An ideal biofortification program would strike a balance between practicality, efficiency and economic viability. In the case of Se, variables such as soil conditions and climate affect the efficacy of Se uptake into plants, and hence should be considered when optimising fertiliser strategies for Se biofortification. This balance would ensure the maximal nutritional quality of crops with minimal financial inputs, which would be a significant financial incentive for all stakeholders – from farmers to governments – to adopt proposed Se fertilisation strategies. Another aspect to consider prior to setting up biofortification programs is the residual impact of added fertilisers on the environment and on subsequent crops. Recently, Se biofortification studies have established that, although the utilisation rate of added Se by plants is < 50%, the residual Se is not available for plant uptake by the second season due to strong retention in soil and/or leaching. (Chilimba et al., 2012a; Lyons, 2018; Mäkelä et al., 1993; Mathers et al., 2017). The poor residual fate of Se fertilisers in the soil highlights the relative safety and effectiveness of agronomic biofortification. However, it also implies that annual fertiliser applications are required to reach the targeted Se concentrations in plants grown for human consumption, hence increasing the costs associated with this strategy (Mathers et al., 2017).

In light of existing literature about Se biofortification strategies, this study will aim to develop novel formulations and methods of applications in order to optimise Se uptake by staple crops. At the same time, it will also investigate the mechanisms of Se

retention in soils varying in physicochemical properties. The aggregation of such knowledge will be of importance in refining biofortification programs across different soil and climatic regimes.

1.2 Selenium in the environment

Soil concentrations of Se are highly variable, ranging from nearly zero to several thousand mg Se kg^{-1} (Hartikainen, 2005; Oldfield, 1999). Most soils contain 0.1 – 2.0 mg Se kg^{-1} (world mean of 0.4 mg kg^{-1}), and generally, soils containing < 0.6 mg Se kg^{-1} – low Se areas – are likely to produce crops with insufficient Se (< 0.1 mg kg^{-1}) (Fig. 1.1). On the other hand, concentrations up to 1200 mg Se kg^{-1} can be observed in seleniferous areas, for example, in Ireland (Fleming and Walsh, 1956). The unevenness of soil Se distribution around the world highlights the variability of soil types.

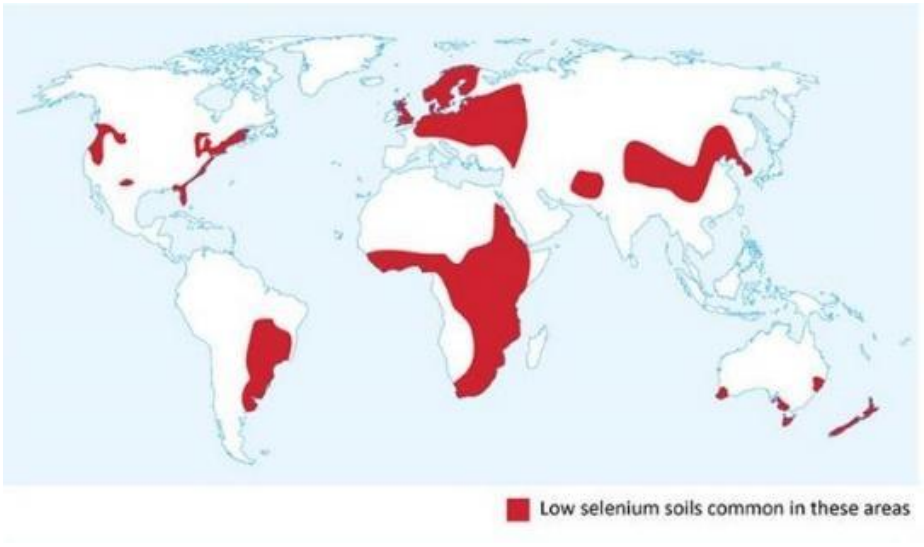


Fig. 1.1: Selenium-poor areas around the world, taken from Lyons et al. (2018).

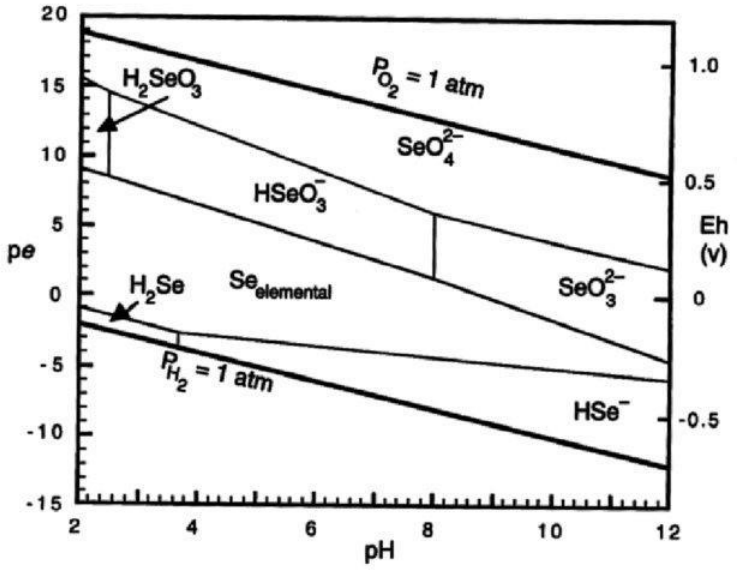
Selenium is mainly derived from parent material and, hence, its content in the soil depends on the nature and composition of the parent material; Se-deficient soils tend to be derived from igneous rocks while Se-rich areas are usually formed of sedimentary deposits (Fordyce, 2007). Additional factors include processes that add or remove Se, such as groundwater flow, precipitation, leaching and anthropogenic activities such as

458 the burning of coal (Shamberger, 1981; Sun et al., 2016). Although geology primarily
459 influences the total Se concentration of soils, the *bioavailability* of Se depends on the
460 chemical speciation of Se and soil characteristics such as pH, redox potential, texture
461 and the presence of competitive ions (Fordyce, 2007).

462 1.3 Chemistry of Se in soils

463 As with any other trace metal, when Se is freshly added to the soil, the
464 partitioning between soil solution and the solid phase will change with time until an
465 equilibrium is reached (Hamon et al., 2007). This process is referred to as the natural
466 attenuation of metal availability, or fixation and ageing. The rate at with Se fixation
467 occurs is likely to depend not only on time, but also, on the chemical behaviour of Se
468 and soil properties.

469 Selenium can exist in soil as fully oxidised selenate (SeO_4^{2-}, Se^{VI}), selenite
470 (SeO_3^{2-}, Se^{IV}), reduced elemental Se (Se^0), selenide (Se^{2-}), methylated Se species and
471 organically-bound forms (Elrashidi et al., 1987). The organic species include both
472 'pure' compounds such as selenocysteine and poorly characterised humus-bound
473 forms (Kang et al., 1991). The chemical speciation of Se, influenced by soil variables
474 such as pH and redox potential, determines its mobility and bioavailability in the soil
475 (Elrashidi et al., 1987) (Fig. 1.2).



476

477 Fig. 1.2: Stability of individual Se species as a function of pH and redox potential
478 (Drever, 1997).

479 With respect to the *inorganic* Se species, under fully oxidizing conditions and
480 high pH (pH + pe > 15.0), SeO_4^{2-} tends to predominate in the soil solution. Within the
481 mid redox range (pH + pe = 7.5 – 15.0), the formation of SeO_3^{2-} or hydrogen selenite
482 ($HSeO_3^-$) ions (pKa = 7.3) would be favoured; in more reducing conditions, metal
483 selenides or Se^0 may be prevalent (Masscheleyn et al., 1990). Despite both ions being
484 anionic oxyacids, Se^{VI} is generally more mobile and more bioavailable than Se^{IV-} as it
485 is much more weakly bound to soil surfaces than Se^{IV} (Keskinen et al., 2009).

486 Selenium ions bind to soil surfaces by different mechanisms, and the strength
487 of the bonds formed between Se and soil components depends on factors such as the
488 chemical speciation of Se, properties of the sorbent phase and the ionic strength of the
489 soil solution. Generally, Se^{VI} tends to adsorb primarily by outer-sphere complexation,
490 although a mixture of inner- and outer-sphere complexation can occur at low pH, while
491 Se^{IV} adsorbs more strongly by an inner-sphere mechanism on sesquioxides and clay
492 mineral edges (Peak and Sparks, 2002). In alkaline calcareous soils, Se can be
493 retained by precipitation onto surfaces of calcite. Early structural studies by Lamble et
494 al. (1995) showed that the Se^{VI} ions can be retained on calcite by substitution with the

carbonate group. More recent studies contradicted these findings by showing that only Se^{IV} formed precipitate on calcite, forming calcium selenite ($CaSeO_3$. H_2O), and not Se^{VI}. They attributed the selective retention of Se ions to the fact that Se^{VI} is tetrahedral in shape while Se^{IV} assumes a pyramidal-trigonal coordination, which makes the substitution with a carbonate (planar-trigonal shape) easier (Putnis et al., 2013; Renard et al., 2013). Selenite is less mobile and bioavailable than Se^{VI}, not only because of its stronger adsorption capacity, but also because it desorbs at a much slower rate from sorbent phases than Se^{VI} (Hingston et al., 1967). Hydroponic experiments seem to suggest that, when supplied with both ions at the same rate, plants preferentially take up Se^{IV} over Se^{VI} (Li et al., 2008; Longchamp et al., 2013). However, in the soil, assuming that leaching is minimal, the net effect is that Se^{IV} would be rather rapidly adsorbed and made unavailable for plant uptake compared to Se^{VI} (Mayland et al., 1991).

The presence of soil organic matter (SOM) strongly affects Se availability for plant uptake as Se can rapidly form associations with SOM (Tolu et al., 2014). Gustafsson and Johnsson (1994) showed that microbial reduction of Se to lower valence states (lower than Se^{IV}) and subsequent incorporation into humic substances was the primary driving mechanism for Se retention in SOM-rich soils. In such environments, the abiotic surface adsorption of inorganic Se^{IV} was a relatively unimportant, transitory step compared to microbially-assisted Se fixation in SOM fractions. This research also highlighted that, in reality, Se sorption into SOM did not occur by one straightforward mechanism, but rather by several processes operating simultaneously (Gustafsson and Johnsson, 1994). Selenium associates with the different fractions of SOM, such as humic acids (HA) and fulvic acids (FA) by different bonds, and depending on the strength of the bonds formed, SOM can be a source (solubilisation of SOM-Se) or sink (immobilisation) of Se in the soil (Wang et al., 2012).

Selenium binds more weakly to fulvic acids (FA-Se) and forms somewhat unstable complexes, which could solubilise to form a source of bioavailable Se. In comparison, humic acid bound-selenium (HA-Se) complexes are stronger, less easily broken down, and not a source of bioavailable Se (Qin et al., 2012) (Fig. 1.3) According to Li et al. (2017), the source-sink effect of SOM-Se in natural soils exists simultaneously; however, compared to immobilisation, Se release from SOM is a slow process which is relatively hard to detect.

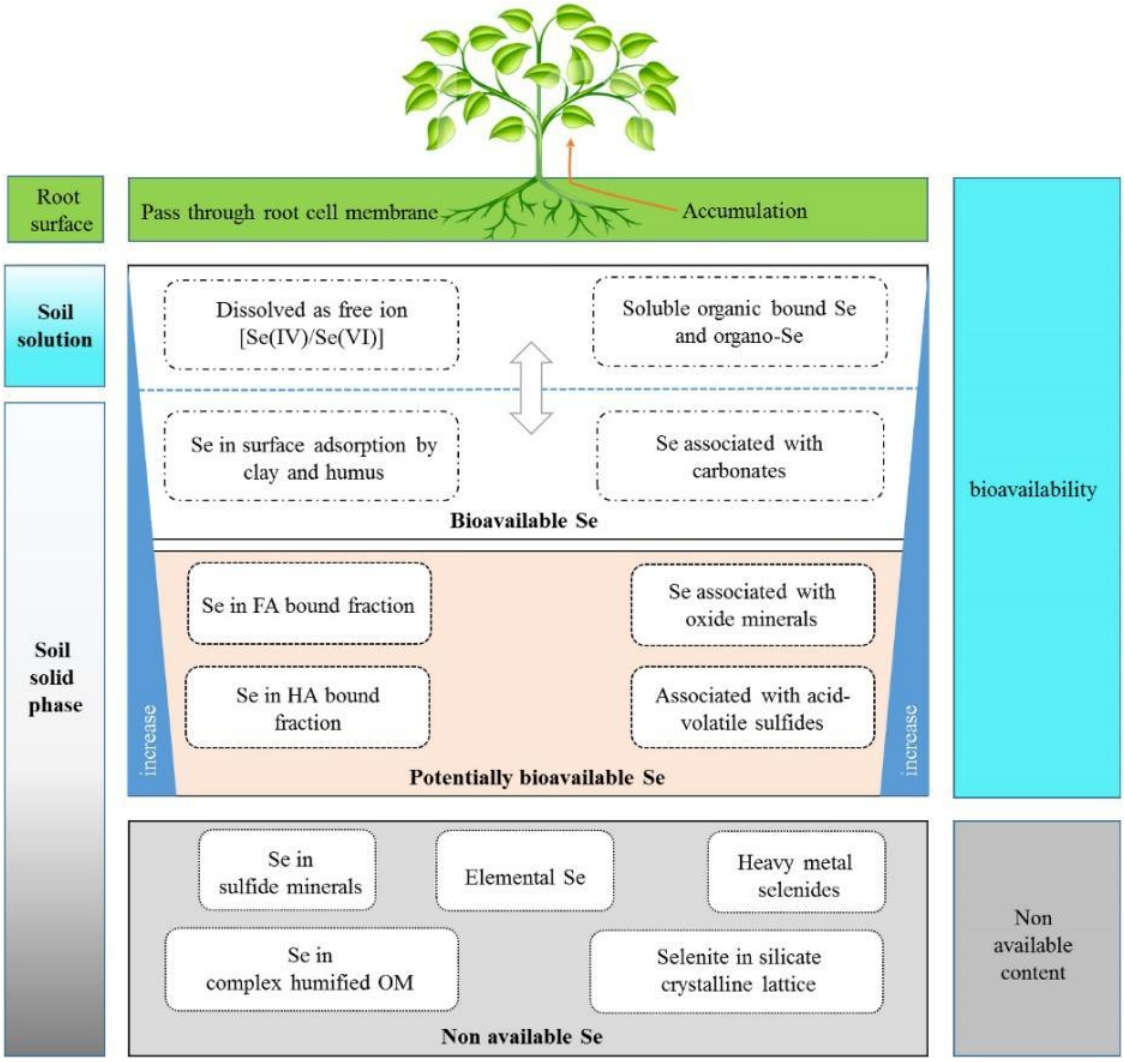


Fig. 1.3: The different processes affecting Se bioavailability in the soil-plant system (Dinh et al., 2019).

Given the nature of agronomic biofortification programs, understanding and potentially also predicting how the mobility of added soluble Se fertilisers varies with time and other factors such as soil characteristics and climate, is pivotal to the success

534 of the program. The bioavailability of metals in soil can be assessed using both
535 chemical and biological methods, although chemical methods are usually appraised
536 for their simplicity and rapidity compared to their biological counterparts (Hamon et al.,
537 2007). The chemical methods to used assess metal availability in soil are single or
538 sequential extractions, or more recently, isotope dilution (ID) techniques and diffusive
539 gradients in thin-films (DGT). The isotope dilution technique uses radioactive isotopes
540 to analyse chemically reactive, or “labile” fractions of metals in soil and discriminate
541 between the native and the exogenous metal source (Young et al., 2000); it is relatively
542 simple, rapid and efficient as only a small spike is required to be measurable in soil
543 extracts (*E* value) as well as plant samples (*L* value) (Smolders et al., 1999). On the
544 other hand, this technique is restricted to elements with suitable isotopes (Atkinson et
545 al., 2011). The DGT technique provides a true reflection of metal lability in soil as it is
546 founded on kinetics rather than equilibria: using a layer to metal resin to locally lower
547 metal concentration in soil and mimic plant uptake, as well as a gel layer to allow the
548 resupply of ions and complexes from the solid phase, it accounts for the ability of soil
549 to sustain solution concentration following depletion by uptake (Zhang et al., 2001).
550 Although more arbitrary than ID or DGT, conventional methods, especially sequential
551 extraction procedures (SEPs), are still widely used to classify the metal fractions in
552 soils and assess fixation over time (Hamon et al., 2007).

553 Measuring the total Se concentration in soil may not accurately represent the
554 bioavailable Se pool (Christophersen et al., 2012); instead, chemical fractionation of
555 Se could be carried out for more precise assessment. Simple extraction methods which
556 dissolve soluble and weakly adsorbed fractions, such as hot water, neutral salts (e.g.
557 $CaCl_2$) and competing anions (e.g. phosphate) can theoretically extract soil Se that is
558 ‘readily available’ for plant uptake (Wright et al., 2003). While single extractions are
559 useful to quantify the amount of Se that can be directly taken up by plants, they do not

reflect the dynamic equilibrium between the various fractions of Se in the soil – e.g. the distribution between the solid and solution phases. As plants take up soluble Se from the soil solution, exchangeable Se is desorbed from the solid phase to restore the pre-existing equilibrium (Dinh et al., 2019). Furthermore, some weakly bound SOM-Se may mineralise to replenish the source of plant-available Se in the soil, as observed by Wang et al. (2012). Alternatively, SEPs, developed by Wright et al. (2003) and adapted in later studies, purport to quantify Se associated with different soil fractions and thereby provide a more holistic picture of soil Se dynamics and the fate of applied Se in the soil. The use of SEPs to determine the different fractions of Se in the soil is discussed in greater detail in Chapter 3.

1.4 Plant uptake and accumulation of selenium

1.4.1 Plant uptake of selenium

Although some algae require Se to make selenoproteins, higher plants have shown no such requirement for Se (Pilon-Smits and Quinn, 2010). However, given that plants are often the primary dietary source of Se for humans and animals, it is necessary to understand the processes behind Se uptake and metabolism in plants (Dumont et al., 2006; Gupta and Gupta, 2000). The effectiveness of soil-to-plant transfer depends on a myriad of factors, which can be grouped into two sets:

(1) soil variables such as pH, redox potential, concentration of competitor ions such as sulphate (SO_4^{2-}) and phosphate (PO_4^{3-}), speciation and concentration of Se;

(2) plant characteristics, such as species, developmental stage and the activity of Se transporters in root membranes (Gupta and Gupta, 2000).

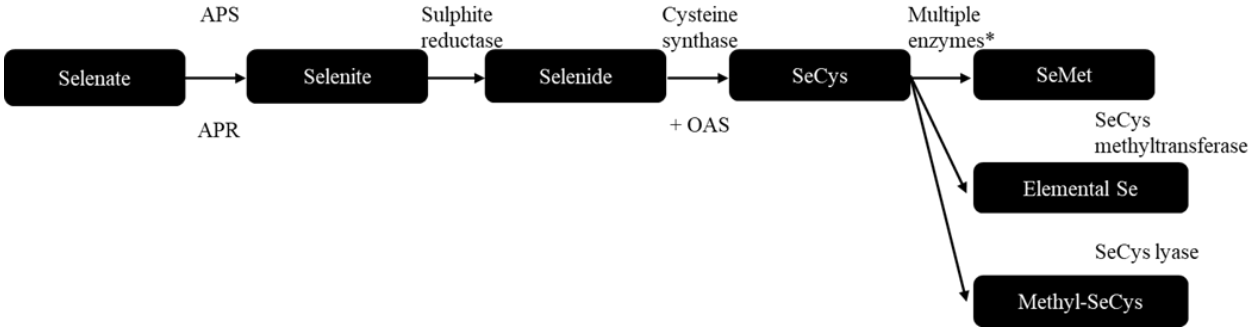
As discussed earlier, even though both Se^{IV} and Se^{VI} tend to occur in agricultural soils, Se^{VI} is more mobile and hence more bioavailable than Se^{IV}. Given its chemical similarity to sulphate, Se^{VI} is taken up into roots by SO_4^{2-} transporters (Sors

et al., 2005; Terry et al., 2000). The preference for transporters to select Se^{VI} or SO_4^{2-} depends on the nutritional status inside and outside of the plant: under high external SO_4^{2-} concentrations, the selectivity of transporters for Se decreases while upon S starvation, the expression of SO_4^{2-} transporter genes is induced and upregulated, leading to an increase in Se^{VI} uptake from the external medium (Li et al., 2017). The S-nutritional status of the plant and its environment affect Se uptake because inducible SO_4^{2-} transporters have a higher affinity for SO_4^{2-} over Se^{VI} compared with constitutively active SO_4^{2-} transporters (White et al., 2004). The SO_4^{2-}- Se^{VI} interaction has significant implications because SO_4^{2-} is usually added as fertiliser to agricultural soils in much higher concentrations (c. 0.2 – 1.0 mM SO_4^{2-}) (Barber, 1995; Gupta and Gupta, 2000) than Se^{VI} (*c.* 42 nM Se^{VI}, assuming an application rate of 10 g Se ha^{-1}).

A possible antagonism between PO_4^{3-} and Se^{IV} has been reported despite their chemical dissimilarity; a 10-fold increase in PO_4^{3-} concentration in nutrient solution caused up to 50% reduction in Se^{IV} uptake in ryegrass in studies by Hopper and Parker (1999). While certain studies suggested that Se^{IV} uptake occurs through passive diffusion into plant roots (Ellis and Salt, 2003; Terry et al., 2000), molecular studies have been able to show that Se^{IV} and PO_4^{3-} transporters share similar uptake mechanisms, suggesting active transport rather than diffusion as the primary uptake mechanism. Under phosphorus (P)-starvation conditions, the gene expression of PO_4^{3-} transporters is upregulated, causing significant increases in Se accumulation in both roots and shoots (Li et al., 2008; Zhang et al., 2014). However, it is noteworthy that the antagonism between SO_4^{2-} and Se^{VI} is stronger than that between PO_4^{3-} and Se^{IV} (Hopper and Parker, 1999).

1.4.2 Plant Se metabolism

In plants, both Se ions are metabolised to form organic selenocompounds such as selenomethionine (SeMet) and selenocysteine (SeCys), as shown in Fig. 1.4.



611

612 Fig. 1.4: Schematic flow of Se metabolism in plants. Acronyms include APS (ATP
613 sulphurylase), APR (APS reductase), OAS (O-Acetyleserine). *Multiple enzymes
614 include Cystathcysthathionine-γ-synthase, cystathionine-β-lyase and methionine
615 synthase (adapted from Pilon-Smits and Quinn (2010) and Gupta and Gupta (2017)).

616 The rate-limiting step in Se assimilation is the conversion of Se^{VI} to Se^{IV}.
617 Selenite is then further reduced, and the first organic compound formed is SeCys.
618 Selenocysteine then has multiple possible pathways: (1) it can be non-specifically
619 incorporated into proteins by replacing cysteine (Cys), which could lead to toxicity; (2)
620 it can transform to SeMet, which would be incorporated into proteins with beneficial or
621 less harmful consequences (Gupta and Gupta, 2017). In turn, SeMet could be further
622 enzymatically methylated to methyl-SeMet and volatile compounds such as dimethyl
623 selenide (DMSe). The volatilisation of methylated Se compounds from plants offers an
624 excretion pathway for excess Se (Gupta and Gupta, 2017; Lewis et al., 1974); SeCys
625 can transform to elemental Se, which would be another pathway of Se detoxification
626 in plants (Pilon-Smits and Quinn, 2010).

627 The chemical speciation of Se influences its translocation within plants. When
628 supplied as Se^{VI}, most Se remains unchanged and translocates to the shoots quickly,
629 while a small portion converts to SeMet-like species. Selenate is assimilated to
630 selenoproteins in leaves *via* the S-assimilation pathway to either SeCys or SeMet, as
631 described above (Gupta and Gupta, 2017). By comparison, Se^{IV} is rapidly reduced and
632 metabolised into organic compounds, which tend to accumulate in roots; the lower
633 translocation rate of Se in plants fertilised with Se^{IV} compared to Se^{VI} seems to verify

634 this statement (Renkema et al., 2012). Zayed et al. (1998) showed that the chemical
635 speciation of Se affects not only its accumulation in plants but also its rate of
636 volatilisation from plants. Volatilisation rates are linearly correlated with the Se
637 concentrations in roots (de Souza et al., 1998), which explains which higher
638 volatilisation is observed when plants were supplied with Se^{IV} rather than Se^{VI} (Lewis
639 et al., 1974; Zayed et al., 1998).

640 The amount of Se accumulated in plants also depends on the plant species.
641 Plants have been classified as hyperaccumulators, secondary accumulators and non-
642 accumulators, depending upon their capacity to accumulate Se in their cells.
643 Hyperaccumulators thrive in seleniferous soils and can accumulate Se concentrations
644 up to several thousand mg kg^{-1} dry weight (DW). Examples of hyperaccumulators
645 include *Astralagus* sp., *Stanleya genus* and *Codonopsis*. These plants accumulate
646 predominantly methylated forms of SeMet and SeCys, which can be further
647 metabolised to volatile DMSe. The high concentrations of Se in hyperaccumulators are
648 likely to have an essential role in the protection of the plants against a variety of
649 herbivores and pathogens as herbivores ingesting hyperaccumulator material would
650 likely convert the ingested methylselenocysteine into cysteine, which is toxic upon
651 incorporation into proteins. Secondary accumulators rarely accumulate more than 50-
652 100 mg Se kg^{-1} when grown on seleniferous soils, for example, *Brassica* sp. such as
653 mustard, cabbage and broccoli, *Aster* and *Carmelina*. On the other hand, non-
654 accumulators are plants that cannot tolerate >100 mg Se kg^{-1} DW; they may volatilise
655 Se as DMSe, and when grown on high Se soils, they show retarded growth and can
656 eventually die. Examples of non-accumulators are most grasses and cereal crops
657 (Pilon-Smits and Quinn, 2010).

1.5 Selenium and Health

1.5.1 Recommended intake levels

The reference nutrient intake (RNI) for Se in the UK is 60 and 75 μg day^{-1} for females and males, respectively (Broadley et al., 2010). More recently, the European Food Safety Authority (EFSA) in the European Union (EU) has set the recommended daily intake of Se at 70 μg. In the United States (US), the recommended daily allowance (RDA) for men and women is 55 μg, which increases to 60-70 μg during pregnancy and lactation (Stoffaneller and Morse, 2015). Biomarkers such as blood (serum and plasma) and breast milk are commonly used to assess the Se nutritional status of a population.

1.5.2 Animals

Selenium is involved in the growth and development as well as the regulation of enzymatic processes and reproduction capacities of animals (Hosnedlova et al., 2017). Hence, inadequate levels of Se intake in livestock can cause deficiency diseases such as the white muscle disease (WMD), which is characterised by breakdown of skeletal and heart muscles and affects livestock including lambs, calves, horses and poultry amongst others (Oldfield, 1990). In New Zealand alone, about 10-15 million sheep, or 10-20% of the total stock, were estimated to be at risk of WMD (Wolf et al., 1963).

Prolonged exposure to high levels of Se (0.28 – 0.8 mg Se kg^{-1} bodyweight) in the diet, for example through ingestion of seleniferous plants, could lead to Se toxicity in livestock, causing the alkali disease (O'Toole and Raisbeck, 1995). The latter is characterised by abnormal posture, difficulty in respiration, abdominal pain and eventually death of livestock (James and Shupe, 1984). However, low concentration of Se in pasture land is more widespread than an excess of Se, which means that livestock are at risk of Se deficiency more so than toxicity (Reilly, 2006).

1.5.3 Humans

The nutritional function of Se in the body is through the formation of 25 selenoproteins that are important enzymes required for the proper functioning of the immune system. High Se status has been associated with low disease risk and low overall mortality (Rayman, 2012) (Fig. 1.5). More recently, Se has been appraised as a protective agent against cancer, including lung and colon cancers (Brozmanová et al., 2010).

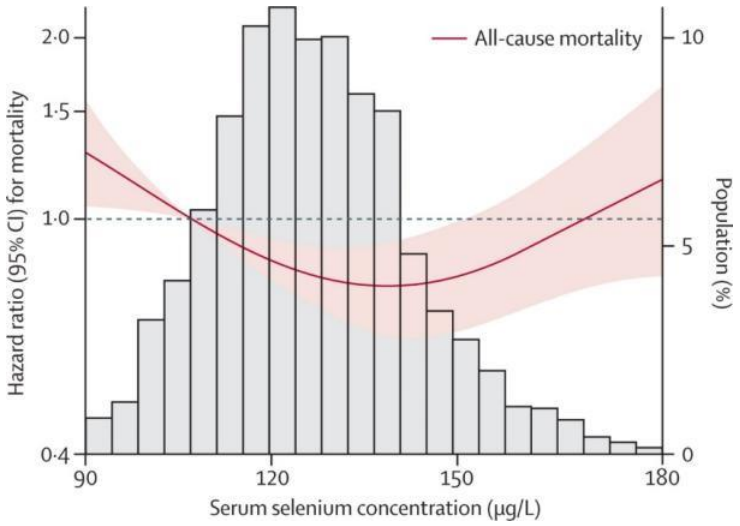


Fig. 1.5: Hazard ratio (adjusted) for all-cause mortality as a function of serum selenium concentration in adult participants of the US (Rayman, 2012).

A low intake of Se in the human diet, which could be due to several reasons, for example, the consumption of low Se-containing foods and poor food choices, could lead to Se deficiency (Broadley et al., 2010). In the Se-deficient areas of China, where deficiency diseases such as Keshan and Kashin-Beck (KBD) are prevalent, a mean dietary intake of 27.6 µg Se day^{-1} by residents was observed, as they rely almost entirely on local foods, which are grown on low Se soil, with little or no access to imported goods (Wang et al., 2017).

Keshan is an endemic cardiomyopathy, characterised by a disorder of the blood circulation, an abnormal endocardium and myocardium necrosis, while Kashin-Beck is a deforming arthritis disease, causing chronic joint disease and reduced growth in

humans. In children, acute KBD can cause dwarfism (Rayman, 2000). Less overt deficiency of Se can lead to reduced immune system function, which would increase disease susceptibility and cause general poor health (Rayman, 2000).

Although less common than inadequacy, an excessive intake of Se, selenosis, can also occur in humans. It is characterised by hair, finger and toe nail losses, extended bleeding time, lesions of the skin and 'garlic breath'. Selenosis is likely to be prevalent in areas where prolonged exposure to dietary Se intakes occurs, for example in certain villages of China where diets can supply, on average, >1200 µg day^{-1} (Wang et al., 2018).

Currently, populations of about 40 countries in the world are Se-deficient, with 15% of the global population exhibiting Se deficiency signs to different degrees (Tan et al., 2016; Wang et al., 2017). Even though populations living in geographically low-Se areas will be prone to Se deficiency as a result of the low Se content of local produce, this problem is likely to be exacerbated in underprivileged populations as a result of limited dietary choices. Over the last few decades, research has focused on addressing Se deficiency concerns in ways that are reliable, affordable and environmentally sound as well. The next part of the literature review will give an overview of the methods proposed by previous researchers to improve the Se status of human populations.

1.6 Methods to ensure adequate dietary Se intake

Several strategies have been suggested to improve human Se intake, including the consumption of natural higher-Se content foods, germinating seeds in high Se media, the application of Se fertilisers to staple crops or plant breeding for enhanced Se uptake and the supplementation of livestock and individuals. A few of these methods are briefly described below.

1.6.1 Diet diversification

Selenium enters the food chain mainly *via* plants; intake *via* drinking water or the air is comparatively trivial (Rayman, 2008). The Se content of foods is variable, depending on the Se content of their geological origins as well as their capacity to accumulate Se. Educating a population about Se sources and their contribution to their diet is necessary to ensure an optimal intake of Se. For example, consumption of a mixed diet containing a variety of foods from Table 1.1 would ensure an adequate Se intake of 55 – 70 µg person^{-1} daily, levels set by the United States Department of Agriculture (USDA) (Constantinescu-Aruxandei et al., 2018).

Table 1.1: Typical Se concentrations in foods (WHO, 2016).

Food sources	Se concentration (mg kg^{-1})
Organ meats and seafood	0.4 – 1.5
Muscle meats	0.1 – 0.4
Cereals and grains	< 1.0
Dairy products	< 0.1 – 0.3
Fruits and vegetables	< 0.1

Even in developed countries, where most people consume a varied diet, there is evidence that the intake of Se in some population groups, while not deficient, is low enough to pose health risks (Rayman, 2008). Moreover, the chemical speciation of Se in foods and supplements affects the efficiency of Se assimilation in the body and hence impacts on the overall Se status of the individual. Selenite and Se^{VI} appear to be well absorbed but less well retained in the body than organic Se forms such as SeMet and SeCys. As a result of their efficient absorption and retention in proteins in the body, organic forms of Se, especially SeMet (accumulated in cereals and high-Se yeast *Saccharomyces cerevisiae*), are the preferred form of Se intake for humans (Schrauzer, 2000).

Non-accumulator plants such as cereals and legumes accumulate predominantly bioavailable SeMet species, which makes them a good option for

biofortification (Lyons, 2018). There is far less information available about the speciation of Se in animal produce, but it appears that the primary forms of Se are SeMet and SeCys, which are incorporated non-specifically into muscle protein (Huerta et al., 2004). Sound knowledge of the typical Se species accumulated by different foods and supplements is crucial in addressing adequate dietary Se intake concerns for optimal health.

1.6.2 Oral supplementation

The consumption of Se pills is both fast and efficient for increasing Se intake in individuals. They have been used extensively in Se-deficient areas in northeastern China to reduce the incidence of Keshan disease in the population. Supplements can either be inorganic in the form of sodium selenite (Na_2SeO_3) and sodium selenate (Na_2SeO_4) or organic, for example, Se-enriched yeast (Se-yeast) (*Saccharomyces cerevisiae*) and SeMet (Xia et al., 2005). Even though Se supplements have proven to be efficient, the reliance of populations on such supplements to reach an optimal Se intake is not sustainable; this strategy is rather targeted and potentially financially viable only in natural high-Se areas, which could produce high Se foods at a lower cost. Instead, ways to intrinsically improve the ability of plants to take up Se (through breeding), to enrich seeds with Se during germination (through coating or soaking) or to apply Se fertilisers to crops to improve Se uptake can be useful to optimise Se intake.

1.6.3 Biofortification

Biofortification refers to the process of deliberately enhancing the nutritional quality of food crops by increasing the density of nutrients in edible parts of foods through different approaches, namely transgenic, plant breeding and agronomic practices, to improve the nutrient intake of the human population in a safe, sufficient and sustainable manner (Garg et al., 2018; Welch and Graham, 2004).

1.6.3.1 Transgenic and crop breeding techniques

Transgenic approaches are valid when the genetic variability of food crops is limited or cannot be efficiently exploited. It consists of introducing desirable traits into the genome of selected plants. In the case of vitamins and amino acids, the desired compounds are synthesised within the plant with some help from metabolic engineering by either increasing their concentrations in the plant or reducing the number of competitive compounds or even altering the metabolic pathway in a way to catalyse the production of novel products (Zhu et al., 2007). For essential nutrients, the technique is different because these are usually taken up from the environment. Hence transgenic approaches aim to improve the micronutrient content of plants by increasing uptake, mobility and accumulation in edible parts (Zhu et al., 2007). In the case of Se, it appears that the use of transgenic plants so far has been restricted to phytoremediation purposes. For example, genetically-modified Indian mustard grown on Se-contaminated land in California accumulated 4.3 times as much Se compared to wild-type Indian mustard plants (Peplow, 2005).

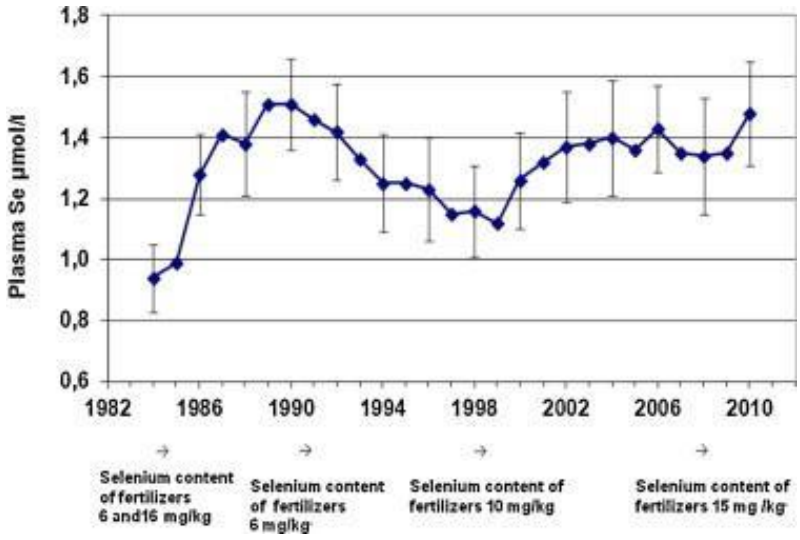
Another approach to improve the nutritional quality of foods is through conventional breeding. Plants can accumulate Se to different extents, and exploring the genetic variability for Se accumulation can be an effective method to sustainably improve Se dietary intake (Haug et al., 2007). The mechanism behind conventional breeding is that cultivars showing enhanced Se-accumulation and Se-retention traits may be bred and grown in areas with low -to-average levels of Se where, otherwise, uptake would be suboptimal. Even though the practical application of conventionally-bred crops is greater than transgenic ones, traditional breeding is at a disadvantage as the process of identifying useful traits to then test on elite cultivars, is time-consuming (Zhu et al., 2007). Moreover, with staple crops such as wheat, limited genetic variability has been observed, which makes increasing the micronutrient

density via traditional breeding very difficult. Lyons et al. (2003) suggested that agronomic biofortification is likely to be a more practical and efficient approach to increase Se uptake and translocation in crops such as wheat.

1.6.3.2 Agronomic biofortification

Biofortification of food crops by agronomic practices requires the physical application of nutrients in the form of fertilisers to improve the nutritional quality of edible parts of the crops grown for human consumption (Garg et al., 2018). In the case of Se, agronomic biofortification has proven to be highly effective because: (1) plants absorb applied inorganic Se fertilisers and transform them into highly bioavailable organic compounds, such as selenomethionine (SeMet), (2) since staple crops are usually non-accumulators, they can only take up safe levels of Se and hence can act as a buffer for humans, and (3) it is a reliable, relatively inexpensive and straightforward way of improving micronutrient intake in the human diet.

One success story of Se agronomic biofortification is the policy implemented by Finland. For climatic and geological reasons, the soils of Finland were low in available Se, which produced low-Se agricultural products. In 1984, the Finnish government aimed to improve the Se content of cereal grains to a safe concentration of 0.1 mg kg^{-1} through the mandatory addition of Se at a rate of 6 mg kg^{-1} (which later increased to 10 mg kg^{-1} in 1988) to all compound fertilisers (Eurola et al., 2000). The results from this supplementation were very positive as the increase in the Se concentration of most basic foods was reflected in a concomitant increase in the daily dietary intake of Se by at least 20% as well as in the mean plasma Se levels, from 0.75 μmol L^{-1} to 1.4 μmol L^{-1} (Alfthan et al., 2015; Eurola et al., 2000).



827

828 Fig. 1.6: Mean (± standard deviation) annual Se concentrations in the blood plasma
829 of healthy Finns before and during the Se fertilisation program (Alfthan et al., 2015).

830 In common with Finland, the soils in Denmark, New Zealand, central Siberia,
831 parts (north-east to south-central) of China, as well as areas of Africa, are also
832 considered Se-deficient, with average total concentrations of soil Se of 0.1 – 0.6 mg
833 kg^{-1}(Fordyce, 2013). Although very few of those low-Se regions have set up Se
834 supplementation programs similar to that of Finland, extensive research about the
835 potential of Se fertiliser application to staple crops as a means of improving Se levels
836 in the diet has been carried out over the last decades.

837 Inorganic Se fertilisation, through the application of Na_2SeO_4 or Na_2SeO_3, has
838 been the most common strategy for Se agronomic biofortification, as the application of
839 elemental Se or organic Se has been shown to be less effective in raising plant Se
840 levels (Ros et al. (2016) and references therein). More specifically, the application of
841 Se^{VI} is, on average, 33 times more effective in raising plant Se concentrations, than
842 Se^{IV}, even when Se^{IV} is applied at higher rates (Fig. 1.7). For example, in studies by
843 Ylaranta (1983), grain Se concentrations of 0.1 – 0.2 mg kg^{-1} were achieved by
844 application of 10 – 20 g ha^{-1} of Se^{VI}, but up to 100 g ha^{-1} was required to reach this
845 level when Se was supplied as Se^{IV} (Lyons, 2004). Comparing the efficiency of both
846 ions for biofortification in rice, Chen et al. (2002) also observed than grain Se

concentrations were 36% higher when Se^{VI}-fertiliser was applied, compared to Se^{IV}. A dry climate, low SOM content and a high soil pH are all factors that are likely to increase the Se^{VI}:Se^{IV} ratio in the soil, and consequently plant Se uptake (Combs, 2001). Moreover, the mobility of Se^{VI} within plants is higher, which leads to its rapid translocation and accumulation in the edible parts of crops in the highly bioavailable SeMet form. In contrast, Se^{IV} transforms into organic Se compounds relatively rapidly and accumulates, mostly in the roots (Cakmak and Lyons, 2009; de Souza et al., 1998).

Other types of Se fertilisers tested include 'slow-release' forms such as $BaSeO_4$, $CuSeO_4$ and 'Selcote Two Year'® (Na_2SeO_4: $BaSeO_4$ 1:1; 10 g Se kg^{-1}), which showed longer-lasting effects on Se uptake by grazing animals such as sheep, in comparison to soluble forms such as Na_2SeO_4 (Lyons et al., 2003). Due to the low rates of Se application advised for biofortification, Se is often co-applied with macronutrients such as nitrogen (N) or compound fertilisers to facilitate its uniform application in the field. Such fertilisers are called Se 'carriers'. More details about Se carriers are given in Chapter 2.

Selenium fertilisers can be applied by two methods: to the soil directly or to the leaves/canopy, a method called foliar fertilisation. In common with micronutrients such as Zn, foliar application of Se has been observed to be more efficient than soil Se application (Mao et al., 2014; Ylaranta, 1984). There are two main advantages of foliar application: (1) it obviates contact with soil constituents, such soil organic matter and mineral oxides, that would reduce Se availability for plant uptake and, (2) it generally leads to higher Se recoveries in the aboveground biomass. The meta-analysis by Ros et al. (2016) showed that plant response to foliar fertilisation is almost double that of soil-applied Se fertilisers (Fig. 1.7).

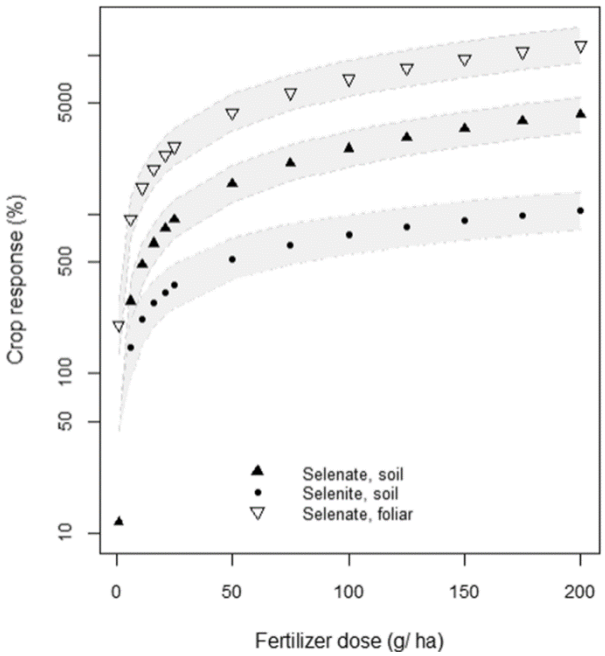


Fig. 1.7: Crop response to soil-applied selenate and selenite, and foliar selenate fertilisers (Ros et al., 2016).

Studies by Boldrin et al. (2013) were one of the few to show that Se concentration in rice grains was greater (by 450%) when fertilised by soil-applied Se compared to a foliar Se spray. Given that foliar application was performed at the flowering stage, they suggested that not enough time was available for applied Se to be transported to the grains from the leaves *via* the phloem. In comparison, when applied to the soil, Se had a longer contact time with plant roots, which led to greater uptake. On the other hand, Cakmak (2008) showed that the effectiveness of foliar Zn fertilisers in increasing wheat grain Zn concentrations could be improved by their application at a late growth stage, This is because grain accumulated the highest concentration of Zn during the milk stage (Ozturk et al., 2006). These studies were useful in highlighting the importance of Se fertiliser application timing in maximising grain Se concentrations.

Little information is currently available on the effect that fertiliser application timing (soil or foliar) could have on the efficiency of Se uptake into plants. For example, soil-application of Se during spring usually leads to greater uptake of Se by plants than

its application in wet winters, during which leaching can occur, especially when (soluble) Se^{VI} is applied (Curtin et al., 2006). With respect to foliar fertilisers, it seems logical to suggest that they should be applied when plants are at a growth stage that allows for rapid utilisation and mobilisation of the applied nutrient and maximum fertiliser interception by the canopy (Lyons, 2018) Moreover, care should be taken not to apply foliar fertilisers in periods of rainfall and not to irrigate crops straight after foliar application. However, more information is required to assess time-dependent changes on Se uptake and translocation through crops and determine whether there is a window of opportunity during which plants absorb the applied Se with the greatest efficiency. Chapter 4 of the present study covers such information.

1.6.3.3 Efficiency of agronomic biofortification

Biofortification programs aim to improve fertiliser use efficiency through the optimisation of fertilisation strategies by managing variables such as soil properties, climatic conditions and farming practices. The uptake efficiency- recovery - of applied Se ranges from 5 – 35% in cereal grains (Broadley et al., 2010; Chilimba et al., 2012a; Curtin et al., 2006; Mathers et al., 2017; Stroud et al., 2010a). Apart from soil physicochemical properties such as soil pH, SOM and mineral oxides contents affecting Se mobility in soil and its uptake efficiency into crops (section 1.3), the presence of competitor ions such as SO_4^{2-} and PO_4^{3-} also influences Se uptake efficiency (Lee et al., 2011). As discussed in Section 1.4.1, there is a strong antagonism observed between SO_4^{2-} and SeO_4^{2-} when both ions were supplied to plants grown hydroponically (Hopper and Parker, 1999; Li et al., 2008). Moreover, Dhillon and Dhillon (2000) observed that the Se concentration of wheat grain decreased by 3 - 64% with gypsum application of 0.2 – 3.2 t ha^{-1} to alkaline calcareous soils in Punjab.

915 The effect of PO_4^{3-} fertilisation on Se uptake efficiency has been more
916 controversial than SO_4^{2-} fertilisation. Nakamaru and Sekine (2008) suggested that P
917 can desorb Se from soil surfaces and increase its concentration in soil solution,
918 although the trend was more pronounced for SeO_3^{2-} than SeO_4^{2-}, as PO_4^{3-} and SeO_3^{2-}
919 are chemically similar (Eich - Greatorex et al., 2010). On the other hand, Lee et al.
920 (2011) observed a decrease in grain Se concentration with the application of PO_4^{3-},
921 which was attributed to a dilution effect arising from an increased grain yield as a result
922 of P fertilisation. A recent meta-analysis by Ros et al. (2016) observed that P and
923 potassium (K) fertilisation had a small effect on Se uptake within the common range of
924 P and K fertiliser application rates. In comparison, crop response to Se fertilisation
925 decreased with S application, except when S was applied at > 150 kg ha^{-1}.

926 Understanding the different mechanisms by which P and S fertiliser influence
927 Se dynamics in the soil and its uptake efficiency into plants is therefore crucial to
928 predict how (in what form and at what rate) Se fertilisers should be applied to maximise
929 grain Se concentration. The relevance of this dynamic is dependent on
930 agroecosystems, for example, it is particularly important in tropical soils, which receive
931 large amounts of PO_4^{3-} and SO_4^{2-} fertilisers (Lopes et al., 2017). Similarly, uncultivated
932 soils adsorb more Se than cultivated soils, presumably due to addition of SO_4^{2-} and
933 PO_4^{3-} with cultivation (Lessa et al., 2016).

934 Improving the fertiliser use efficiency of Se would cut down on, not only costs
935 (lower application rates required), but also on environmental losses. With current
936 recovery of applied Se being < 30% in the aboveground biomass of crops, a large
937 proportion of the applied Se is unaccounted for (Broadley et al., 2010). By using ^{74}Se-
938 labelled Na_2SeO_4 fertiliser to biofortify maize, Chilimba et al. (2012a) showed that the
939 recovery of the applied Se in second-season crops was only 0.78 – 2.0%, suggesting
940 that the residual benefits from Se fertiliser application are measurable but very small,

potentially due to losses of applied Se fertiliser either by leaching or incorporation of Se into soil sorbent phases such as SOM. More recent studies by Mathers et al. (2017) seem to confirm these results as they recovered 12-15% of applied Se fertiliser in wheat, with up to 40% loss potentially through leaching, and negligible uptake in the second harvest crops. They concluded that at a realistic rate of 10 g ha^{-1}, Se application would have to be repeated annually due to the low carry-over effect of the applied Se in the soil. Hence, there seems to be a need to research ways in which the efficiency of soil-applied Se fertilisers can be improved. More information about the influence of N, P, K and S fertilisers on the uptake efficiency of Se is required. More information about ways of improving Se fertilisation practices is given in Chapter 2.

1.6.3.4 Target crops for biofortification

Wheat is one of the most important sources of calories for humans and is the most widely cultivated food crop in the world (Awika, 2011). It represents about 30% of the total energy intake in the UK diet (Broadley et al., 2010) and 15-25% in Australian diets (Lyons, 2004), about 26% in the Finnish diet (Eurola et al., 2000) and more than 50% of the Turkish diet (Cakmak, 2008). The impact of the Se content of wheat on the nutritional status of a population has been demonstrated before in the UK, where the change in flour consumption from high-Se wheat imported from the US and Canada to low-Se European wheat led to a 50% fall in the dietary intake of Se (Broadley et al., 2010; Rayman, 2000). Similarly, the drop in blood Se levels in South Australians during 1977-1987 was attributed partly to the consumption of low-Se South Australian wheat (Lyons, 2004). Wheat is the most efficient accumulator of Se out of the conventional cereal crops (wheat > rice > maize > barley > oats), and accumulates Se predominantly in the form of SeMet, which is the most bioavailable form of organic Se for humans (Gupta and Gupta, 2017).

Rice is the staple food for > 50% of the world's population and provides > 80% of the daily calorie intake for more than 3 billion people around the world. It is an especially important source of Se in China, where people rely on it heavily for nutritional requirements (Boldrin et al., 2013; Meng et al., 2005). Despite China being ranked 4th in the world for its Se reserves (after Canada, the United States and Belgium), it also has geographical belts of very low-Se areas, which give rise to very low-Se agricultural products. Unsurprisingly, cardiomyopathy is very common among the inhabitants of such regions. Hence, research has focused on developing Se-enriched products such as Se-enriched rice, tea, salt and vegetables are to supplement dietary Se intakes in those areas (Chen et al., 2002; Zhu et al., 2009).

Cereals such as wheat and rice are slightly more responsive to Se fertilisation than grasses and corn (Ros et al., 2016). Nevertheless, research has also shown that Se biofortification of other food products such as peas (Poblaciones et al., 2013), carrots (Kápolna et al., 2009), buckwheat and pumpkin (Stibilj et al., 2004), tomatoes (Schiavon et al., 2013) and fruits such as peaches and pears (Pezzarossa et al., 2012), can effectively be achieved, especially by foliar Se fertilisation (Puccinelli et al., 2017).

1.7 Research gaps

In the light of the above literature, two main research gaps have been identified that, if addressed, will improve one's understanding of Se dynamics in arable systems. Firstly, there is a need to optimise Se fertilisation strategies in order to improve the efficiency of Se fertilisers in the year of application. Improving the uptake efficiency of Se into crops would help reduce costs, by lowering the rate of fertiliser required to biofortify crops. There is a particular need to try and improve the efficiency of soil-applied Se fertilisers, either by improving their uptake into plant roots or minimising their adsorption tendency so that they remain bioavailable for a longer period, or a combination of both. Moreover, gathering information about potential changes in the

translocation of applied Se fertiliser through plants in response to different application timings and formulations can help optimise the process. Secondly, the fate of residual applied Se in relation to soil properties is still somewhat poorly understood, especially when Se is applied in the form of Se^{VI}, which is the preferred form of Se application in biofortification programs (Lyons, 2018).

Hence, the present study will investigate the mechanisms of Se uptake into wheat from various fertiliser formulations, application methods and timings. Novel techniques including the use of enriched stable isotope labelling and chemical speciation analysis, by coupled LC-ICP-MS, were used to trace the transfer of applied Se from foliar application to the rest of the plant and the effect of time on the efficiency of Se uptake. The knowledge of such mechanisms can be used to tailor fertiliser strategies to maximise Se uptake into wheat. Moreover, the processes governing Se, especially Se^{VI}, ageing (or fixation) following its application to soils varying in physicochemical properties will be investigated using a combined chemical and biological approach.

1.8 Aims and Objectives

1.8.1 Aims

The overall aims of the project are to improve the Se biofortification process by optimising fertilisation strategies, and to assess the residual fate and impact of Se fertilisers in soil with time.

1.8.2 Objectives

- To investigate the potential for using macronutrients N, P, K or S from commonly used fertilisers, as carriers of Se for wheat biofortification.
- To assess the effect of soil properties and contact time on Se ageing when soluble Se^{VI} is applied at a realistic rate.

- To determine time-dependent changes in the translocation of foliar-applied Se^{VI} fertilisers from the point of application to the rest of the plant.
- To determine the effect of fertiliser application timing on the efficiency of Se accumulation in wheat.

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2 Improving the efficacy of selenium fertilisers for wheat biofortification

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2.1 Introduction

The essentiality of selenium (Se) as a nutrient for humans and animals was first established in the 1950s by Schwarz and Foltz (1957). Since then, its active role as an antioxidant, thyroid hormone and general immune function regulator has been highlighted, such that a low intake of Se in the diet would result in poor health and in extreme cases, deficiency diseases (Rayman, 2000). Although less common, an excess of Se can also be detrimental to human health (Fordyce, 2013). There is a narrow margin between Se deficiency and toxicity and so it is essential that the daily dietary Se intake for humans falls within a restricted range. Currently, the recommended dietary intake is 50–55 µg day^{1-}(WHO, 1996), but it is estimated that 0.5-1 billion people around the world do not consume sufficient Se and are at risk of disease (Combs, 2001).

Agronomic biofortification is the practice of increasing the nutrient concentration of the edible parts of staple crops through fertilisation practices (Broadley et al., 2010). In recent decades it has been identified as an effective long-term strategy to alleviate micronutrient deficiency because it is relatively easy, efficient and affordable (Premarathna et al., 2012). Cereals, such as wheat and rice, are ideal for Se biofortification because they are widely consumed by the general population and they can act as effective buffers for humans since they accumulate no more than 1.0 mg Se kg^{-1} of dry matter (Hartikainen, 2005).

The form in which Se is applied affects its effectiveness for biofortification. Both selenate (Se^{VI}) and selenite (Se^{IV}) are bioavailable species but the uptake rate of Se^{VI} may be up to 33 times higher than that of Se^{IV} (Ros et al., 2016). This is because Se^{IV} is adsorbed more strongly by inner-sphere complexation onto soil mineral oxides/hydroxides surfaces, which limits its mobility and hence plant uptake (Neal et al., 1987b). Moreover, Se^{IV} has limited translocation through plants and tends to

accumulate in roots, compared to Se^{VI} which is highly mobile in the xylem (Li et al., 2008). The predominance of the different species in soils in turn depends on *in-situ* factors such as the soil geocolloidal phases present, pH and redox potential. Under high pH and well aerated conditions, such as arable soils, Se^{VI} is expected to be the dominant inorganic Se species while in more acidic well-drained soils or under anaerobic conditions, Se^{IV} concentrations are expected to be greater (Elrashidi et al., 1987).

Selenium fertilisers are typically applied at low rates of 10-20 g Se ha^{-1} in biofortification studies (Ylaranta, 1983). To ease the application of such a small amount of Se in the field, it is usually added to other fertiliser matrices, supplying either a mix of nutrients, for example Selcote Ultra and Top Stock (Broadley et al., 2010), or predominantly macronutrients, such as urea and calcium nitrate (Singh, 1994). These fertiliser matrices are referred to as “carriers” of Se. In 1993, Gupta et al. investigated the application of N fertilisers ammonium nitrate (NH_4NO_3) and urea doped with either Se^{IV} and Se^{VI} to improve the Se levels of livestock. While their main findings focused on the superiority of Se^{VI} compared to Se^{IV} in increasing plant Se levels, they also pointed out that both N fertilisers were effective as carriers for Se. Additionally, Premarathna et al. (2012) reported that Se-enriched urea granules were very effective in raising Se concentration of rice, hence highlighting the potential of N as a carrier for Se. Rice however has different growth conditions to cereals crops such as wheat, such that findings from such an experiment may or may not be transposed onto other crops. To our knowledge, no study had either investigated this carrier effect with wheat or compared the efficiency of different macronutrients as Se carriers.

A few studies have compared the efficiency of applying Se by different methods – to the soil or to the leaves (foliar). Results showed that, while both are effective in raising plant Se concentrations, foliar fertilisation is up to 8 times more efficient than

soil Se application (Ros et al., 2016). This greater efficiency of foliar-applied fertilisers may be ascribed to (1) rapid uptake and assimilation due to application at a later growth stage, (2) less influence of root-to-shoot ratio on translocation to the edible parts of crops and (3) the avoidance of losses through fixation in soils. On average, only 12% of soil-applied Se fertilisers is taken up by plants; most Se applied is retained and immobilized in the soil (Broadley et al., 2010), with very little residual value for subsequent crops (Mathers et al., 2017). This means that repeated applications of Se fertilisers are required for each growth period, unless the efficacy of Se fertilisers can be improved.

In this study, the potential for enriching commonly used fertilisers supplying macronutrients nitrogen, phosphorus (P), potassium (K) and sulfur (S), with Se to biofortify crops was investigated. It was hypothesised that macronutrients can act as effective carriers for Se and help improve fertiliser use efficiency in the field. To the best of my knowledge, this is the first study investigating the efficiency of N, P, K and S as well as water as carriers for Se, applied either to the soil or to the leaves, with the aim of increasing Se levels in wheat grains. In addition Se speciation analysis of the wheat grains, to determine whether the different fertiliser formulations had an effect on the bioavailable Se content of the wheat grain, was carried out.

2.2 Materials and methods

2.2.1 Soils

The experiment used three Australian soils, Kangaroo Island (KI), Mallala and Black Point, air-dried and sieved to < 2 mm. They were chosen to provide a range of physical and chemical characteristics likely to affect Se dynamics (Table 2.1).

1386 Table 2.1: Physicochemical properties of the three soils used in this pot experiment.

Soils	[a]EC	pH	$CaCO_3$	Clay	Sand	Organic C	Exchangeable cations ($cmol_c\ kg^{-1}$)				
	$dS\ m^{-1}$		%	%	%	%	[b]ECEC	Ca	Mg	Na	K
Kangaroo Island	0.07	5.5	<0.5	5	94	1.6	2.71	2.09	0.62	<0.1	<0.2
Mallala	0.13	8.5	4.7	11	47	1.6	30.8	26.2	2.58	0.16	1.85
Black Point	0.07	8.3	<0.2	18	73	0.4	17.9	14.2	2.54	0.17	0.97

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1388 [a]: Electrical conductivity (EC) of soils

1389 [b]: Effective cation exchange capacity (ECEC) of soils

Soil pH and electrical conductivity (EC) were measured in a 1:5 soil-to-solution suspension on an automated Skalar pH/EC system. Soil organic carbon (C) content was measured using a dry combustion method (Matejovic, 1997). The textural classification of the soils were determined using mid-infrared spectroscopy and R code to generate the classification from the Australian soil textural triangle. To determine the exchangeable cations contents and effective cation exchange capacity (ECEC), the soil samples were shaken with a 1 M ammonium acetate solution at pH 7 in a 1:10 soil-to-solution ratio and the extracts were analyzed for elemental concentrations using inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 8300; PerkinElmer Inc., Waltham, Massachusetts). For the soils with pH < 5.5 and pH > 7.0, ECEC was determined by first pre-treating the soils with aqueous ethanol prior to extraction of cations and ions by 1 M ammonium chloride (Rayment and Lyons, 2011).

2.2.2 Selenium fertilisers

Based on application suggestions from previous biofortification studies (Curtin et al., 2008), Se was applied as sodium selenate (Na_2SeO_4) at a single rate of 3.33 µg Se kg^{-1} (equivalent to 10 g ha^{-1}, based on a 20 cm depth and 1.5 g cm^{-3} bulk density). There were nine treatments for each soil, each replicated four times. Treatments included: (i) a control without added Se, (ii) a treatment with Se added to soil as sodium selenate solution, (iii) four treatments with Se-enriched granular fertilisers and (iv) three treatments with foliar Se fertiliser.

The granular fertilisers used were urea, di-ammonium phosphate (DAP), muriate of potash (MOP) and sulfate of ammonia (SOA), supplying the macronutrients N, P, K and S respectively (Supp. Info.Table 2.3). To enrich these fertilisers with Se, a sodium selenate solution was added to powdered commercial fertiliser and mixed thoroughly to ensure homogeneity. The paste was then oven-dried overnight at 30°C and ground to a fine homogenous powder using a pestle and mortar. The Se-enriched

fertiliser powder was then pressed into tablets (5 mm diameter, ca. 2 mm height) using a tablet press (TDP-5, Shanghai Develop Machinery Co., China). For the treatment with the soil-applied selenate only, a Na_2SeO_4 solution containing 0.042 g Se L^{-1} was applied to the soil as 3 x 26 µL droplets, in the same position as the granular fertilisers.

Foliar treatments included a Se-only solution (water as carrier), Se + N in the form of either 2% w/v urea or 2% v/v urea ammonium nitrate (UAN). All three solutions contained Se as sodium selenate at a concentration of 0.083 g Se L^{-1} (rate equivalent to 3.33 µg Se kg^{-1}) and were mixed with 0.5% “Spreadwet 1000” (SST Australia PTY LTD., Victoria, Australia) surfactant prior to application.

2.2.3 Pot trial

All soils were mixed with the following nutrients (mg kg^{-1} of soil): Ca (10), Mg (10), B (1.0), Cu (2.0), Mn (2.0), Mo (0.1) and Zn (2.0) and left to equilibrate overnight prior to potting into 1 kg pots. Macronutrients were also supplied, including 80 mg kg^{-1} N as a split application, 20 mg kg^{-1} P and S, and 40 mg kg^{-1} K. The application method of the macronutrients depended on the treatment; when enriched with Se, the macronutrient fertiliser was applied as granules (3-4 per pot) in a circle at a distance of 1 cm from the side of the pot halfway through potting. The other macronutrients were then applied as part of the basal solution, such that, regardless of their form of application, all nutrients were balanced in all the soil pots. After fertilisation, five pre-germinated wheat seedlings (*Triticum aestivum cv.* Axe) were transplanted into each pot and thinned to two plants after two weeks. The soils were maintained close to field capacity by watering the soil surface regularly with reverse osmosis (RO) water. At heading stage, foliar solutions were applied to the youngest flag leaf as four 5-µL drops per plant using a micropipette. The soil surface was covered with cling film to avoid any contamination during foliar application and care was taken to water the plants at the soil surface only, avoiding irrigation of leaves. Plants were grown to grain maturity

under controlled conditions (temperature of 23.2°C, humidity of 72% and 12 h daylight cycle).

Harvest

At grain maturity, shoots and heads were harvested separately. Marked treated leaves were also separated from the rest of the biomass and washed in dilute hydrochloric acid (HCl; 0.1 M) and then rinsed with reverse osmosis (RO) water; acid rinses were saved and analyzed for Se. All plant biomass was dried at 60°C for 72 h, after which wheat heads were hand-threshed to separate grains. Prior to analyses, the grains were ground to fine powder using a pestle and mortar, and the rest of the head biomass was combined with the shoots and ground using a laboratory grade grinder.

2.2.4 Analyses

2.2.4.1 Fertilisers

Total Se concentration in the fertilisers was measured following acid digestion. Two mL of concentrated nitric acid (HNO_3) and 0.5 mL of 30% hydrogen peroxide (H_2O_2) was added to 0.25 g of Se-enriched fertiliser and left to stand overnight. The samples were then heated to 80°C for 45 min followed by 125°C for 160 min on a block digester. After acid digestion, the samples were cooled for 30 min then made to 10 mL volume using ultrapure Milli-Q water. To measure water-soluble Se in the fertiliser, 0.5 g of granular Se-enriched fertiliser samples was dissolved in 10 mL of Milli-Q water and the mixture was shaken end-over-end for 4 h. The samples were then centrifuged (15 min at 3000 *g*) and filtered through 0.22 µm filters (Sartorius, Göttingen, Germany). All solutions were analyzed for total Se by ICP-OES.

The water solubility test of our Se-enriched fertilisers indicated that they were highly soluble, releasing 100±10% of the added Se in water (Table 2.2).

Table 2.2: Water solubility of Se-enriched macronutrient fertilisers.

Se-enriched fertiliser	Water-soluble Se	Acid-soluble Se	[a]Water solubility
	mg kg^{-1}	mg kg^{-1}	%
Urea	34.7	32.3	107 ± 0.92
MOP	29.3	31.6	93 ± 0.36
DAP	25.8	26.7	96 ± 0.24
SOA	37.7	36.3	104 ± 0.25

[a]: Water solubility is presented as a percentage of the total Se released by acid digestion.

2.2.4.2 Plants

Approximately 0.25 g of plant sample (4 replicates) were weighed into 50 mL digestion tubes (Axygen, Thermo Fisher Scientific, New York) and left overnight in 2 mL of HNO_3 acid and 0.5 mL of H_2O_2 to predigest. The samples were digested using the same method as for the fertilisers, cooled and made to a final volume of 20 mL with Milli-Q water.

The acid digests were analyzed after hydride generation using a Multimode Sample Introduction System (MSIS) (Agilent Technologies, Victoria, Australia) mounted onto conventional ICP-OES (Amorin, 2016). Since only selenite forms hydrides, all samples were pre-reduced to Se^{IV} by heating an aliquot (5 mL) of the acid digest with an equal volume of concentrated HCl at 90°C for 30 min prior to analysis. Other elements (Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn) were analyzed by conventional ICP-OES, after a 5-fold dilution of the plant acid digests.

Analytical accuracy was verified through the analysis of wheat flour certified reference materials, NIST 8437 and NIST 1567b (National Institute of Standards and Technology, Maryland). The total Se concentration of the reference materials was within the range 90 - 110% recovery of the certified values.

After initial analysis, grain samples with the highest measured Se concentration (from foliar and soil-applied selenate-only treatments) were analysed for total N content and Se speciation. Grain nitrogen was determined by the combustion (Dumas) method, as described by Horneck and Miller (1998), and analysed on an N analyser (Model Leco FP-528L 601-500-100; Leco Corporation, St Joseph, Michigan). For Se speciation, 0.2 g of ground grain was weighed into 15 mL polypropylene tubes with 20 mg of protease XIV enzyme (Sigma-Aldrich, Queensland, Australia) and dissolved in 5 mL of 30 mM TRIS-HCl buffer solution. The solution pH was adjusted to 5.5 using ammonia (NH_3) solution. The samples were shaken end-over-end in an incubator at 37°C for 24 h, centrifuged at 3000 *g* for 30 min and filtered through 0.22 µm filters. The resulting solutions were analyzed for Se^{IV}, Se^{VI} and SeMet using high-performance liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC-ICPMS, Agilent 7500ce, Agilent Technologies). The operating conditions were adapted from Premarathna et al. (2012) (Table 2.4). The concentration of Se species in the samples was determined by comparison of their retention times with those of standards, prepared from individual and mixed stock solutions of sodium selenite (Na_2SeO_3), Na_2SeO_4 and selenomethionine (SeMet).

Recovery of the applied Se in the plants ($Se_{recovery}$; µg pot^{-1}) was calculated as the total amount of Se measured in the aboveground biomass as a percentage of the applied Se fertiliser (Eq. 1).

$$Se_{recovery} = \frac{[(Se_{shoots} - Se_{ctrl,shoots}) + (Se_{grain} - Se_{ctrl,grain})] \times 100}{Se_{applied}} \quad \text{Eq. 1}$$

where Se_{shoots} and Se_{grain} are the amounts of Se (µg pot^{-1}) measured in the shoots and grains respectively (as calculated from the dry weight and tissue Se concentration) and $Se_{ctrl,shoots}$ and $Se_{ctrl,grain}$ are the Se amounts in shoots and grain of the control plants.

Statistical analyses

The effects of different fertilisation treatments on grain yield and Se concentrations were determined using the analysis of variance (ANOVA) procedure in SPSS (IBM SPSS Statistics for Windows, Version 24.0., IBM Corp, Armonk, New York), with a significance threshold of 5%. Duncan's and Tukey's post-hoc tests were used to compare treatment means.

2.3 Results

2.3.1 Macronutrient concentration

Despite the application of macronutrient fertilisers in different ways (either as granules or as a basal solution) in this experiment, all treatments received the same rate of macronutrients' N, P, K and S application. Hence, no significant differences were observed in the macronutrient content of the grain, except for the granular DAP-Se treatment in the KI soil, which showed a higher grain P concentration (3.51 ± 0.17 g kg^{-1}) than when P was applied in the basal solution (2.70 ± 0.07 g kg^{-1}) (Supp. Info. Fig. 2.6).Slight, although statistically significant, differences in grain K concentration were observed between some treatments in KI and Mallala soils, whereby foliar treatments seemed generally higher than soil-applied ones. However, in all these treatments, a similar rate and method of K fertiliser (MOP in basal solution) was applied; any differences observed were therefore attributed to random effects.

2.3.2 Yield and Se concentration

Irrespective of their formulation and method of application, the different Se fertilisers employed in the study did not significantly affect grain yield, which ranged from 3.5–4.2 g pot^{-1} for the three soils (Supp. Info. Fig. 2.7), but significantly increased grain Se concentrations above control levels (Fig. 2.1).

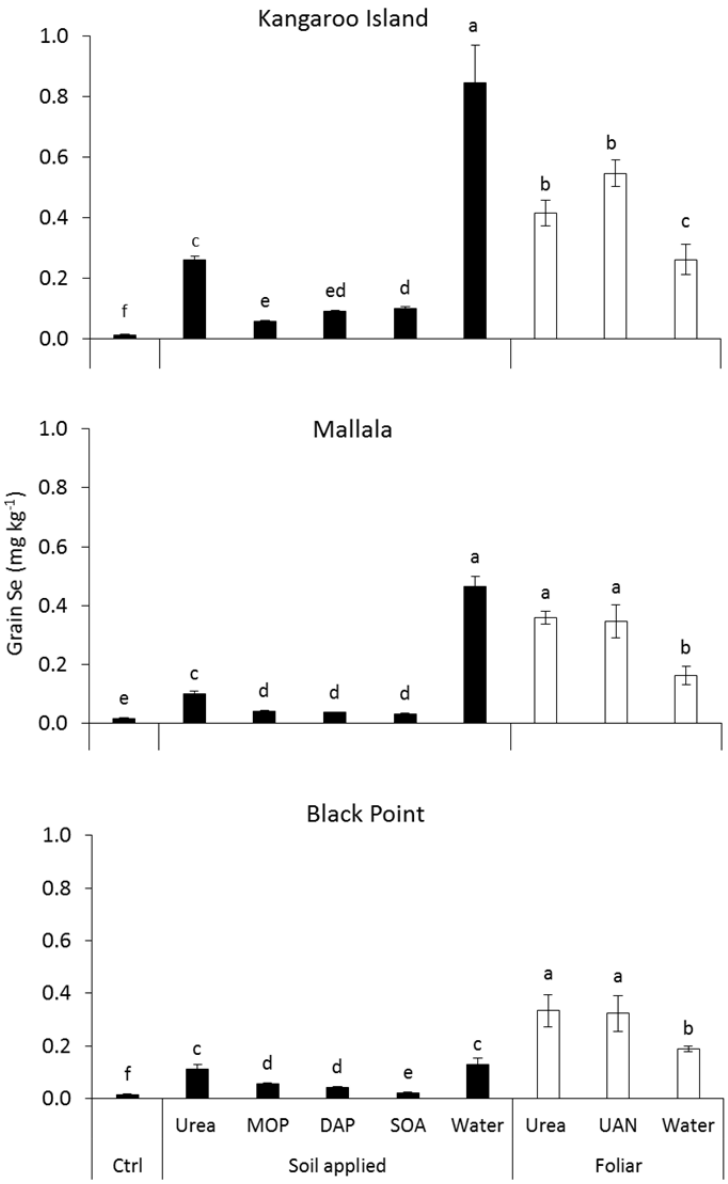

Fig. 2.1: Grain Se concentration across different Se fertilisation treatments used in the three soils. Under soil-application, Se was applied with N, K, P and S fertiliser carriers as Se-enriched urea, MOP, DAP and SOA granules respectively. Also a treatment with water as carrier (spot-applied sodium selenate solution) was included. Error bars show standard errors (n=4). Different letters above the bars indicate significant differences between treatments ($p < 0.05$).

A similar pattern in Se accumulation across the treatments was observed in the three soils, although plants grown in the KI soil generally had higher Se concentrations than Mallala- and Black Point-grown ones. For the soil-applied treatments, the application of Se on its own was the most effective (0.84 ± 0.01 mg kg^{-1} in KI; 0.46 ± 0.04 mg kg^{-1} in Mallala and 0.13 ± 0.02 mg kg^{-1} in Black Point) followed by granular

Se+urea treatments (0.26 ± 0.11 mg kg^{-1} in KI; 0.10 ± 0.01 mg kg^{-1} in Mallala and 0.11 ± 0.02 mg kg^{-1} in Black Point) (Fig. 2.1). In comparison, soil application of Se with the other macronutrients P, K and S had a much smaller effect on Se accumulation in the plants. Grain accumulation of Se following foliar fertilisation was consistently higher when 2% w/v N, in the form of urea or UAN, was added to the foliar Se solutions (Fig. 2.1): grain Se concentrations under the foliar Se only treatment averaged at 0.20 ± 0.02 mg kg^{-1}, which compared to 0.37 ± 0.02 mg kg^{-1} and 0.41 ± 0.07 mg kg^{-1} when foliar Se was co-applied with urea and UAN, respectively. The use of either liquid urea or UAN were equally effective in enhancing grain Se accumulation. No Se was measured in the foliar rinses of the treated leaves, suggesting that the surface-applied Se had been absorbed into the leaves.

2.3.3 Nitrogen content and Se speciation

Grain N was around 2.1% of the total weight across the different treatments where N was analysed, except when Se-enriched urea granules were soil-applied in KI soil, which resulted in higher grain N content (3.53%) (Supp. Info. Fig. 2.7). Protease hydrolysis of the grains measured 104 ± 4.39 % of the total Se, suggesting that it was a reliable way of releasing Se from the grains (Fig. 2.2), the majority of which was in SeMet form (average 97 ± 6%).

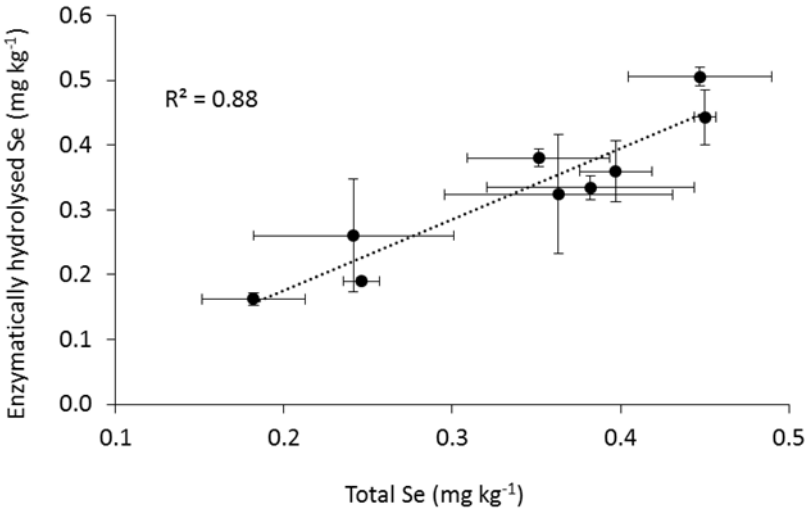

Fig. 2.2: Correlation between total grain Se concentrations measured by two methods: acid digestion and enzymatic hydrolysis.

The distribution of SeMet therefore followed that of the total Se (Supp. Info. Fig. 2.9), suggesting that the use of different carriers and methods of application did not affect speciation of Se in the grains. Other Se species such as selenocysteine (SeCys) and Se-methyl-selenocysteine (MeSeCys) generally found in wheat grains were not quantified in this study, but it can be assumed that the small percentage of unidentified Se species in the grains was in the organic form (Whanger, 2002).

2.3.4 Selenium recovery and translocation to grains

Generally, the recovery of fertiliser in the aboveground biomass was less than 50% when Se fertilisers were applied to the soil, except for soil-applied selenate-only in KI and Mallala soils (100% and 56% respectively; Fig. 2.3).

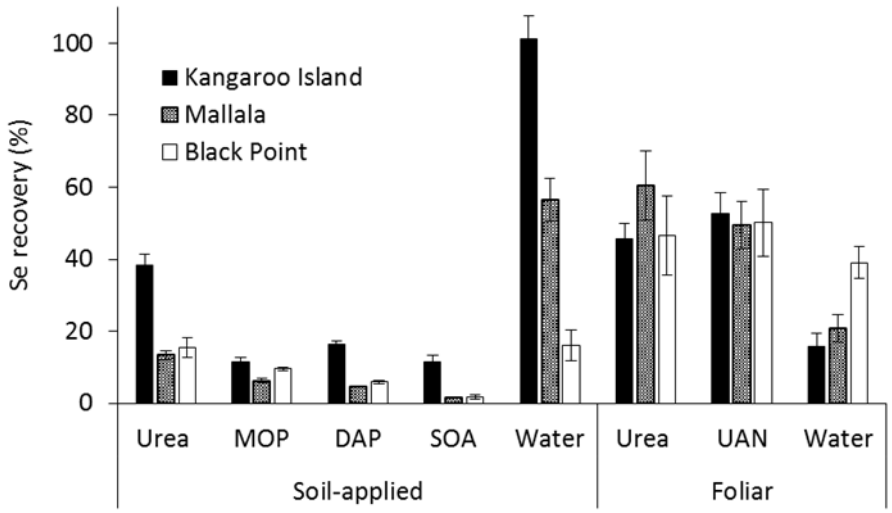

Fig. 2.3: Percentage of applied Se fertiliser recovered in aboveground biomass. Error bars show standard errors (n=4).

Although the roots or the soils were not analysed for Se concentrations in this study, it is assumed that the rest of the applied Se might either be stored in the roots or lost to the environment either through a retention mechanism onto soil particles or volatilization from the plants (Broadley et al., 2010; Zieve and Peterson, 1984). Crop Se recovery was especially low (2–38%) when Se was applied to the soil with macronutrient fertilisers, with the highest recovery recorded for the soil-applied Se+urea treatment in KI. The foliar Se fertilisers were more efficient in accumulating Se in crops with 19-30% and 46-61% Se recovered in the harvested biomass when Se was applied on its own and with an N carrier, respectively.

To examine translocation of Se into grain, the uptake (Se concentration x grain dry weight) of Se by wheat grains was expressed as a percentage of the total amount of Se accumulated in the aboveground biomass (grains+shoots).

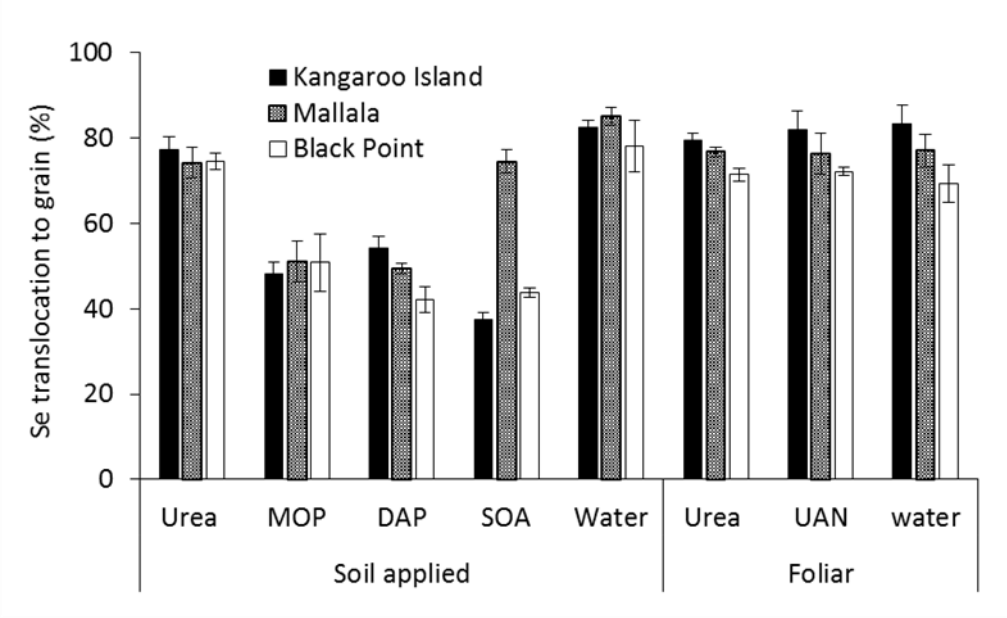

Fig. 2.4: Percentage of Se translocated to the grain across the different fertiliser treatments used in the three soils. Error bars show standard errors (n=4).

Our results showed that when Se fertiliser was soil-applied with water or with an N carrier, > 75% of the Se fertiliser taken up in the aboveground biomass was translocated to the grains (Fig. 2.4). On the other hand, limited translocation (< 50%) was observed when Se was applied with MOP, DAP and SOA (except in Mallala). The foliar applications, both with and without N, showed a large translocation to the grain.

2.4 Discussion

Yield did not differ significantly across treatments in this study, in agreement with previous studies when rates of up to 100 g ha^{-1} of Se have been applied (Broadley et al., 2010; Curtin et al., 2006; Lyons et al., 2005) (Supp. Info. Fig. 2.7). In other, albeit fewer, instances where a positive relationship between Se application and plant yield was observed, the response was attributed to a stimulation of antioxidant activity and subsequent plant protection from abiotic stresses such as cold, desiccation and the presence of toxic metals (Gupta and Gupta, 2017). The essentiality of Se for higher plants is still unconfirmed; it is generally thought to be beneficial for several physiological processes but is not a limiting factor for growth (White, 2016).

Grain Se concentration of control plants in this study was very low, averaging 0.015 ± 0.00 mg Se kg^{-1}, which is below the target Se concentration of 0.1 mg kg^{-1},suggested to be adequate for human consumption (Eurola et al., 1990) (Fig. 2.1). Under soil application treatments, the effectiveness of the Se fertilisers depended on the macronutrient carrier as well as the soil characteristics. When Se was co-applied with macronutrient fertilisers such as MOP, DAP and SOA as granules to the soil, most (> 90%) of it remained unutilized by the crop. Recovery rates of Se in those soil-applied treatments were lower than the average 12–27% reported by Stroud et al. (2010a) and Broadley et al. (2010) but compared favorably with rates in the field trial by Stephen et al. (1989) who reported 6.9% to 4.9% recovery in autumn-grown wheat (Fig. 2.3). However, unlike their autumn field trial, where considerable amounts of the applied Se fertiliser might have been lost by leaching (Stephen et al., 1989), this pot trial was conducted under controlled conditions. This suggests that mechanisms other than leaching, for example, sorption by soil, were responsible for the poor efficiency of Se-enriched macronutrient fertilisers. The exact mechanism explaining their poor efficiency compared to the application of selenate on its own to the soil is not known yet, but a possible explanation might be that the reduction of Se^{VI} to Se^{IV} was faster for the granular treatments. Since Se^{IV} is more strongly sorbed to soil hydrous oxides and organic matter and has a relatively low root-to-shoot translocation compared to Se^{VI} (Johnsson, 1991; Masscheleyn et al., 1990), its predominance in the soil would explain the low Se uptake in the plants. A positive relationship between Se translocation and Se recovery was observed (Fig. 2.5), which supports this hypothesis.

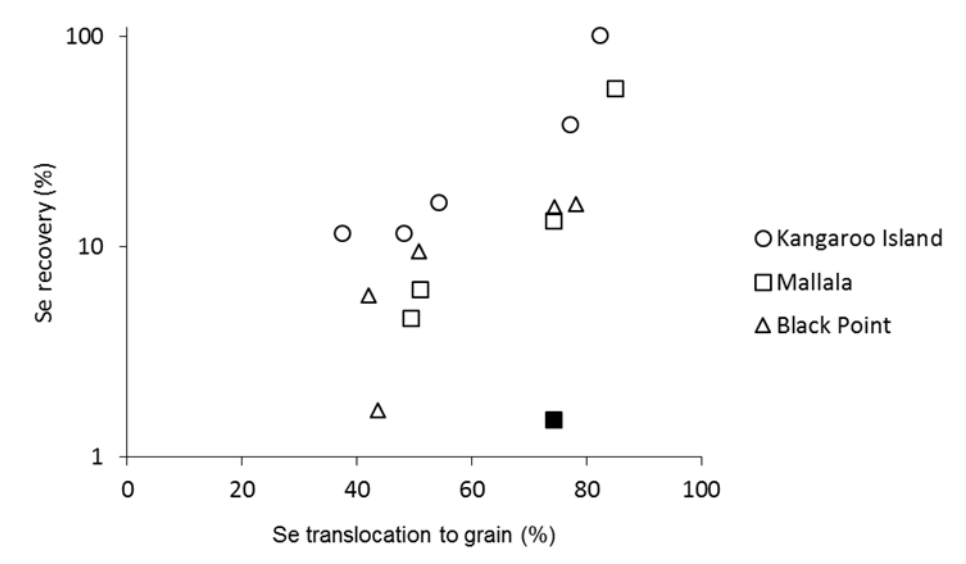

Fig. 2.5: Percentage of Se recovered in the aboveground biomass vs. % of Se translocated to the grain of plants fertilised with soil-applied Se-enriched macronutrient fertilisers (urea, MOP, DAP and SOA). The single filled data point indicates the Se-enriched SOA treatment in Mallala soil.

The low Se translocation for the treatments with low recovery (with the exception of Se-enriched SOA in Mallala soil) suggests that Se^{IV} was the predominant species available for plant uptake in these treatments. This change in Se chemical speciation could have been because, as the fertiliser granule dissolved in the soil and salt concentration built up, water would flow towards the granule as a result of the high osmotic pressure (Hettiarachchi et al., 2006), and that could create a locally reducing environment. For Se-enriched urea granules, this mechanism might be less relevant because urea is initially uncharged and even though its hydrolysis is rapid (Martens and Bremner, 1984), the urea would already have started to diffuse away from the application site before hydrolysis, resulting in less osmotically-driven water flow towards the application site. Moreover, the consumption of H^+ ions during urea hydrolysis (NO_3^- assimilation) is usually accompanied by a temporary increase in soil pH (Kirkby and Mengel, 1967). All these conditions would tend to favor the predominance of Se^{VI} ions, which could explain the higher Se uptake when urea was co-applied with Se compared to the other macronutrient fertilisers.

For the treatment with Se-enriched SOA granules in the Mallala soil, a very low Se recovery (2%) was recorded in the aboveground biomass of these plants despite the high translocation of Se to the grain (Fig. 2.5). While the high translocation rate suggests that Se^{VI} was the predominant species available for uptake, probably because roots were exposed to alkaline aerobic conditions (Elrashidi et al., 1987; Sors et al., 2005), the low Se recovery suggests that the uptake of Se from the soil was restricted. The negative effect of S fertiliser on grain Se uptake has been documented before (Stroud et al., 2010b); the antagonism arises as a result of the competition between chemically similar selenate and sulfate ions for uptake transporters in the root, where sulfate is preferentially taken up to selenate due to its higher affinity for the transporters (Sors et al., 2005; Terry et al., 2000). More recent studies by Tan et al. (2018), investigating novel mechanisms behind the competitive relationship between sulfate and selenate showed that the reduced plant uptake of selenate in the presence of sulfate ions could also be due to a suppression in microbial ability to assimilate Se^{VI}. In our study, even though sulfate and selenate were applied at the same rate for all treatments, their close proximity in Se-enriched SOA granules potentially enhanced the competition, thus reducing the uptake of Se.

In comparison to the application of Se with macronutrient carriers, the application of Se^{VI} on its own to the soil was far more effective in increasing grain Se concentration (high Se recovery and high translocation to grain), especially in the KI and Mallala soils. There could be three possible explanations for this phenomenon: (1) there was potentially a lower propensity for Se^{VI} to be reduced to Se^{IV} as a result of the lower osmotic pressure (no granule dissolving); (2) there was a lack of competition between ions since Se^{VI} was applied in pure form and; (3) there was no added physical restriction of Se having to diffuse out of the granule when it was applied in pure fluid form to the soil. Despite the granular fertilisers being highly soluble in water (Table

2.2), the dissolution of the individual granule in the soil might have been slower than expected, hence restricting Se release.

Under soil-applied Se treatments, plants grown in KI soil accumulated more Se compared to those grown in Black Point or Mallala soils (Fig. 2.1), indicating that soil properties affected the effectiveness of the fertilisers. Soil properties can affect mobility and availability of Se for plant uptake through their effect on soil conditions (e.g. pH and pe), which in turn affects Se chemical speciation and sorption behavior. Under high soil pH and aerobic conditions, Se^{VI} ions would predominate in the soil, which would favor plant uptake because Se^{VI} is adsorbed to a much lesser extent on geocolloids compared to Se^{IV}, which makes it more mobile and bioavailable (Mayland et al., 1991). However, in soils with such conditions (good aeration and high pH) for example Mallala, Se uptake was lower than expected, suggesting that other factors, such as $CaCO_3$, might have limited Se bioavailability. Previous studies have shown that Se^{IV} can get adsorbed onto calcite surfaces via an anion exchange mechanism as CO_3^{2-} and SeO_3^{2-} have a similar charge and ionic radius (Cowan et al., 1990b). Soil texture and organic matter content are also factors which can influence Se bioavailability. With only 5% clay content, KI soil is very sandy, which, not only makes it more likely to be well aerated, hence promoting the predominance of mobile Se^{VI} ions, but also lowers its adsorption capacity, compared to the Black Point and Mallala soils (Table 2.1).

The foliar application of Se fertilisers tended to be more efficient than the soil application, with higher Se uptake and recovery rate in the plants (Fig. 2.3). In this study, a foliar application equivalent to 10 g Se ha^{-1} led to grain concentrations of 0.1–0.3 mg kg^{-1} when Se was applied on its own and up to 0.5 mg kg^{-1} when Se was applied with an N carrier to the leaves (Fig. 2.1). These concentrations compare favorably with the average Se concentration of 0.4 – 0.5 mg kg^{-1} measured in studies by Curtin et al.

(2008) and Ducsay et al. (2016), where twice the amount of Se (20 g ha^{-1}) was applied to the leaves. Thus there was clearly greater efficiency in co-applying foliar Se with an N carrier to enrich wheat grain with Se, although the reasons for this have not yet been established. In studies looking at the effect of co-applying trace elements such as Fe and Zn with N, the N nutritional status of the plants was given as an explanation for improved grain micronutrient uptake because proteins can act as a sink for micronutrients and aid their re-translocation from shoots to the grain (Aciksoz et al., 2011; Kutman et al., 2011). However, the present study showed that the addition of 2% w/v N in foliar solutions did not significantly alter grain N (protein) content (Supp. Info. Fig. 2.8), suggesting that a physiological mechanism may instead be responsible for the improved plant uptake when foliar Se was co-applied with N. The exact mechanism behind the positive impact of N in foliar Se solutions in increasing grain Se concentration is not known, but it is possible that urea improved the absorption of Se through the the cuticular membrane of the leaf, leaving a smaller window of opportunity for losses by (phyto)volatilization (Minorsky, 2004). Effectively, the recovery of applied Se fertiliser for the foliar Se+N –treated plants was twice as much as that for the foliar Se treatment (Fig. 2.3). In a study using foliar Fe (± urea) solutions labelled with ^{59}Fe to trace the pathway of Fe from the treated leaf to the rest of the plant, it was shown that adding urea to foliar Fe solutions not only improved the absorption of applied Fe into the leaf, but aided the translocation of absorbed Fe into the grain (Aciksoz et al., 2014). In this case, additional studies exploring Se speciation in the foliar-treated leaf and the rest of the plant would be required to test the hypothesis that N in foliar Se solutions improved Se absorption into the leaf and/or assimilation of into organic Se compounds, which were subsequently translocated to the grain. A follow-up experiment was undertaken to verify this hypothesis and is described in Chapter 4. Nevertheless, this study was the first one showing the benefit of co-applying foliar Se with a N source to improve grain Se concentration.

2.5 Conclusions

This study aimed to determine whether fertilisation strategies for Se biofortification could be made more cost-effective by co-applying Se with commonly used macronutrient fertilisers. It was observed that the effectiveness of those Se-enriched fertilisers was highly dependent on soil properties and that the co-application of Se with macronutrients in granular form generally led to poor Se uptake and translocation within the plant. In two of the three soils used in this experiment, the application of selenate on its own to the soil was more effective in increasing grain Se concentrations than any other soil-applied fertiliser strategy. This study also showed that foliar application of Se with 2% w/v N can lead to twice as much Se uptake and recovery in plants, compared to foliar application of Se only. It should be noted that foliar solutions were applied as targeted droplets on specific leaves in this pot trial, and that, in the field where foliar sprays would be used, lower Se recovery rates can be expected. However, it appears likely that foliar co-application of Se with a N carrier would still be more effective in raising grain Se concentrations compared to foliar Se only or soil-applied Se-enriched macronutrient fertilisers.

2.6 Supplementary Information

Table 2.3: The concentrations of nutrients and Se supplied to soil pots either as basal solution or in granular form.

Fertiliser	Nutrient supplied	Concentration of		Concentration of Se in fertiliser	
		Nutrient	Fertiliser	As Se	As Na_2SeO_4
		mg kg^{-1}	mg kg^{-1}	g kg^{-1}	g kg^{-1}
Urea	N	43‡	92	0.036	0.086
MOP	K	40	76	0.043	0.104
DAP	P	20	100	0.033	0.079
SOA	S	20	82	0.040	0.096

‡Since DAP and SOA supply 21% N each, N supplied as urea either through nutrient solution or as granular fertilisers, was adjusted so that the overall N application was 80 mg kg^{-1}.

1755 Table 2.4: HPLC-ICPMS operating conditions for Se speciation of grain samples

Isotopes monitored	^{76}Se, ^{77}Se, ^{78}Se and ^{82}Se
Analytical column	Hamilton PRP-X100 anion exchange column (Phenomenex) (250 x 4.6 mm, 10 µm)
Column temperature	25°C
Mobile phase	10 mM citric acid with 2 % methanol (v/v); pH= 5
Flow rate	0.8 mL min^{-1}
Injection volume	100 µL
Tune conditions	H_2 reaction gas

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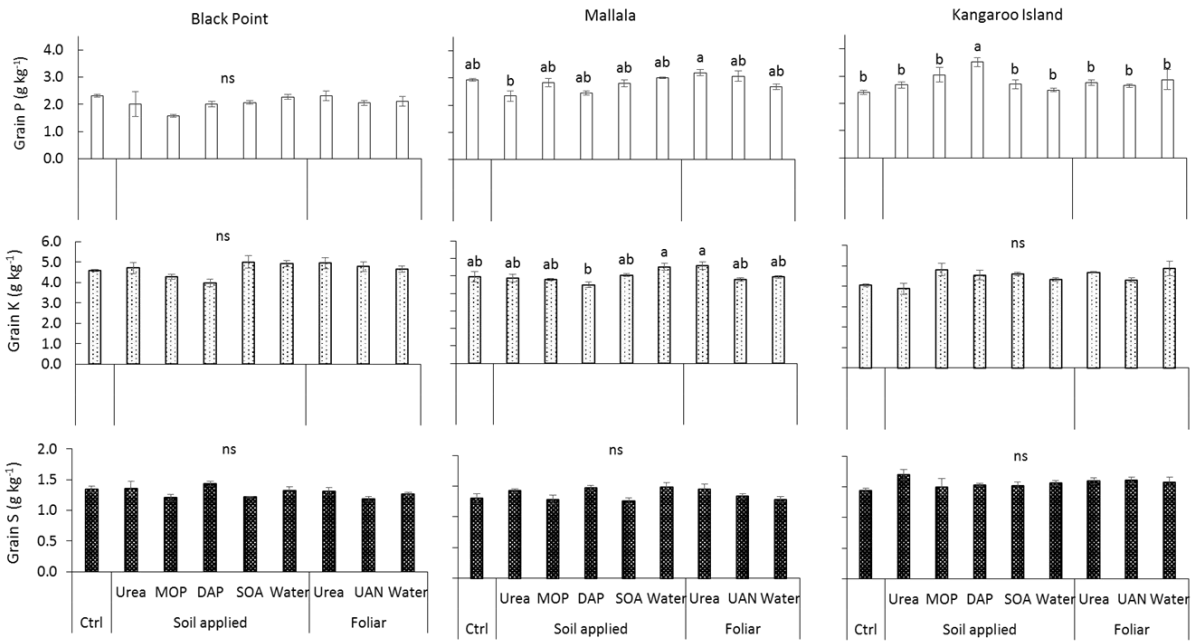

Fig. 2.6: Average concentrations of P, K and S in grains of plants grown under different fertilisation treatments (soil-applied and foliar) in three soils. Under soil-applied treatments, fertilisations urea, MOP, DAP and SOA supplying the macronutrients N, K, P and S respectively, were applied in granular form to the soil directly. In all other treatments, macronutrients were applied at the same rate in liquid form as a basal solution mixed into the soil prior to potting. Control treatment (no Se applied) is denoted here as 'Ctrl'. The error bars represent standard errors (n=4); a,b represent significant differences in macronutrient concentration of grains under the different treatments, using a Tukey's test at 5% significance level and; ns denotes no statistical differences.

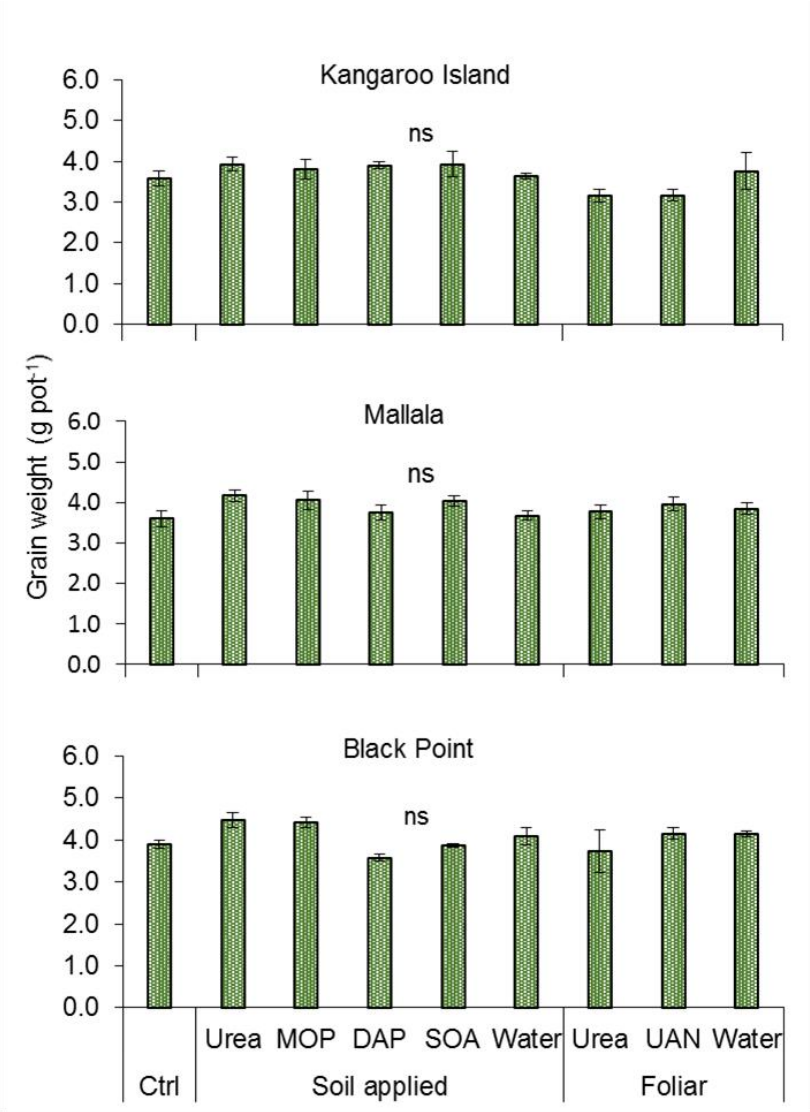

Fig. 2.7: Grain yield (dry weight) measured as the weight of wheat grains per pot, across the different treatments for plants grown in three soils. The error bars represent standard errors (n=4) and ns denotes no statistical differences ($p > 0.05$).

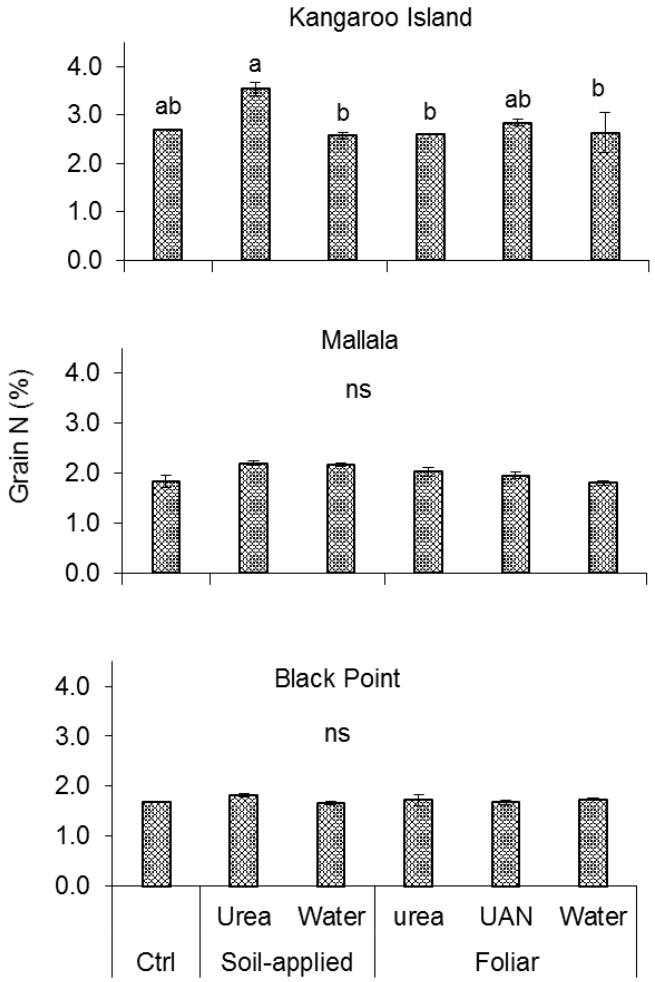

Fig. 2.8: Nitrogen content of grains of plants that were treated with Se-enriched N fertilisations as well as Se on its own (water as carrier) either to the soil or to the leaves, and grown in three different soils. The error bars show standard errors (n=4). Letters above the bar denote statistical differences ($p < 0.05$). 'ns' denotes no significant differences.

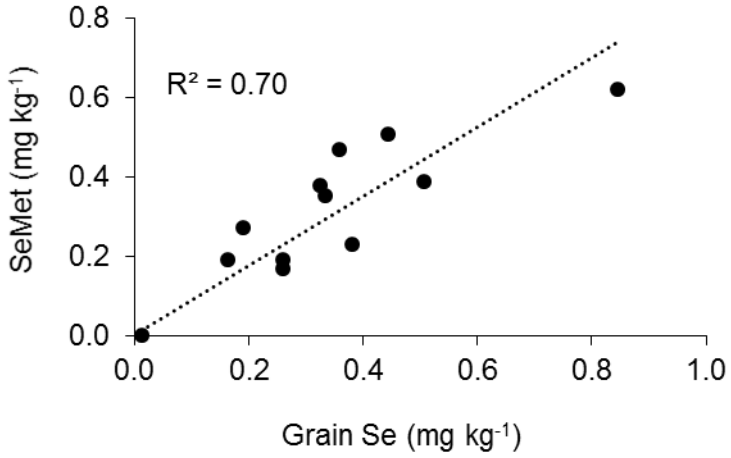

Fig. 2.9: Correlation between total Se and selenomethionine concentration of grains for soil-applied urea (KI only) and foliar Se (± N) treatments. Grains from the selected treatments only were analysed for speciation as they showed Se concentration of > 0.2 mg kg^{-1}, deemed effective for biofortification Gupta and Gupta (2017).

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3 Effect of soil properties and contact time on the ageing of selenate

Statement of Authorship

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Statement of Authorship

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Name of Principal Author (Candidate)	Chandnee Ramkissoon		
Contribution to the Paper	Experimental set up Data collection, analysis and interpretation Manuscript preparation		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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3.1 Introduction

Selenium (Se) is an essential nutrient required for the proper functioning of the human immune system. In recent years, a decline in human dietary Se levels has triggered research into ways by which Se consumption can be increased in a sustainable manner. Applying Se fertilisers to staple crops, a process known as biofortification, is highly effective in increasing Se concentrations in edible crop parts, such as wheat grain, to a safe concentration of 0.1 mg kg^{-1} (Gupta and Gupta, 2000). However, an excessive dietary intake of Se can also be detrimental to human health. It is therefore vital to understand how the applied Se transfers within the soil-plant system and determine how factors such as soil conditions and contact time influence that transfer.

Selenium exists mainly as oxygenated species in most agricultural soils, in the form of selenate (SeO_4^{2-} or Se^{VI}) and selenite ($HSeO_3^-$ or Se^{IV}) (Banuelos and Schrale, 1989; Gupta and Gupta, 2000). In alkaline aerobic soils, Se^{VI} tends to predominate while in more reducing acidic conditions, Se^{IV} is usually prevalent (Elrashidi et al., 1987). Selenium ions have different sorptive behaviour in soil, which results in a difference in their mobility as well. Anions can adsorb onto soil geocolloidal phases by different mechanisms, including inner- and outer-sphere complexation. Outer sphere complexes, formed by weak electrostatic forces of attraction between ions and the functional groups of soil sorbent phases, are usually reversible. On the other hand, inner-sphere complexation, which occurs by covalent bonding between the ions and the functional groups, results in stronger sorption behaviour. Selenite ions adsorb specifically by inner-sphere complexation onto surfaces of soil components such as oxides of aluminium (Al) and iron (Fe) as well as clay minerals (Peak, 2006; Zhang and Sparks, 1990). Selenate, on the other hand, tends to form mostly outer-sphere complexes, which explains its lower sorption and higher mobility compared to Se^{IV} in

soils (Peak and Sparks, 2002; Sparks, 2003). Soil pH is another factor influencing Se mobility through its influence on the charges of the functional groups of minerals, for example, greater sorption of Se^{IV} onto soil mineral phases is observed under acidic compared to neutral/alkaline conditions (Dhillon and Dhillon, 1999; Neal et al., 1987a). Studies by Gissel-Nielsen and Hamdy (1977) also highlighted the importance of the type of minerals on the Se sorption capacity of soils, for example, the adsorption of selenite on 1:1 minerals such as kaolinite is higher than that on 2:1 minerals (vermiculite and montmorillonite). The presence of soil organic matter (SOM) also influences Se bioavailability. Depending on the type of complexes formed between Se and SOM fractions, Se is irreversibly immobilised or weakly retained (Smažíková et al., 2017). As described by Qin et al. (2012), Se is more weakly bound to fulvic acids (FA) compared to humic acids (HA), with which it forms stronger bonds. Given the weak bonds, there is the potential for FA-bound Se to solubilise and become bioavailable (Qin et al., 2012; Supriatin et al., 2016).

The total content of Se in soil is not a good indicator of its bioavailability (Gupta and Gupta, 2000). Instead, chemical extraction methods (single or sequential) can be employed to fractionate Se in soils and provide a better estimate for Se bioavailability. Soluble Se is usually extracted using hot water or a simple salt solution based on anion exchange and mass action (Wright et al., 2003), while adsorbed Se is extracted using a phosphate extractant on the grounds of ligand exchange since phosphate adsorbs more strongly than selenite on soil surfaces (Hingston et al., 1967; Keskinen et al., 2009). The organically-bound Se fraction is rather difficult to separate from inorganic Se species, but reagents such as sodium hydroxide (NaOH) and tetramethylammonium hydroxide (TMAH) have the ability to solubilise organic matter (OM) and release OM-bound Se (Hingston et al., 1967). An advantage of TMAH over inorganic extractants such as NaOH is that pH can be increased without concurrently

increasing the salt concentration of the solution, which could otherwise lead to precipitation in the nebuliser and torch of the inductively coupled plasma mass spectroscopy (ICP-MS) during sample analysis (Hassan, 2011). Although laborious and operationally defined, sequential extraction procedures (SEPs) are useful to assess differences in extractability between samples over time.

Ageing of metals in soils describes the processes by which added metals to soil become less soluble over time (Degryse et al., 2009). In this chapter, ageing of Se in soils will refer to the decline in the soluble Se fraction in soil with time. The processes influencing Se ageing will be discussed in greater detail, more specifically, *adsorption* which refers to the retention of Se ions onto the surfaces of mineral oxides (potentially reversible), *immobilisation* by OM, which is the retention of microbially-transformed organic Se into organic pools in the soil and, *fixation*, which refers to a stronger retention mechanism by nucleation/precipitation.

Few studies have been carried out on the ageing of Se in soils, even though field trials have indicated that ageing of Se in soil is considerable, causing very low recovery of fertiliser in second-season crops (Broadley et al., 2006; Chilimba et al., 2012a; Mathers et al., 2017). Using a combined approach of chemical extractions and a biological trial to assess the ageing of Se following the addition of Se^{IV} to three distinct soils, Li et al. (2016) determined that Se ageing was controlled by a multitude of processes. Peng et al. (2019) compared the ageing of Se^{IV} and Se^{VI} in two soils using chemical analyses (sequential extraction and the diffusive gradients in thin film technique) and plant uptake measurements over a period of 100 d. Both forms of Se showed a decrease in availability with ageing time, but Se^{VI} was more available than Se^{IV} at all sampling times. To the best of our knowledge, no other studies have investigated the factors controlling ageing of Se.

This experiment assessed the effect of soil properties and contact time on Se ageing over 300 days (d), following a single application of Se^{VI} to eight soils varying in physicochemical properties. Selenate was used as this is the more commonly applied form of Se fertiliser for biofortification (Lyons, 2018). Sequential extraction as well as a biological test with wheat was carried out to assess the changes in Se availability over time. This also allowed to determine which chemical extraction method could predict Se bioavailability in soil most accurately. Finally, the soil properties mainly responsible for driving Se fixation in this set of soils were determined.

3.2 Materials and Methods

3.2.1 Soils

Eight soils, originating from South Australia and Queensland (Table 1, Appendix), were chosen to represent a range of physicochemical properties that were likely to affect Se dynamics. The soils were air-dried, sieved to < 2 mm and homogenised prior to the experimental setup. The physical and chemical properties of the soils, listed in Table 3.2, were determined using the following methods: soil pH and electrical conductivity (EC) was determined in a 1:5 soil-to-water suspension (Rayment and Lyons, 2011); the effective cation exchange capacity (ECEC) in soils within the pH range of 5.5-7.0 was determined by the ammonium acetate method in a 1:10 soil-to-solution ratio at pH 7 (Rayment and Higginson, 1992); the rest of the soils were pre-treated with aqueous ethanol, followed by extraction with 1 M ammonium chloride at pH 7, as described in Rayment and Lyons (2011); the $CaCO_3$ content was determined by a manometric procedure (Martin and Reeve, 1955); total organic carbon (C) (TOC) was quantified by a dry combustion method (Matejovic, 1997); the oxalate Al and Fe contents were determined according to Rayment and Higginson (1992); the textural classification of the soils was determined using a mid-infrared spectroscopy method (Janik et al., 2016); extractable phosphorus (P) and sulphur (S) in the soil were

measured after extraction with sodium bicarbonate and potassium chloride solutions respectively (Lefroy et al., 1993; Olsen et al., 1954). The water holding capacity (WHC) of each soil was determined using a ceramic tension plate and a hanging water column with 100 cm suction.

3.2.2 Soil spiking and incubation

All soils were spiked with 0.5 mg Se kg^{-1} in the form of sodium selenate (Na_2SeO_4). After spiking, the soils were watered with deionised (DI) water to 60% WHC and mixed to homogeneity. They were left to equilibrate overnight and then split into four replicates of 500 g in plastic containers. These soil pots were then transferred into a 25°C oven and incubated under aerobic conditions. During the incubation period, soils were watered regularly to maintain 60% WHC. Control soil pots (- Se) were also included. The soils were incubated for 1, 30, 60, 90 and 300 d prior to extraction and plant growth.

3.2.3 Soil extractions

After incubation, the soils were dried in an oven at 40°C for 72 hours then ground to a homogenous fine powder prior to analytical procedures. Sequential extraction was carried out to determine the partitioning across different fractions, namely: labile or soluble Se (Se-sol) extracted by 0.01 M calcium chloride ($CaCl_2$), adsorbed Se (Se-ads) extracted by 0.016 M potassium dihydrogen phosphate (KH_2PO_4) and organically-bound Se (Se-OM) extracted by 10% TMAH, adapted from methods developed by Wright et al. (2003), Supriatin et al. (2016) and Mathers et al. (2017). Briefly, 0.5 g of soil was shaken on an end-over-end shaker with 5 mL of extractant at 100 rpm overnight, followed by centrifugation at 3000 *g* for 30 minutes and finally filtration of the supernatant with a syringe through 0.45 µm filters into 10-mL tubes. In between the extractions, the weight of the soil samples was recorded to account for the amount of Se carried over from the previous extraction. A separate

single Se extraction by aqua regia digestion, adapted from the British standard method ISO 11 466, was also carried out to quantify pseudo-total Se in the soil (Se-tot): 0.5 g of soil sample was cold digested overnight with concentrated hydrochloric acid (HCl), nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) in a ratio of 3:1:1. Subsequently, the samples were digested at 125°C for 4 hours in a closed system on a block digester. All samples were made up to 50 mL volume with Milli-Q water and stored at 5°C in polypropylene bottles pending analysis.

The method used for the single *aqua regia* extraction was verified through the use of the certified reference material NCS DC 73326 from the China National Analysis Centre for Iron and Steel (Beijing China, 2008). Our results were within 10% of the certified value (1.6 ± 0.2 mg Se kg^{-1}), with a mean of 1.64 ± 0.15 mg Se kg^{-1}.

3.2.4 Pot trial

In parallel to chemical extractions, wheat (*Triticum aestivum cv.* Axe) was grown in 250 g pots filled with the incubated soils. Prior to potting, the soils were mixed with a basal nutrient solution supplying the following nutrients (mg kg^{-1} of soil): N (80, split application) as urea, P (20) as di-ammonium phosphate, K (40) as muriate of potash, S (40) as sulphate of ammonia, Ca (10) as calcium nitrate, Mg (10) as magnesium nitrate, B (1.0) as boric acid, Cu (2.0) as copper chloride, Mn (2.0) manganese chloride, Mo (0.1) as ammonium molybdate and Zn (2.0) as zinc chloride, and left to equilibrate overnight. After potting, two pre-germinated wheat seedlings were sown into the pots and thinned to one plant per pot two weeks later. The soils were watered regularly with DI water to maintain soil moisture at 60% WHC. Wheat was grown under controlled conditions (temperature of 23.2°C, humidity of 72% and 12 h daylight cycle) for six weeks.

The aboveground biomass of the wheat plants was hand-harvested and dried in an oven at 50°C for 72 h, or until a constant weight was achieved. The dried samples

were finely ground using a laboratory-grade grinder and stored dry until total Se analysis. The plant Se concentration (Se-plant) was determined after acid digestion: approximately 0.25 g of plant sample (4 replicates) was weighed into 50-mL digestion tubes (Axygen, Thermo Fisher Scientific, New York) and left overnight in 2 mL of HNO_3 acid and 0.5 mL of H_2O_2 to predigest. The samples were then heated to 80°C for 45 min, followed by 125°C for 160 min on a block digester. After acid digestion, the samples were cooled for 30 min then made to 20 mL volume using ultrapure Milli-Q water.

3.2.5 Sample analysis

Total Se in soil and plant samples were analysed by an inductively coupled plasma – optical emission spectrometer, ICP-OES (Optima 4300, Perkin Elmer) fitted with a continuous flow hydride generator (Hyd-ICPOES). Since only Se^{IV} is reduced to a hydride, a pre-reduction step of Se^{VI} to Se^{IV} was carried out: 3 mL of the different Se extracts ($CaCl_2$, KH_2PO_4 and TMAH) were heated with an equal volume of HCl at 90°C for 30 minutes, prior to analysis. All standards and blanks were subjected to the same treatment before injection into Hyd-ICPOES.

For data quality control, detection limits were calculated for each extraction method as three times the standard deviation of the blanks' measurements (Shrivastava and Gupta, 2011). Concentrations of Se above the highest calibration standard were re-analysed after a 10- or 100-fold dilution of the sample with Milli-Q water. The detection limits for Se were: 0.19, 0.15, and 0.18 µg L^{-1} for $CaCl_2$, KH_2PO_4, TMAH extractions, respectively. Due to the consistently low recovery of the Se-OM fraction by this method, a separate single TMAH extraction, using the same protocol as above, was repeated on all soil samples and the extracts were analysed for total Se by ICPMS (Agilent 7500ce) with H_2 gas added to the collision cell at a flow rate of 4 mL min^{-1}.

The reason for the low recovery of sequentially-extracted Se-OM fraction was potentially due to either (1) TMAH not solubilising the organically-bound Se or, (2) the Hyd-ICPOES not detecting the TMAH-solubilised Se-OM. Interestingly, when TMAH-extracted Se was analysed on the ICP-MS, all (109 ± 3%) of the total added Se was recovered, suggesting that TMAH was effective in solubilising soil-bound Se and that it was rather the method of analysis (Hyd-ICPOES) that was hampering Se-OM determination. Although hydride generation helps to lower the detection limits for Se analysis, it appears to be unsuitable for samples with high organic matter content. Zhang et al. (1999) attributed this ineffectiveness to the reaction between OM and boron hydride (BH_4^-), which produces a foam/effervescence and interferes with Se determination.

The Se-OM fraction in the present study was therefore calculated as the difference between total Se extracted by the single separate TMAH extraction (Se-TMAH) and the sum of soluble and adsorbed Se (Se-sol + Se-ads). Other elements (Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn) in plant samples were analysed by conventional ICP-OES after a 5-fold dilution of the plant acid digests with Milli Q water.

3.2.6 Kinetics models

The kinetics models that are often used to describe the change in nutrient availability over time in soils are order (zero, first and second) reactions, Elovich, power function and parabolic diffusion (Boostani et al., 2019). Different kinetics models were tested to predict the change in the soluble fraction of Se with ageing time, defined as Se ageing (Table 3.1).

Table 3.1: Kinetics models for Se ageing in different soils (Boostani et al., 2019; Islas-Espinoza et al., 2014; Li et al., 2016).

Models	Equations	Parameters
Reversible First Order	Eq.1 (text)	
Second order	$\frac{1}{Se_t} = \frac{1}{Se_{eq}} + k_2 t$	Se_t is the concentration of soluble Se (mg kg^{-1}) at specific ageing time, t (days); Se_{eq} is the concentration of Se soluble at equilibrium (mg kg^{-1}); k_2 is the second-order rate constant
Power function	$\ln Se_t = \ln a + \mathrm{b} \ln t$	a and b are both constants
Elovich equation	$Se_t = a + b \ln t$	
Parabolic diffusion	$\frac{Se_t}{Se_{eq}} = a + b\sqrt{t}$	

The most comprehensive model, the Reversible First Order (RFO) – Eq. 1 – was adapted from Buekers et al. (2008) and Crout et al. (2006) as follows:

$$f = f_{eq} + (f_0 - f_{eq})\, x\, e^{-t/T_c} \qquad \text{Eq. 1}$$

where f, f_0 and f_{eq} is the fraction of added Se that is soluble at time t, time 0 and at equilibrium, respectively and T_c is the response time of the reaction (d). The model parameters (f_0, f_{eq} and T_c) were optimised by minimising the residual standard deviations (RSD) and the goodness-of-fit of the different models was assessed using Pearson's correlation coefficient (r) and the RSD. The RSD was calculated as follows:

$$RSD = \sqrt{\frac{\sum(Se_{meas} - Se_{model})^2}{n-x}} \qquad \text{Eq. 2}$$

where, Se_{meas} and Se_{model} were the measured and modelled values of Se-sol (mg kg^{-1}), respectively, released at specific time intervals; n is the number of data points, and x is the number of model parameters.

3.2.7 Statistical analyses

The changes in Se concentrations in various soil fractions over time were assessed for statistical significance using an analysis of variance (ANOVA) at a 5% significance threshold. Simple correlation analysis was carried out to assess which chemical extraction method for Se best correlated with plant Se concentrations. A

multiple linear regression analysis was also used to determine which soil properties were primarily driving Se ageing. Pearson's correlation coefficients (r) were used to calculate the percentage variation in Se fixation explained by the different predictors. The statistical analyses were conducted in SPSS (IBM SPSS Statistics for Windows, Version 24.0., IBM Corp, Armonk, New York). The parameters of the different kinetics models were fitted to the data using the Microsoft Excel Solver function.

3.3 Results and Discussion

3.3.1 Change in soil Se fractions with ageing

The background concentrations of total Se in the soils used in this study ranged from 0.02 – 0.23 mg kg^{-1}, with the highest concentrations observed in soils with high organic matter content such as Charleston and Inman Valley (5.1 and 5.7% TOC, respectively) (Table 3.2).

Table 3.2: The physicochemical characteristics of the soils used in this study. ‘b.d’ denotes concentrations that were below analytical detection limits.

Soils	pH	EC	ECEC	$CaCO_3$	Org C	Oxal Al	Oxal Fe	Clay	Sand	Available P	Available S	Total Se
	water	dS m^{-1}	c.mol kg^{-1}	%	%	g kg^{-1}	g kg^{-1}	%	%	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Mt Compass	5.4	0.01	1.7	b.d	0.2	0.1	0.1	1.0	98	5	3	0.02
Inman Valley	5.6	0.14	16.0	b.d	5.7	1.4	5.2	26.0	41	41	19	0.18
Charleston	5.8	0.09	7.8	b.d	5.1	0.4	4.6	3.6	85	190	14	0.23
Kingaroy	6.8	0.09	8.3	b.d	1.9	2.5	3.7	52.0	20	10	61	0.13
Balaklava	8.0	0.17	27.0	3.1	1.7	1.2	0.8	12.0	46	35	13	0.10
Black Point	8.0	0.12	15.9	2.7	1.1	1.0	0.8	15.0	70	5	10	0.10
Mallala	8.2	0.13	25.9	11.0	0.9	1.2	0.9	13.0	62	16	9	0.14
Monarto	8.9	0.09	5.1	1.1	0.4	0.3	0.2	2.6	95	5	5	0.05

The solubility of Se generally decreased over time, but at various rates depending on the type of soils (Fig. 3.2). Sandy soils such as Monarto and Mt Compass showed no significant change in their Se-sol fractions over 300 d, as the fraction of the added Se that was soluble decreased from 96% to 71% in Monarto and from 103% to 68% in Mt Compass. For the other soils, a rapid initial decrease in Se-sol was observed, followed by a more gradual partitioning into non-soluble pools. The most rapid decrease in the Se-sol fraction occurred in Kingaroy, where the majority of the added Se (> 50%) was not extractable by $CaCl_2$ within the first 24 h. The Kingaroy soil is rich in clay (52%) and Al and Fe oxides (2.54 and 3.69 g kg^{-1} respectively), explaining its high Se retention capacity. The second fastest decrease in soluble Se was observed in the alkaline calcareous Mallala soil (11% $CaCO_3$), as solubility decreased by 95% within the first 30 d, followed by a more gradual decrease. These results compare favourably with those in studies by Singh et al. (1981), which showed higher Se retention in calcareous compared to non-calcareous, saline and alkaline ones.

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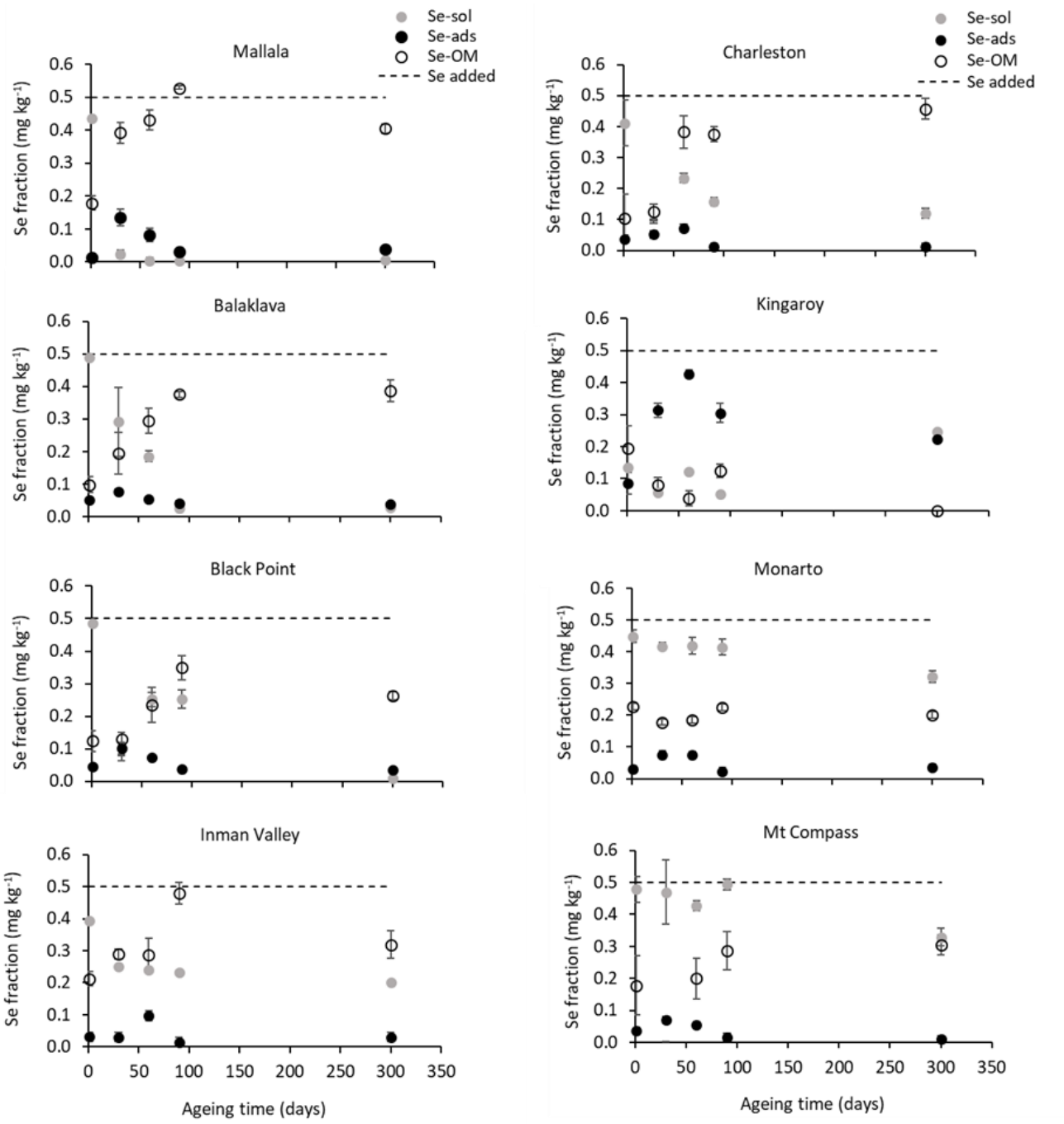

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2172 Fig. 3.1: The soluble, adsorbed and OM-bound (Se-sol, Se-ads and Se-OM,
2173 respectively) Se fractions for different soils measured over an ageing period of 300 d.
2174 Error bars indicate standard errors (n = 4).

The adsorbed Se fraction desorbed by KH_2PO_4 extraction (Se-ads) showed a similar pattern of distribution in most soils (Fig. 3.1). A slight increase in Se-ads was observed for the first 60 days, followed by a decrease and stabilisation over a longer-term. Except for the Kingaroy soil, the adsorbed fraction was small at d 1 (7 ± 0.88% of the added Se), increased to 16 ± 2.35% at d 30 and gradually decreased to 6 ± 0.95% at d 300, showing that Se-ads was not the dominant fraction in those soils. This trend suggests that Se fixation in most of the soils was primarily driven by processes other than strong surface adsorption. Comparatively, Kingaroy was the only soil where Se-ads was higher than Se-sol at every ageing time, which suggests that specific adsorption onto mineral soil fractions was the primary mechanism responsible for the loss of solubility in that soil (Fig. 3.2). A similarly high Se-ads:Se-sol ratio was observed in soils with comparable mineral oxides contents to Kingaroy in a study by Li et al. (2016). This highlights the efficacy of active sites such as those of Al and Fe oxides as well as clay minerals to rapidly adsorb Se applied either as soluble Se^{VI} or Se^{IV}.

At t=1 d, Se-OM averaged at 0.03 ± 0.01 mg kg^{-1} in most soils, which gradually increased with ageing time (Fig. 3.1). High-OM soils such as Inman Valley and Charleston were expected to have higher Se-OM fractions compared to others; however, Se-OM was the highest in alkaline calcareous soils Mallala, Black Point and Balaklava, suggesting that TMAH was not specifically solubilising organically-bound Se, and was potentially also dissolving Se bound to carbonates. Since calcite forms precipitates within a pH range of 5.5 to 10, adding a powerful alkaline reagent such as TMAH (pH 13 at 25% v/v) to calcareous soils may have caused calcite dissolution and the release of previously-bound Se. Similar overestimation of organically-bound Se in soils has been observed with other alkaline oxidising agents such as NaOH, sodium hypochlorite (NaOCl) and potassium persulfate ($K_2S_2O_8$). For example, Wright et al. (2003) found that the NaOCl extraction of sediments resulted in overestimation of the

Se-OM fraction, as the extract was also solubilising metal selenides such as iron selenide (FeSe). Kinetics models for Se ageing in soils

For most soils, except Kingaroy, the best goodness-of-fit between measured and modelled Se-sol values was given the reversible first order (RFO) equation model. Kingaroy was best modelled by a Power Function (Table 3.3). Given the superiority of the RFO in modelling Se solubility with ageing compared to the other models, it will be further discussed in this section and onwards.

Table 3.3: Residual standard deviation (RSD) and Pearson's correlation coefficients (r) of kinetics models for change in soluble Se fraction over time in different soils. The numbers highlighted in bold show the best goodness-of-fit between modelled and measured Se-sol values.

Kinetic models	Mt Compass		Inman Valley		Charleston		Kingaroy		Balaklava		Black Point		Mallala		Monarto	
	RSD	r	RSD	r	RSD	r	RSD	r	RSD	r	RSD	r	RSD	r	RSD	r
Reversible first order	**0.04**	**0.88**	**0.01**	**0.99**	**0.08**	**0.91**	0.11	0.00	**0.05**	**0.99**	**0.04**	**0.94**	**0.00**	**1.00**	**0.01**	**0.98**
Second order	0.04	0.80	0.06	0.73	0.10	0.75	0.09	0.00	0.06	0.95	0.14	0.73	0.01	1.00	0.01	0.97
Power Function	0.06	0.00	0.02	0.98	0.22	0.79	**0.08**	**0.55**	0.10	0.88	0.12	0.77	0.01	1.00	0.04	0.45
Elovich	0.06	0.00	0.01	0.98	0.08	0.79	0.09	0.00	0.07	0.94	0.08	0.94	0.08	0.93	0.04	0.57
Parabolic Diffusion	0.06	0.67	0.06	0.75	0.14	0.62	0.09	0.00	0.13	0.86	0.17	0.86	0.20	0.57	0.02	0.95

Table 3.4: The estimated model parameters derived from the Reversible First Order model to predict Se solubility over time. Kingaroy was not well represented by the model.

Soils	f_0	f_{eq}	T_c (d^{-1})	Soil type
Mallala	0.97	0.01	9.40	Calcareous
Balaklava	1.00	0.02	48.3	
Black Point	0.98	0.01	110	
Monarto	0.89	0.00	943	
Inman Valley	0.80	0.44	18.9	Non calcareous
Charleston	0.84	0.23	56.5	
Mt Compass	0.98	0.66	121	
Kingaroy	0.25	-	-	Oxisol

The RFO model parameters indicate whether there was immediate sorption of Se onto soil surfaces ($f_0 < 1$), the fraction of the added Se that remains soluble at equilibrium (f_{eq}) and the "response time" of the ageing reaction (T_c, the time required to reach 63% of the difference between initial and equilibrium value) (Table 3.4). The estimated proportion of Se instantly fixed ($1-f_0$) was not an indication of the extent of Se ageing in the soil: despite their low proportion of immediately-fixed Se (< 5%), calcareous soils such as Mallala, Balaklava and Black Point showed more pronounced Se ageing (higher f_{eq}) than the other soils (Table 3.4). For all calcareous soils, almost all of the added Se was predicted to be fixed at equilibrium, but the rate of fixation increased with increasing $CaCO_3$ content of soils, following the order of Mallala ($CaCO_3$ = 11%) > Balaklava ($CaCO_3$ = 3.1) > Black Point ($CaCO_3$ = 2.7) > Monarto ($CaCO_3$ = 1.1%) (Fig. 3.2).

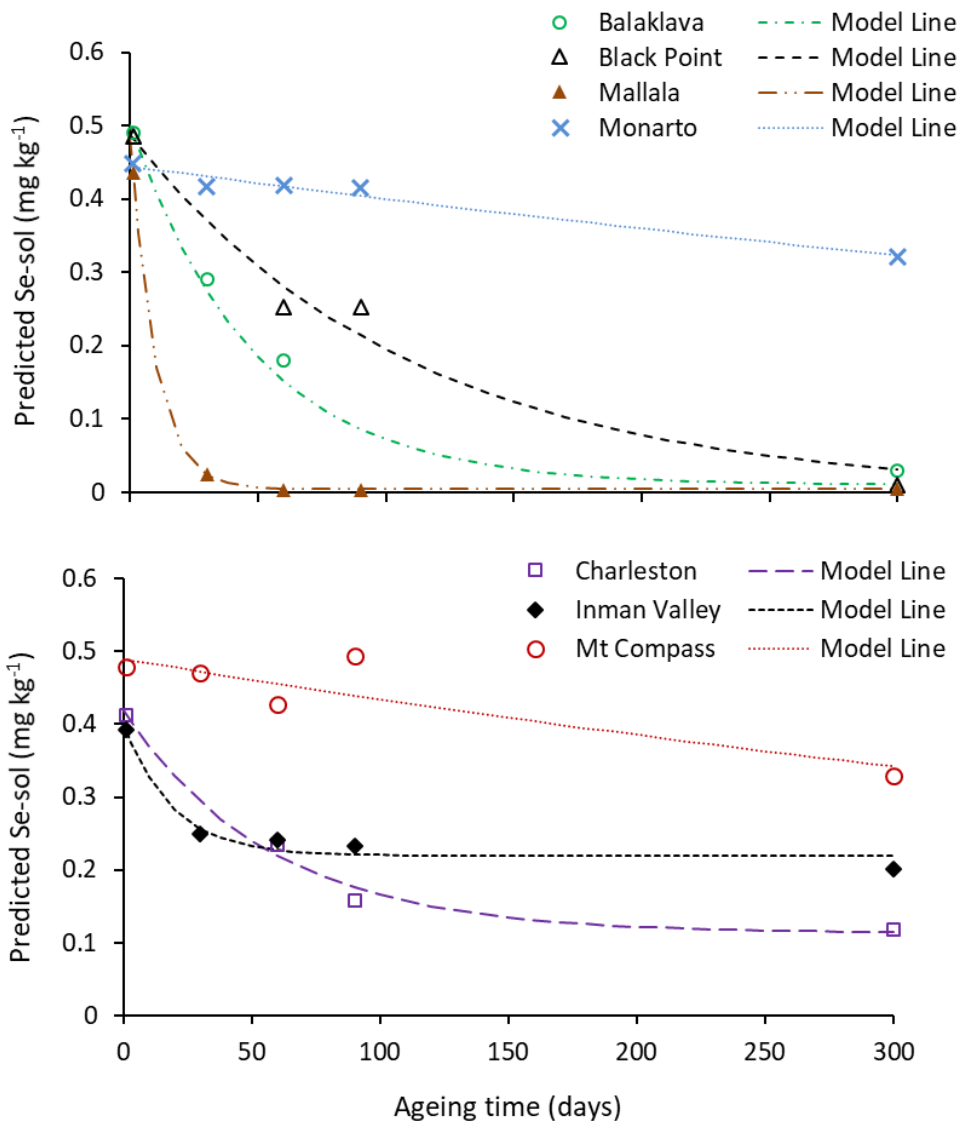


Fig. 3.2: The Se-sol fraction in different soils aged for a period of 300 d, modelled by the reversible first order equation model. Calcareous and non-calcareous soils are shown on the first and second graphs, respectively.

The lack of immediate fixation in the calcareous soils suggest that more Se remained bioavailable shortly after addition probably because the alkaline, aerobic soil conditions favour the predominance of mobile Se^{VI} ions (Gupta and Gupta, 2000). However, over time, more pronounced Se ageing occurred in the calcareous soils, pointing to a strong binding mechanism, possibly by ion exchange with CO_3^{2-} on calcite surfaces (Goldberg and Glaubig, 1988). The non-calcareous soils showed relatively less pronounced ageing of Se (Table 3.4). Selenium immobilisation occurred a higher rate in the high-OM soils such as Inman Valley and Charleston (T_c of 19 and 57 d),

compared to low OM soils such as Mt Compass and Monarto (T_c of 120 and 942 d respectively).

Despite the calcareous nature of Monarto, albeit with lower $CaCO_3$ content (1.1%) compared to the other calcareous soils, the f_{eq} in Monarto was predicted to be 0 (Table 3.4). The model seems to suggest that fixation of Se in Monarto would occur at a very slow rate over a long period of time (estimated 942 d). Given that the ageing period for this study was 300 d, it was not possible to verify this assumption. However, for all the other soils, f_{eq} was estimated to be reached within the 300 d period (T_c < 300 d; Table 3.4) and the good correlation observed between f_{eq} and Se-sol concentrations at d 300 for those soils (r=0.99) (data not shown) suggests that the model prediction was reliable. Hence, it is reasonable to assume the predicted long fixation process of Se in Monarto was also realistic.

The Kingaroy soil was not well represented by kinetic models because most sorption occurred in the first day (> 75% of the added Se adsorbed with 24 h) and there was little change in Se solubility afterwards. This soil was, therefore, omitted from the statistical analysis determining which soil fractions were primarily driving Se fixation (next section).

3.3.2 Effect of soil properties on Se ageing

A multiple linear regression analysis between the estimated soluble Se fraction at equilibrium (f_{eq}) and soil properties showed that soil pH was the main property influencing the loss in Se solubility with ageing, explaining 81% of the variance among the different soils ($p < 0.05$) (Fig. 3.3).

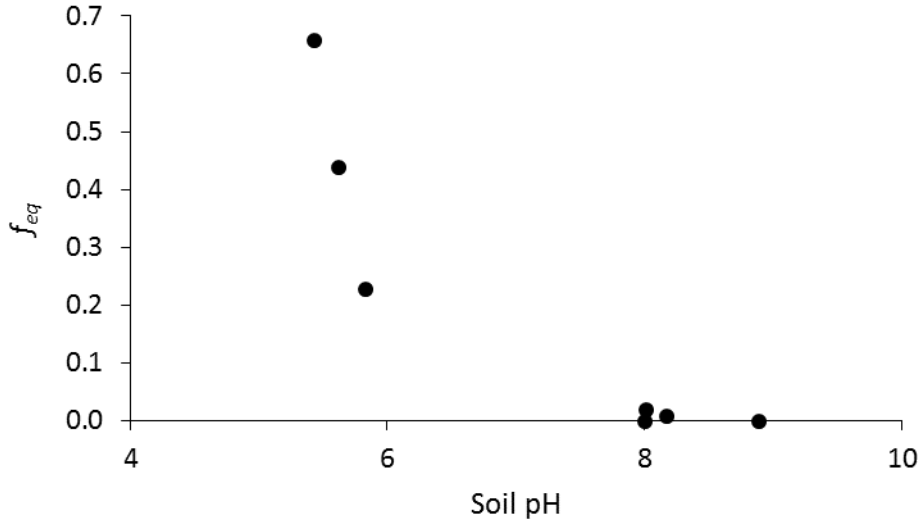


Fig. 3.3: The relationship between the fraction of Se that was soluble at equilibrium and soil pH.

Under aerated alkaline conditions, Se^{VI} is expected to predominate, and this species is more mobile and bioavailable than Se^{IV} (Mayland et al., 1991). Hence, the more pronounced ageing at higher pH may seem surprising. However, the alkaline soils were also calcareous, and the rate of fixation increased with increasing $CaCO_3$ content of the soils. It seems therefore likely that retention of Se on $CaCO_3$ was the primary mechanism responsible for Se fixation in the alkaline soils.

Compared to the number of studies about the sorption of Se ions on mineral oxides and clay surfaces (Balistrieri and Chao, 1990; Gissel-Nielsen and Hamdy, 1977; Goldberg, 2014; Peak, 2006), studies investigating Se sorption in calcareous soils are fewer (Cowan et al., 1990a; Jones and Belling, 1967; Renard et al., 2013; Singh et al., 1981). Singh et al. (1981) observed calcareous soils to retain more Se^{VI} and Se^{IV} than other soil types. Goldberg and Glaubig (1988) showed that the retention behaviour of Se on calcite, especially Se^{IV}, was highly dependent on soil pH as sorption increased from pH 6 to 9, peaked between pH 8 and 9, and decreased with at pH values > 9. However, the mechanisms behind Se sorption on calcite are still poorly understood. Studies have used the chemical similarity of sulphate (SO_4^{2-}) and phosphate (PO_4^{3-})

ions with Se^{VI} and Se^{IV}, respectively, to infer information about the mechanisms governing Se retention in calcareous soils. For example, Cowan et al. (1990a) showed Se^{IV} was adsorbed on $CaCO_3$ by its exchange with CO_3^{2-}/HCO_3^- ions on anion exchange surface sites of calcite, in a similar manner to PO_4, as both SeO_3^{2-} and PO_4^{3-} have similar charge, ionic size and structure (Shock and Helgeson, 1988). However, it should be noted that in the present study, and it would potentially be in the field as well, PO_4^{3-} was applied to the soil at a much higher concentration (20 mg P kg^{-1}) than SeO_4^{2-} (0.5 mg Se kg^{-1}). A realistic rate of Se application for biofortification in the field is estimated to be 10 g ha^{-1} (Mathers et al., 2017), which is equivalent to 3.33 µg kg^{-1} (based on a 20 cm depth and 1.5 g cm^{-3} bulk density), and hence > 6000 times lower the concentration of P fertiliser application in the soil. It could be argued that the precipitation of SeO_4^{2-} ions at such low levels on calcite surfaces would be unlikely. Structural studies by Lamble et al. (1995) using XAFS provided direct evidence that SeO_4^{2-} can substitute CO_3^{2-} in calcite; however, high concentrations of aqueous Se (1006 ppm) were used in that study as well (Lamble et al., 1995). Therefore, the retention of Se onto calcite surfaces through an ion exchange mechanism seems possible, but it would require substantial distortion of the site, which could potentially occur as a result of an increasing Se concentration in the soil (Reeder et al., 1994). There is, however, no way of verifying which mechanism was Se retained by in the soil as no speciation analysis of Se was carried out for the aged soils.

In non-calcareous soils, Se retention occurs predominantly by an adsorption mechanism (Goldberg, 2014), and adsorption is, in turn, primarily dictated by the contents of Fe/Mn and Al oxides as a result of their specific surface area and strong chelating ability (Dinh et al., 2019; Muller et al., 2012). In this study, the most rapid decline (> 75% within 24 h) of soluble Se was observed in the Kingaroy soil (Fig. 3.1), which is an Oxisol with high Al/Fe oxides and clay contents (Table 3.2), suggesting

2309 that the oxides were responsible for Se sorption in this soil. In the other non-calcareous
2310 soils, fixation was most pronounced in the high-OM soils (Fig. 3.2), suggesting that
2311 binding onto or incorporation into OM played a role in the ageing process. However, it
2312 is hard to infer the type of bonding responsible for the retention of Se in OM as the
2313 chemical fractionation method employed in this study did not selectively speciate HA-
2314 and FA-bound Se (Qin et al., 2012; Supriatin et al., 2016).

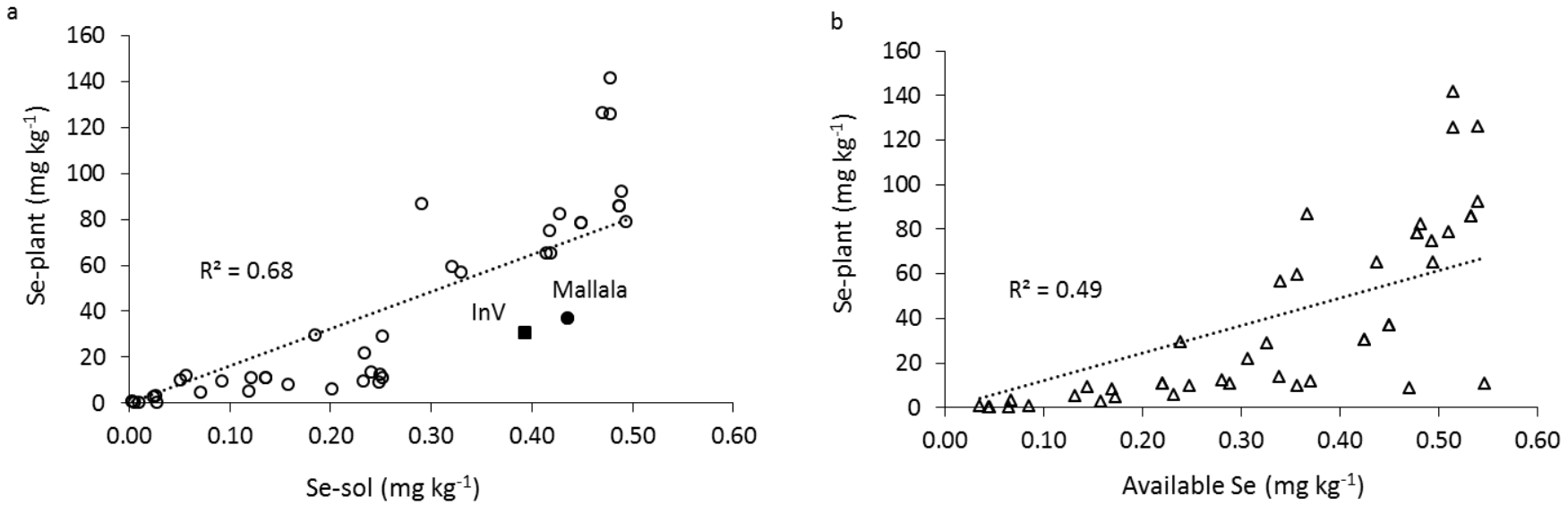
2315 **3.3.3 Soil extract to predict Se bioavailability**

2316 The Se concentrations of plants grown in soils aged with Se for different lengths
2317 of time over a 300 d period are shown in Table 3.5. The plant Se concentrations
2318 generally declined with time of incubation after Se addition, but the rate of decrease
2319 varied significantly among the different soils. Initial (t=30 d) plant Se concentrations
2320 were highest in non-calcareous, low-OM sandy soils (average 110 ± 31.6 mg kg^{-1}),
2321 followed by calcareous soils (average 71.9 ± 17.5 mg kg^{-1}) and were lowest in high-
2322 OM soils (average 17.3 ± 6.74 mg kg^{-1}). By the end of the ageing period, plant Se
2323 concentration decreased to < 10 mg kg^{-1} in all soils, with the exception of low OM, non-
2324 calcareous (sandy) soils (average 58.3 ± 1.40 mg kg^{-1}). The lowest concentrations
2325 were measured in plants grown in the calcareous soils Mallala, Black Point and
2326 Balaklava (average 0.45 ± 0.03 mg kg^{-1}) (Table 3.5).

Table 3.5: Concentrations of Se in plants grown in soils that were aged with Se for 1, 30, 60, 90 and 300 days. Results show averages ± standard errors (n=4). The highlighted data points were outliers as Se-plant concentrations were unusually low, which stemmed from the very poor growth of plants at that stage, presumably due to external factors such as low nutrient availability despite basal fertilisation. An ageing factor (AF_{plant}) was calculated as the ratio of Se-plant at d 1 to Se-plant at d 300.

Soils	Se concentrations in plants (mg kg^{-1}) grown in soils aged with Se for *t* days					AF_{plant}
	1	*30*	*60*	*90*	*300*	
Mt Compass	142 ± 16.0	127 ± 16	82.3 ± 4.5	78.9 ± 0.6	56.9 ± 1.0	2.49
Inman Valley	30.8 ± 3.9	12.7 ± 1.3	13.8 ± 1.5	9.84 ± 0.7	6.06 ± 0.2	5.08
Charleston	**1.33 ± 0.2**	22.0 ± 1.2	9.6 ± 0.8	8.4 ± 0.2	5.3 ± 0.2	0.264
Kingaroy	10.9 ± 0.6	12.0 ± 5.5	11.2 ± 0.5	10.0 ± 0.4	9.05 ± 1.0	1.20
Balaklava	92.5 ± 14.0	87.0 ± 3.1	29.8 ± 2.2	3.47 ± 0.2	0.27 ± 0.1	338
Black Point	85.9 ± 5.9	**4.85 ± 0.7**	29.1 ± 4.2	11.3 ± 1.0	0.31 ± 0.0	280
Mallala	37.0 ± 2.7	2.94 ± 0.2	0.80 ± 0.02	0.76 ± 0.1	0.36 ± 0.0	102
Monarto	78.7 ± 7.8	75.0 ± 9.0	65.6 ± 4.5	65.2 ± 4.5	59.7 ± 2.9	1.32

To verify which chemical extraction method was the best indicator of bioavailability, concentrations of Se in plants were correlated with Se-sol and plant-available Se (Se-sol + Se-ads) fractions (Fig. 3.4).



2337

2338 Fig. 3.4: The chemically-extractable soil (a) soluble and (b) available Se (Se-sol + Se-ads) fractions of aged soils vs. the measured
2339 Se concentrations of plants grown in the aged soils. The data points for Inman Valley (InV) and Mallala at the first sampling point (t=1
2340 d) are identified separately in (a) to highlight the overestimation of bioavailability by chemical extraction due to considerable ageing
2341 occurring at the beginning at plant growth.

The results indicate that a dilute salt (0.01 M $CaCl_2$) extraction could predict bioavailability reasonably well (r=0.83). However, slight overestimation of bioavailability for some of the freshly spiked soils with high sorption capacity, such as highly calcareous Mallala and high-OM Inman Valley soils, as identified by separate symbols on Fig. 3.4, was observed. It could be argued that using the Se-sol fraction of such soils, especially at d 1, to predict bioavailability was not a true depiction of the pool of soluble Se that was actually available for plant uptake. Nevertheless, the exclusion of the d 1 data Se-plant concentrations did not significantly affect the correlation between Se-plant and Se-sol (r=0.84) (data not shown).These results demonstrated that, although reasonably reliable, the use of chemically-fractionated soluble Se fractions of highly sorptive soils to predict bioavailability, especially shortly after fertiliser addition, should be carried out with caution.

The soluble Se pool in soil solution is continuously replenished as Se gets taken up by plants or lost from the soil-plant system, usually through the desorption of adsorbed Se from surfaces of soil particles (Dhillon et al., 2005). Hence, the Se-ads fraction in soil could be a potential pool of 'plant-available Se'. In this study, correlating available (Se-sol + Se-ads) Se fraction with Se-plant concentrations (r=0.69) did not improve the prediction for bioavailability from Se-sol only (r=0.84) (Fig. 3.4). Our results differ from those by Zhao et al. (2005) which showed a high correlation (r=0.83) between KH_2PO_4-extractable Se and plant Se concentrations. The suitability of a chemical extractant in extracting plant-available Se depends on the type and nature of the soil (Dhillon et al., 2005). Although the physicochemical properties of the three soils used in the study by Zhao et al. (2005) are not listed, they did show that the KH_2PO_4-extractable Se accounted for most (> 95%) of the total Se in the soil, suggesting the prevalence of adsorption sites such as Al/Fe oxides or clay minerals. In contrast, in our study, the Se-ads fraction in most soils, except Kingaroy, was minimal (< 10%) and

showed no change with ageing. This was because mechanisms other than adsorption was responsible for Se retention in this set of soils, as discussed in the previous section. However, the results of the present study were in agreement with those by Dhillon et al. (2005), in which the chemical extraction of seleniferous soils (> 0.5 mg Se kg^{-1}) by either hot water or potassium chloride (KCl), was more effective (r=0.70) in predicting bioavailability in wheat than extraction by KH_2PO_4. Hence, the use of chemical extractants to predict Se bioavailability could be made more reliable by firstly determining the chemical properties of soils in order to ascertain the sorptive behaviour of Se in the soil.

Ageing factors were derived based on both the plant concentrations and chemical-extraction data. They were calculated as the ratio of the Se concentration in the plant or in the $CaCl_2$ extract in freshly spiked soil and that in the soil aged for 300 d. Ageing was much more pronounced for the three soils with $CaCO_3$ > 2%, in which the plant Se concentrations decreased > 100-fold with ageing than in the other soils (Table 3.5). For the non-calcareous soils, the highest ageing factor was in Inman Valley. Since Inman Valley had the highest SOC content (Table 3.2), this suggests that Se incorporation into OM was the primary ageing mechanism in non-calcareous soils. The biological ageing factor (AF_{plant}) was strongly correlated with the chemical extraction-based ageing factor (AF_{extr}) (r=0.93) (Fig. 3.5), hence confirming the possibility of chemically extracting soils with $CaCl_2$ to estimate Se bioavailability.

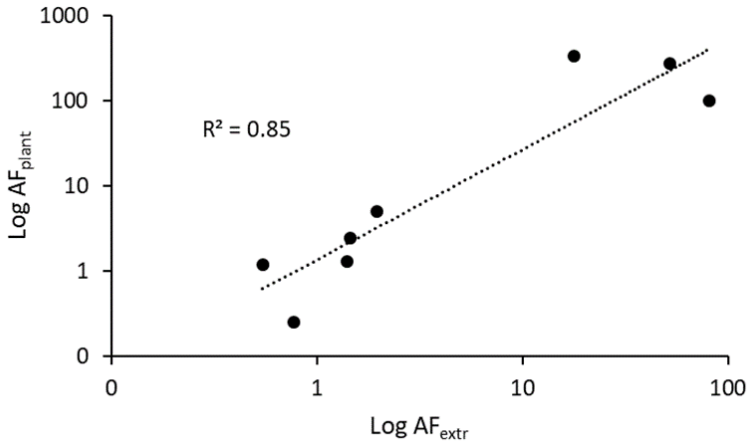


Fig. 3.5: The relationship between AF_{plant} and AF_{extr}, which are the ageing factors (AF) of Se derived from the ratio of plant Se concentrations and chemically-extracted soil soluble Se at the beginning and end of the ageing period. Ageing factors were log-transformed to homogenise variances.

3.4 Conclusions

The availability of added Se^{VI} in soil decreased with ageing time, which was observed both in chemically-extracted Se-sol fractions and concentrations of Se in plants. However, the rate of decrease varied significantly among the different soils. The solubility of Se as a function of ageing time was best represented by a reversible first order equation model. Ageing of Se was most pronounced in calcareous soils, with the predicted soluble fraction at equilibrium close to zero. The rate of Se fixation in the calcareous soils increased as a function of the $CaCO_3$ content of the soil. In non-calcareous soils, ageing was less pronounced. The bioavailability of Se in Oxisols such as Kingaroy could not be modelled kinetically; the majority of the added Se was rapidly adsorbed, while the rest remained more or less at constant solubility. Chemical extraction of soils with $CaCl_2$ was effective in predicting bioavailability, except in soils with a high sorption capacity such as Mallala (highly calcareous) and Inman Valley (high OM) where a significant drop in solubility was observed within the first 30 d.

While the RFO model described the pattern of ageing in most soils well, it does not indicate which mechanisms were driving Se ageing. Moreover, given the lack of

selectivity of certain extractants, such as TMAH, it was not possible to get an accurate insight of the processes that may have caused these changes. Such information could only be speculated on the basis of their relationship with soil properties, for example the retention of Se onto calcite surfaces by an ion exchange mechanism may have been responsible for the more pronounced retention of Se in calcareous soils compared to the others. Nevertheless, the study was one of the few to investigate the influence of soil properties and ageing time on the bioavailability of Se, especially when added as Se^{VI}. It has the merit of a combined chemical and biological approach in estimating the kinetics of ageing and the change in bioavailability over time. Such knowledge could be useful in determining the rate and frequency of Se fertiliser application in biofortification programs. Further work could be undertaken to strengthen the model developed to predict Se fixation and sorption in soils over time, for example, by including a greater variety of soils and introducing additional source terms to account for losses of Se from the soil-plant system, in the form of leaching after rainfall episodes or irrigation.

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4 Using a ^{77}Se tracer to determine how fertiliser formulation, application method and timing affect Se transfer within wheat plants

2554 **Statement of Authorship**

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Statement of Authorship

Title of Paper	Using a ^{77}Se tracer to determine how fertiliser formulation, application method and timing affect Se transfer within wheat plants
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Principal Author

Name of Principal Author (Candidate)	Chandnee Ramkissoon
Contribution to the Paper	Experimental design and set up Data collection and analysis Manuscript preparation
Overall percentage (%)	70
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	DocuSigned by: 8C23DA3E1894499... Date 2/12/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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4.1 Introduction

Micronutrient deficiencies affect one in three people globally (FAO, 2005) as a result of intake or absorption at a rate which falls below the level required to sustain good health and development (Bouis and Saltzman, 2017). Selenium (Se) is one such micronutrient which is currently consumed at lower than recommended levels in many parts of the world. Combs et al. (2001) estimated that 0.5-1 billion people worldwide were at risk of Se deficiency diseases as a result of inadequate dietary Se intake.

Selenium is an essential micronutrient for humans and other animals (Rayman, 2000). It has been shown to have antiviral effects, to be beneficial for reproduction, and to lower autoimmune thyroid disease risks. More recently, its role as an antioxidant and potential anticarcinogen has been appraised (Rayman, 2012; Reid et al., 2008). While inadequate Se intake can cause general poor health, extremely low levels of Se can cause deficiency diseases such as Keshan (cardiomyopathy) and Kashin-Beck (an osteoarthritis disorder), for example in some regions of China and Siberia (Broadley et al., 2006; Fordyce, 2005). However, Se can also be toxic if ingested at higher than recommended levels. An excess of Se in the body, resulting in 'selenosis', is characterised by the loss of hair, nails and general fatigue (Institute of Medicine, 2007) . The current Se daily recommended intake of Se is set at 55 and 70 µg person^{-1} for females and males respectively; more generally, a dietary Se intake range of 40-400 µg day^{-1} is considered safe (Macfarquhar et al., 2010). As a result of increasing concern about the inadequacy of Se intake in many locations around the world, research has, in recent decades, focused on ways to improve dietary Se levels sustainably in order to pre-empt or alleviate Se deficiency.

Agronomic biofortification is a term describing the process through which the concentration of micronutrients in edible parts of staple crops is increased through agronomic practices, such as the application of fertilisers enriched with trace elements

(Bouis and Saltzman, 2017). Finland has successfully implemented an agronomic Se biofortification programme since 1984, through the mandatory addition of Se in the form of sodium selenate to all multi-nutrient fertilisers used in agriculture. By enforcing this policy, the Finnish government aimed to increase cereal-grain Se concentration from 0.01 to 0.1 mg kg^{-1} of dry matter (Venalainen et al., 1997). The results from this biofortification programme were very positive, with Se concentrations improving >10-fold in grain, fruits and vegetables as well as in dairy products, meat and meat products (Eurola et al., 1990). This increase was concomitantly matched with an increase in the Se intake in the Finnish diets from 25 µg day^{-1} per capita in 1975-6 to 124 µg day^{-1} per capita by 1989 (Eurola et al., 1990) as well as an increase in the blood plasma Se levels (Varo et al., 1988). This programme highlighted the efficiency and safety of agronomic biofortification as an intervention strategy intended to improve the Se status of a national population. Although substantial information can be gathered about the Finnish biofortification programme ahead of establishing similar strategies in other parts of the world, more knowledge about the behaviour of the added Se in various environments is required.

Selenium exists as several chemical species; in agricultural soils, it is predominantly present as selenate (SeO_4^{2-}, Se^{VI}) and selenite (SeO_3^{2-}, Se^{IV}) (Gupta and Gupta, 2017). Selenite tends to be less mobile and bioavailable than selenate as it adsorbs onto soil components such as clay minerals and hydrous oxides of Al and Fe (Christophersen et al., 2012). The sorption capacity of these soil components for Se depends on soil characteristics such as pH, redox potential, the presence of competitor ions such as sulphate (SO_4^{2-}) and phosphate (PO_4^{3-}) and the specific surface chemistry of the sorbent phases. Selenium ions also differ in their capacity to travel and accumulate within plants; Se^{VI} usually has higher mobility in the xylem and accumulates in edible parts of plants before converting to organic forms such as

selenomethionine (SeMet); by contrast, Se^{IV}, despite rapid uptake into roots, tends to convert more rapidly to organic forms and accumulates in roots (White et al., 2004). As a result of its higher mobility in the soil as well as in the plant, Se^{VI} is often the preferred form of fertiliser applied. Its higher mobility in soil, however, also means it has higher leaching potential (Mayland et al., 1991). To obviate problems such as leaching, adsorption and immobilisation of Se within soil particles, foliar application of Se has been tested as a potential biofortification strategy. In a recent meta-analysis, Ros et al. (2016) showed that foliar fertilisation could be on average eight times more efficient than soil Se application. For example, they found that an application rate of 30-60 g ha^{-1} Se^{VI} to the soil would be needed to increase grain Se concentration from 0.07 to 0.1 mg kg^{-1} compared to just 4.5-10 g ha^{-1} Se^{VI} when foliar-Se would be applied. Less discussed in the literature is the importance of fertiliser application timing, especially that of foliar-applied fertilisers. Lyons (2018) suggested that the foliar application of nutrients such as Se and I to cereals are best made between the booting and early milk stages, preferably around the heading stage, in order to maximise the area of canopy available for fertiliser uptake. Understanding how Se transfers from the application point to the rest of the crop when different application methods and timing are employed would be useful to plan Se fertilisation tactics to optimise crop uptake.

The use of stable isotope Se tracers, such as enriched ^{77}Se, allows for the reliable, accurate and simultaneous determination of native and applied Se sources in both plant and soil systems (Di Tullo et al., 2016; Mathers et al., 2017). Using a ^{77}Se-selenate fertiliser, an experiment was undertaken to investigate how applied Se fertiliser is transferred into the plant over time and determine whether the rate of transfer could be optimised by adapting fertiliser strategies. The objectives of the study were to: a) examine time-dependent changes in the partitioning of ^{77}Se fertiliser in wheat; b) determine the effect of different fertiliser formulations, application methods –

soil and foliar- as well as timing (different growth stages) on the transfer of Se fertiliser in wheat; c) examine how the different fertiliser strategies affected the chemical speciation of Se within the plant. To achieve these objectives, a pot trial was established in which wheat was supplemented with Se in the form of ^{77}Se-enriched sodium selenate by two different application methods (soil and foliar) and at two growth stages.

4.2 Materials and Methods

4.2.1 Soil

A sandy loam topsoil, from Colchester, United Kingdom, was used for the pot trial. The soil was air-dried, sieved to < 2 mm prior to characterisation (Table 4.1). Soil pH and electrical conductivity (EC) were measured in a 1:2.5 soil-to-solution suspension on an automated Skalar pH/EC system. Soil organic matter content was determined by the loss-on-ignition method (Dean, 1974). Particle size analysis was determined by fractionation as described by McKenzie et al. (2002). Extractable P and S (mg kg^{-1}) were determined by the method developed by Olsen et al. (1954) and Lefroy et al. (1993) (Rayment and Higginson, 1992).

Table 4.1: The physical and chemical properties of the soil used in the experiment.

pH (water)	7.9
Electrical conductivity (µS cm^{-1})	1300
Organic matter (%)	4.1
Clay (%)	13
Sand (%)	72
Extractable P (mg kg^{-1})	3.0
Extractable S (mg kg^{-1})	18

4.2.2 Pot trial

The pot trial was set up in a glasshouse with natural light conditions. Five seeds of spring wheat (*Triticum aestivum cv.* Willow) were sown directly into free-draining pots containing 1.8 kg soil and thinned to two plants per pot three weeks later. Plants

were fertilised with 5 mL of an ammonium nitrate solution (16.4 g L^{-1} NH_4NO_3) at stem extension and 5 mL at head emergence. Pots were arranged in a randomised design and watered to an estimated weight of 60% WHC of the soil using Milli Q water, throughout the experiment. Care was taken during watering not to create leaching. All treatments were replicated four times.

4.2.3 Selenium fertiliser application

Selenium fertilisers (Se_{fert}) were prepared from a ^{77}Se-enriched sodium selenate solution (259 mg L^{-1} $^{77}Se^{VI}$). Selenium was applied at a single, realistic rate of 3.33 µg kg^{-1} (equivalent to approximately 10 g ha^{-1}, based on a 20 cm depth and 1.5 g cm^{-3} bulk density) at either growth stage 1 (**GS1**), which was at stem elongation (growth stage 31/32 on the Zadoks scale and 63 days after sowing (DAS)) – or at head emergence (**GS2**; Zadoks stage 57 and 119 DAS) (Zadoks et al., 1974). Four fertiliser treatments were used: (i) direct soil Se application (Soil Se); (ii) foliar-applied Se (F. Se); (iii) foliar-applied Se with a 2% w/v N source in the form of urea (Sigma-Aldrich, 99-100% purity, United Kingdom) (F. Se+N); (iv) control (Ctrl) where neither Se nor N was applied.

Foliar solutions all contained 0.5% surfactant (Triton-X 100; Sigma-Aldrich), in order to reduce the surface tension between the droplets and the leaf surface. The surface of the soil was covered with cling film for a week following foliar fertiliser application to prevent any potential runoff into the soil. Care was taken not to irrigate the pots straight after foliar fertilisation. The foliar Se+N solution was prepared by dissolving 0.21 g of urea in a solution with a ^{77}Se concentration of 180 mg L^{-1}. Foliar Se and Se+N applications were applied as four drops of 5 µL volume to the two youngest flag leaves of each plant (2 plants per pot). For soil Se application, 5 mL of a 1.44 mg L^{-1} ^{77}Se stock solution was applied to the soil surface. For the control

treatment, water with 0.5 % surfactant was applied in a similar manner to foliar Se solutions.

4.2.4 Plant harvest

The aboveground biomass of the wheat plants was harvested at 3, 10 and 17 days (H3, H10 and H17) after fertiliser application at GS1 and 3, 10 and 34 days (H3, H10 and H34) after fertiliser application at GS2. For the plants treated at GS2, wheat heads were harvested separately from the straw and, for the last sampling (H34), wheat heads were further hand-threshed to separate wheat grains. For all foliar treatments, the foliar-treated leaves were harvested separately from the straw, washed in 0.1% v/v detergent and then rinsed with Milli Q water (Labanauskas, 1968). Water rinses were saved to analyse for any unabsorbed applied Se_{fert}. After harvest, all plant parts were dried at 50°C for 72 h or until constant dry weight was achieved. The dry weights of the different plant parts were recorded. Subsequently, plants were ground using a centrifugal mill (model ZM 200, Retsch, Germany) fitted with a 0.5 mm titanium screen and stored under ambient conditions prior to digestion and chemical analyses.

4.2.5 Selenium analyses

4.2.5.1 Total Se determination

The total Se concentration in plant samples was measured using inductively coupled plasma mass spectrometry (ICP-MS; model iCapQ, Thermo Fisher Scientific, Bremen, Germany) following microwave-assisted acid digestion. Approximately 0.2 g of plant material was weighed into perfluoroalkoxy (PFA) vessels and mixed with 6 mL of concentrated nitric acid (HNO_3) before microwave heating (Model Multiwave 3000, fitted with a 48-place rotor; Anton Paar, Graz, Austria). The digested samples were then made to 20 mL final volume using Milli Q water and further diluted 10-fold with 2% HNO_3 prior to analysis.

4.2.5.2 Speciation analysis

An enzymatic hydrolysis method was employed to determine Se speciation in the foliar treated leaves and in the wheat grain samples. Four Se species were assayed: selenate, selenite, seleno-L-cysteine (SeCys) and seleno-L-methionine (SeMet). A multi-standard solution (10 mL) containing the four Se species nominally at 5 µg L^{-1} concentration was prepared by diluting stock solutions of $^{77}Se^{IV}$ and $^{77}Se^{VI}$ (1000 mg L^{-1}) and SeCys and SeMet (100 mg L^{-1}); the stock solutions with organic Se were prepared by dissolving the individual salts in Milli Q water. The Se concentrations of the individual Se species standards were verified by analysis (direct aspiration) using ICP-MS, with measured Se concentrations of 6.47, 5.37, 5.28 and 5.30 µg L^{-1}, respectively.

Five mL of an enzyme solution containing 0.02 g protease K (Type XIV ≥ 3.5 units mg^{-1} solid from *Streptomyces griseus*) and 0.01 g lipase (Type VII ≥ 700 units mg^{-1} solid from *Candida rugosa*) was added to plant samples (0.2 g) in centrifuge tubes. The samples were incubated in the dark and shaken in a water bath set at 60 rpm at 37°C for 24 h; after incubation, they were centrifuged at 3000 *g* for 30 minutes and filtered through 0.25 µm filters. Enzymatically-hydrolysed samples that were not immediately analysed were stored at 4°C in the dark. Selenium speciation analysis was undertaken using coupled HPLC-ICP-triple quadrupole-MS (ICP-QQQ-MS) instruments. The ICP-QQQ-MS was operated in oxygen cell mode to enable mass shifting of the Se isotopes and thereby minimise interferences (Table 4.2). Standards were run after every block of 12 samples to monitor drift and enable correction of sample concentrations (Mathers et al., 2017).

Table 4.2: The operating conditions of the HPLC-ICP-MS used for Se speciation.

Mobile phase	20 mM (A) and 50 mM (B) ammonium citrate, 2% methanol, adjusted to pH 4.3 using citric acid
Flow rate	1 mL min^{-1}
Gradient conditions	1-5 minutes at 100% A, 5-5.5 minutes up to 100% B, 5.5-12 minutes at 100% B
Injected sample volume	500 µL
Column temperature	30° C
Equipment	Hamilton PRP X-100 anion exchange column (4.1 mm x 250 mm x 10 µm)

Sample processing was undertaken using a version of Chromeleon (Dionex) chromatography software operating within the iCapQ Qtegra software; the peaks generated by the individual Se species were manually integrated for peak area. Raw intensity data (integrated counts-per-second, iCPS) were then imported from the ICP-QQQ-MS at mass:charge (m/z) ratios of 77 and 80.

The enzymatically-hydrolysed plant samples were also analysed for total ^{80}Se and ^{77}Se by ICP-MS, following a 1:10 dilution of the original enzyme extracts with 2% HNO_3 acid. The final concentrations of the individual Se species were calculated from the proportion of the total extract Se that was measured as the peak area of the individual species, as described in Mathers et al. (2017). For example, the concentration (µg L^{-1}) of SeMet (at m/z 77 and 80) was calculated from Eq. 1:

$$SeMet_{conc} = \frac{SeMet_{cps}}{\sum species_{cps}} \; x \; Se_{tot,enz} \qquad (1)$$

where $SeMet_{cps}$ is the signal (iCPS) of SeMet and $\sum species_{cps}$ the sum for all four species ($SeMet_{cps}$, $SeCys_{cps}$, Se^{IV}_{cps} and Se^{VI}_{cps}) and $Se_{tot,enz}$ is the total Se concentration (µg L^{-1}) measured in the enzyme-hydrolysed extracts.

The concentration of individual Se species as well as total Se concentrations were then converted to a gravimetric basis using the dry weights of individual samples and the volume of the different extracts.

4.2.6 Quality control

Replicate samples of a standard reference material (tomato leaves NIST 1573a) were acid digested and analysed for total Se by ICP-MS to provide quality assurance for the analysis of the plant samples. The Se recovery in the reference material was within 100 ± 10% of the certified value.

The extraction efficiency was calculated as follows (Eq. 2).

$$Efficiency = \frac{Se_{tot,enz}}{Se_{tot,acid}} x\ 100 \qquad (2)$$

where $Se_{tot,acid}$ is the total Se concentration measured by acid hydrolysis for individual samples (µg L^{-1}).

4.2.7 Statistical analyses

The effects of the different fertilisation treatments on grain yield and Se concentrations in plants were determined using the analysis of variance (ANOVA) procedure in SPSS (IBM SPSS Statistics for Windows, Version 24.0., IBM Corp, Armonk, New York), with a significance threshold of 5%. Duncan's posthoc tests were used to compare treatment means.

4.3 Results

4.3.1 Plant yield

The yield of plants, calculated as the dry weight of the aboveground biomass, increased significantly with time. Plants harvested at GS2 had a higher biomass than those harvested at GS1 (Table 4.3). No significant differences in yield were observed among the different Se treatments.

Table 4.3: Dry matter yield of aboveground plants harvested 3, 10, and 17 days after Se application at stem elongation (GS1) and 3, 10 and 34 days after Se application at the heading stage (GS2). Results show average ± standard error (SE) (n=16).

Growth Stage (GS)	Days after sowing (DAS) days	Harvest time following Se_{fert} application days	Dry matter yield† g pot^{-1}
1	66	3	3.16 ± 0.1^{e}
	73	10	4.65 ± 0.2^{d}
	80	17	5.92 ± 0.3^{c}
2	122	3	18.9 ± 0.6^{b}
	129	10	21.7 ± 0.5^{a}
	153	34	22.2 ± 0.5^{a}

†Different letters indicate significant differences ($p < 0.05$).

4.3.2 Native Se distribution in plants

The concentration of Se_N in plants averaged 0.07 ± 0.01 µg pot^{-1} at GS1 and 0.28 ± 0.04 µg pot^{-1} at GS2, with no significant differences among Se treatments or harvest time within the same growth stage. The Se_N content of GS2 plants was higher than that of GS1 plants, probably as GS2 plants remained in contact with the soil for a longer time period compared to those harvested 66-80 DAS (GS1). In GS2 plants, wheat heads accumulated twice as much Se_N than straw, the majority of which was recovered in the grain at the last sampling point (Fig. 4.1).

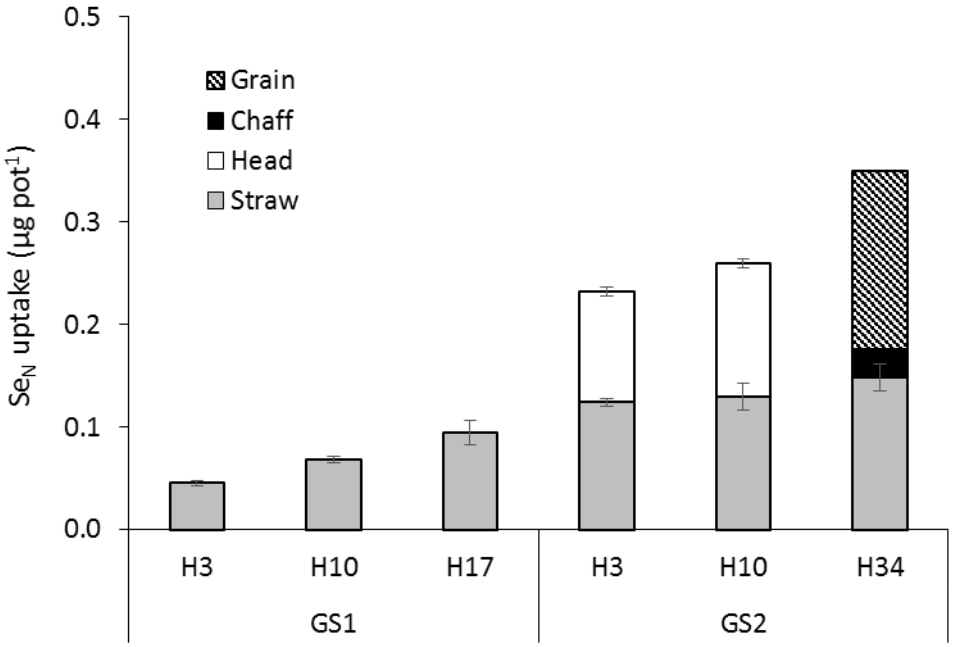


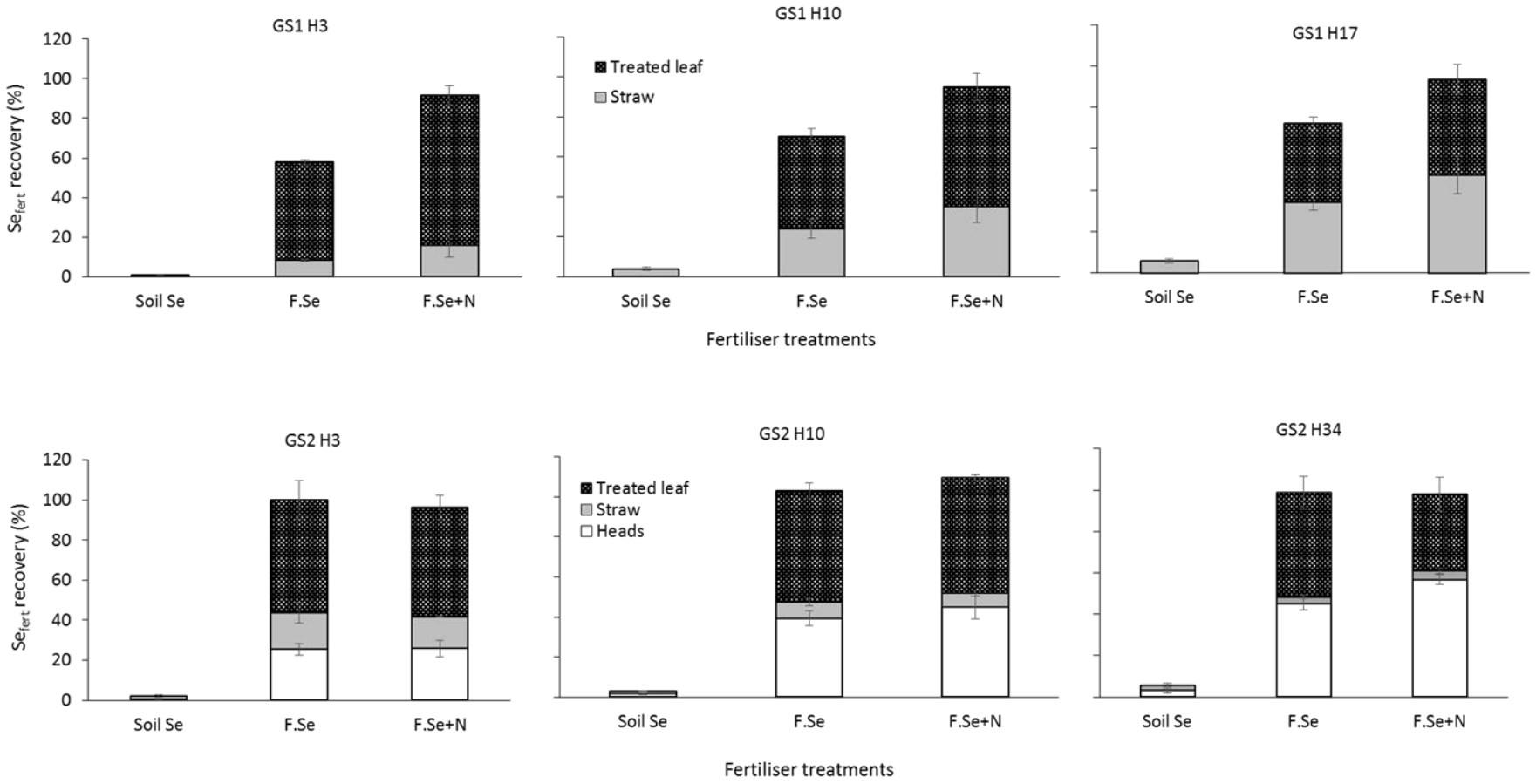
Fig. 4.1: The distribution and partitioning of native Se in the aboveground biomass of plants.

The majority (> 90%) of the total Se recovered in plants fertilised by the foliar method originated from the fertiliser ^{77}Se source; the recovery of native Se (Se_N) in these plants was minimal (average 1.4 ± 0.18% at GS1 and 4.1 ± 0.33% at GS2) (Supp. Info. Table 4.6). In comparison, 70 ± 4.4% of the total Se recovered in plants fertilised with soil-applied Se was derived from the native soil Se. This suggests losses of the added ^{77}Se fertiliser following its addition to the soil through leaching and/or immobilisation in the soil.

4.3.3 Applied Se_{fert} distribution in plants

For treatments applied at GS1, the aboveground biomass was harvested as one fraction, except for foliar treatments where the foliar-treated leaves were harvested and analysed separately. For GS1 plants, the majority (> 63%) of the applied Se_{fert} was measured in the treated leaves up to 10 d after application, which decreased < 50% by d 17, suggesting mobilisation from the leaf to the straw. The recovery of applied Se_{fert} was significantly higher for foliar treatments (± N) compared to soil application, especially when foliar Se was co-applied with N (> 90% recovery), suggesting minimal

losses to the environment. In comparison, lower recovery of Se_{fert} was observed in the F.Se-treated plants at GS1, suggesting that the inclusion of N to foliar Se solutions improved the uptake of Se by plants (Fig. 4.2).



2806

2807 Fig. 4.2: Percentage of applied Se_{fert} that was recovered in the aboveground biomass of plants as a function of harvest time,
2808 application method and timing. Error bars represent standard errors (n=4). The recovery of the applied Se_{fert} in the different plant
2809 parts was calculated as the amount of ^{77}Se in individual parts (µg pot^{-1}) as a percentage of the amount of ^{77}Se applied to each pot
2810 (5.99 µg pot^{-1}).

At GS2, the aboveground biomass was separated into the following parts: straw, heads and Se-applied leaves for foliar treatments (Fig. 4.2). Limited losses of the applied foliar Se (±N) fertilisers to the environment were observed as recovery in the aboveground biomass was > 95%, regardless of harvest time or formulation. This was confirmed by Se_{fert} levels in the foliar rinses being below analytical detection limits (data not shown). Within 3 d of application, 43 ± 0.98% Se_{fert} was translocated from the point of application to the rest of the plant, which was equally distributed between the wheat heads and the straw. At the last sampling time (153 DAS), this translocation increased to 56 ± 5.2% with heads accumulating significantly more Se_{fert} than straw ($p < 0.05$). No significant differences in the recovery of Se_{fert} in the aboveground biomass of plants was observed between foliar Se (± N) treatments at GS2.

4.3.4 Effect of N addition in foliar Se solutions on Se_{fert} uptake

The inclusion of N with foliar Se solutions led to higher Se uptake into plants compared to foliar Se application on its own, when applied at GS1; in comparison, no significant differences in Se uptake was observed in plants treated with F.Se and F.Se+N, when the latter were applied at GS2 (Table 4.4). This suggests that when applied at GS1, foliar Se fertilisers were more effective in raising plant Se concentrations when they were co-applied with urea-N, than foliar Se on its own. At GS2, the uptake of Se_{fert} by plants increased with growth time but was not affected by the different foliar Se formulations.

Table 4.4: The influence of N inclusion with foliar Se solutions and harvest time on the accumulation of Se in the aboveground of biomass (foliar-treated leaves excluded). Results show average ± SE (n=4).

Time after Se_{fert} application (days)	Se_{fert} uptake (µg pot^{-1})			
	GS1		GS2	
	-N	+N	-N	+N
3	0.495 ± 0.03	0.946 ± 0.36	2.40 ± 0.40	2.31 ± 0.30
10	1.44 ± 0.28	2.11 ± 0.49	2.85 ± 0.11	3.12 ± 0.32
17/34‡	2.06 ± 0.25	2.84 ± 0.55	2.90 ± 0.21	4.00 ± 0.51
Two-way ANOVA				
Day	< 0.05		< 0.05	
N	< 0.10		ns	
Day*N	ns		ns	

‡The last sampling was done 17 d and 34 d after Se_{fert} application at GS1 and GS2, respectively.

The effectiveness of foliar Se fertilisers, more specifically, foliar Se application with N, was also observed in the grains (Fig. 4.3). The average grain Se concentrations for soil, foliar and foliar Se+N treatments were: 0.15 ± 0.01, 0.26 ± 0.02, 0.32 ± 0.07 mg kg^{-1}, which accounted for 3%, 44% and 54% of the applied Se_{fert} transferred to the grain, respectively.

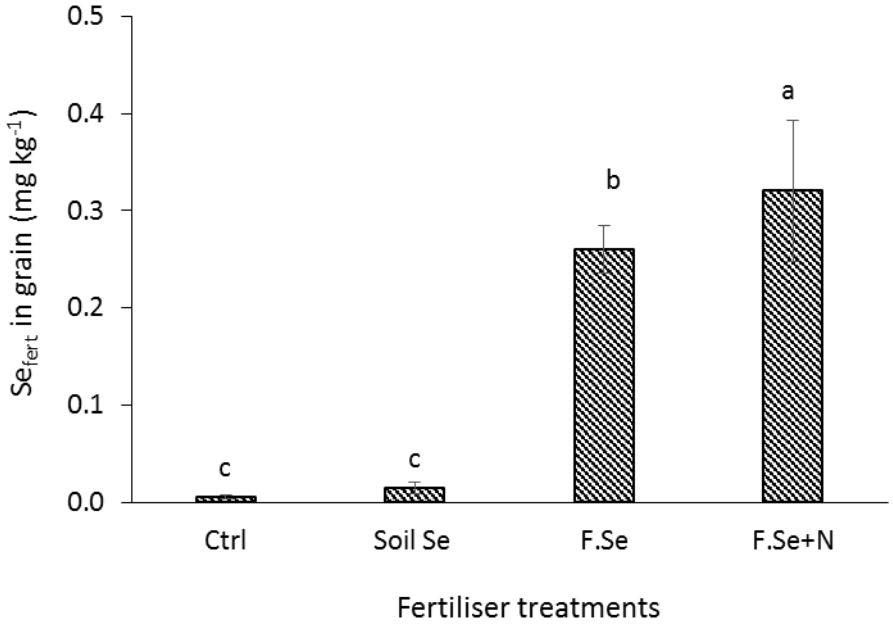


Fig. 4.3: The concentration of Se_{fert} in wheat grain. Results show averages and error bars represent standard errors (n=4). 'a' and 'b' represent statistical differences in means at the 0.05 level.

4.3.5 Selenium speciation

The chemical speciation of Se foliar-treated leaves (± N) and grain was carried out to assess how Se transformed in plants over time.

4.3.5.1 Grain

The protease hydrolysis extracted > 60% of the total Se concentration in the wheat grain (Eq. 2). The distribution of the different Se species was similar across the different treatments, albeit at significantly lower concentrations when Se was soil-applied compared to its foliar application. The different foliar Se formulations (± N) did not affect Se speciation in the grain, as SeMet was the most abundant species, accounting for > 90% of the total Se_{fert} in the grain. A small amount of Se^{VI} (< 10% of the total Se_{fert}) was also detected in the grain, with no significant differences in accumulation among the various treatments; no Se^{IV} or SeCys was measured, irrespective of Se treatments (Table 4.5).

Table 4.5: Distribution of Se species in wheat grain expressed as mean concentration ± SE (% of total grain Se ± SE) (n=4). 'n.d.' denotes non-detectable concentrations of species; 'a' and 'b' show statistical significant differences at the 0.05 level.

Treatments	Se species in grain (mg kg^{-1})‡			
	SeMet	Se^{VI}	Se^{IV}	SeCys
Soil Se	0.04 ± 0.00 (86 ± 4.0)^{b}	0.001 ± 0.00 (14 ± 4.0)^{b}		
F.Se	0.16 ± 0.02 (92 ± 0.3)^{a}	0.014 ± 0.00 (8.3 ± 0.3)^{a}	n.d.	n.d.
F.Se+N	0.21 ± 0.02 (94 ± 2.3)^{a}	0.010 ± 0.00 (6.3 ± 2.3)^{a}		

‡Different letters indicate significant differences ($p < 0.05$) in the accumulation of Se species among different treatments.

4.3.5.2 Leaves treated with foliar Se

The protease hydrolysis extracted 72 ± 2.4% of the total Sefert concentration in the foliar-treated leaves. The main species identified in the extracts were SeVI and SeMet; negligible concentrations of SeIV and SeCys (< 2%) were measured in the leaves (Fig. 4.4). For both F.Se and F.Se+N treatments, the distributions of the Se species were similar. Selenate was the dominant species in the foliar-treated leaves as it formed 91 ± 2.0% of the total Se. The next most abundant species was SeMet (8.0 ± 1.9% of the total Se), followed by Se^{IV} (< 1% of the added Se) (Fig. 4.4).

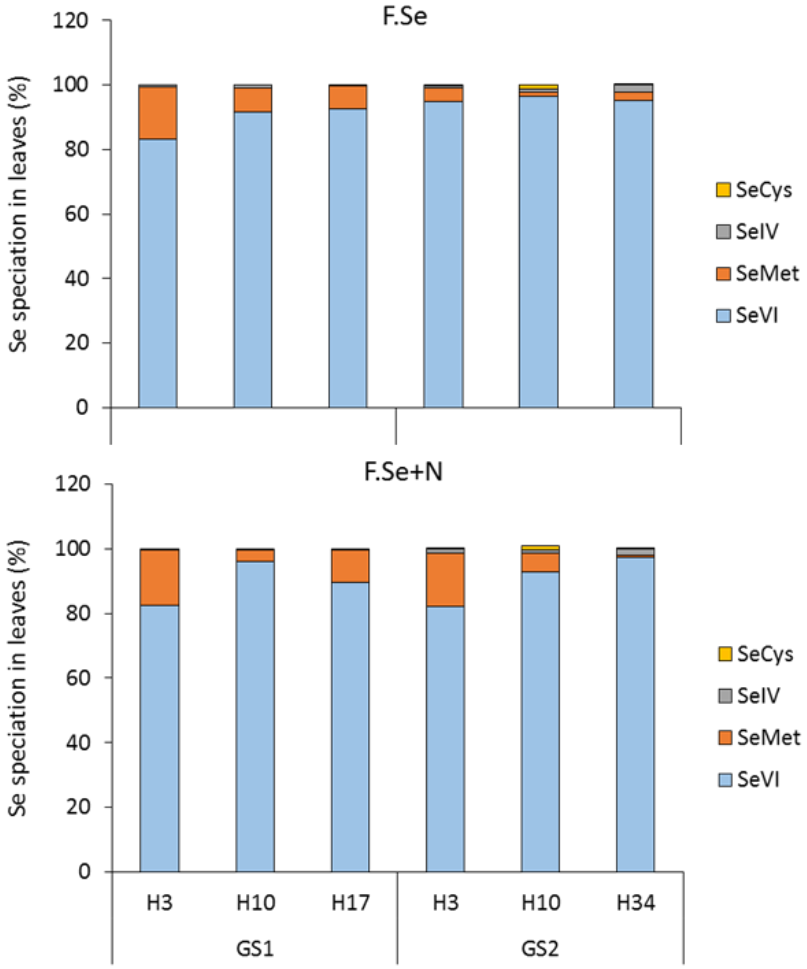


Fig. 4.4: The distribution of Se species as a percentage of the total Se in leaves that were treated with F.Se and F.Se+N and harvested at different times following application at stem elongation (GS1) and at heading (GS2).

The proportion Se^{VI} in the foliar-treated leaves did not change significantly over the 153 d experimental period (91 ± 1.6%). In comparison, the proportion of SeMet decreased significantly with harvest time, in a similar way for both GS1 and GS2, which suggests rapid mobilisation of SeMet from the foliar-treated leaves to the rest of the plant compared to other Se species (Fig. 4.5). However, the influence of N was significant only in GS2-plants: the SeMet content of leaves treated with F.Se+N was significantly higher and decreased more rapidly over time than those treated with F.Se only (Fig. 4.5).

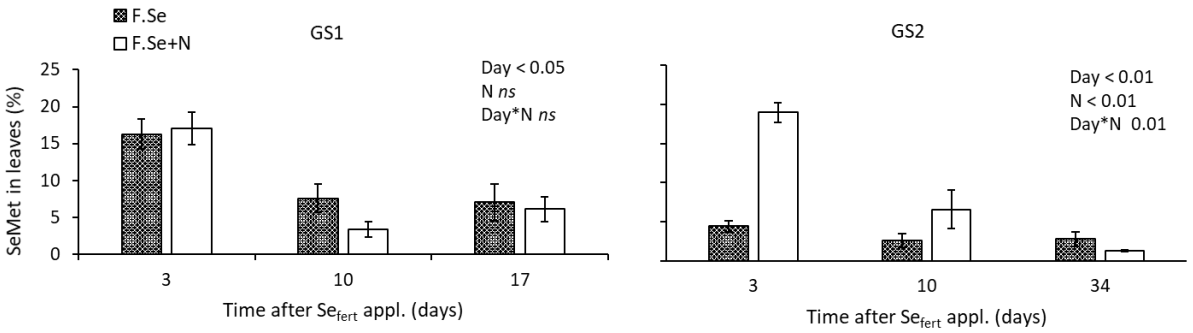


Fig. 4.5: The proportion of total Se measured as SeMet in leaves that were treated with foliar Se and foliar Se+N at stem elongation (GS1) and at heading stage (GS2). Error bars represent standard errors (n=4). The *p*-values displayed on the graph show statistical significance from a two-way ANOVA '*ns*' represents non-significance.

4.4 Discussion

Plant yield, calculated as the dry weight of the aboveground biomass, was not influenced by the different Se treatments (Table 4.3), as expected, given that Se does not play an essential role in plant nutrition. Although Se can mitigate stress in plants by stimulating the activity of antioxidants (Schiavon, 2017), its essentiality in higher plants is not proven (Pilon-Smits et al., 2009).

The Se concentrations of control plants and grains in the experiment were below the target concentration of 0.1 mg kg^{-1} required to reach the lower threshold of adequacy in the diet (Hartikainen, 2005), suggesting very low background Se levels in the soil used in this experiment. The application of Se to the soil, irrespective of application timing, was highly ineffective in increasing plant Se concentrations above control levels. The recovery of the applied Se_{fert} in the aboveground biomass of plants fertilised with soil-applied Se was < 6% across the experiment (Fig. 4.2), 2.4% of which was recovered in the grain, suggesting major losses of the exogenous Se source, either by adsorption/fixation to the soil or leaching. This recovery was much lower than those reported by comparable studies where 10 g ha^{-1} of aqueous selenate was applied to the soil. For example, grain Se_{fert} recovery reported by Mathers et al. (2017)

was 26-45%, 10-17% by Broadley et al. (2010), 14-17% by Curtin et al. (2008) and 7-11% in maize by Chilimba et al. (2012b). The results of the present study compared favourably with Stephen et al. (1989) who reported *c.* 5% recovery of Se_{fert} in wheat grain following its application at sowing in autumn trials. They attributed the poor efficiency of the soil-applied Se to significant immobilisation of Se (mostly likely Se^{IV}) occurring before the plants were able to actively take up Se from the soil in spring. Unlike the field trial by Stephen et al. (1989) , the Se fertiliser in this study was applied in spring, at stages where the plants were potentially actively growing (stem elongation and heading stages). Although care was taken not to water the pots after Se application, there were episodes of rainfall in the early days of spring, where water seeped into the glasshouse. Rainfall data or leachates were not recorded in the present study. Hence the reason for the losses of soil-applied Se_{fert} can only be speculated to be due predominantly to leaching from the free-draining pots rather than immobilisation in the soil. Leaching is certainly a risk for Se^{VI} as it is weakly adsorbed by soil particles and hence, highly mobile (Yamada et al., 1998); given the soil pH (7.9) and aerobic conditions (usual range of 0.40-0.60 V), Se^{VI} was likely to be the stable species in the soil (Fig. 4.6).

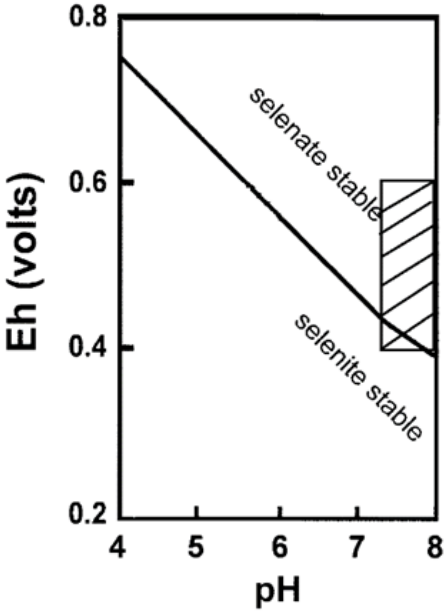


Fig. 4.6: Selenium species in soil as a function of soil pH and redox conditions (adapted from Elrashidi et al. (1987) and Curtin et al. (2008)).

In contrast to Se application to the soil surface, the application of Se by the foliar method was very successful in accumulating Se in crops, as recoveries of Se_{fert} in crops ranged from 60% to 100% (Fig. 4.2), suggesting minimal losses to the environment and highlighting the efficacy of foliar fertilisation. The effect of N inclusion in foliar Se solutions on the total uptake by plants was observed at GS1 stage only, where the recovery of Se_{fert} following foliar Se application on its own was significantly lower than when foliar Se was co-applied with N (Table 4.4). However, the presence of N in foliar solutions at GS1 did not affect the formation of organic Se (SeMet) in the leaves (Fig. 4.5), suggesting that at GS1, N helped improve Se uptake *via* an absorption mechanism rather than assimilation and translocation. Wittwer et al. (1967) reported that foliar urea application facilitates the penetration and absorption of other materials, such as nutrient ions, when applied simultaneously. They attributed this effect to “facilitated diffusion” of the ions through the leaf with co-applied with urea.

At GS2, no significant difference in the total Se uptake in the plants was observed between foliar Se (± N) treatments (Table 4.4). However, N inclusion in foliar

Se solutions led to higher Se concentration in the grain (Fig. 4.3). This suggests that even though total Se uptake in the aboveground biomass was unaffected by N addition in foliar solutions, N improved Se assimilation potentially at the point of application, and translocation of organic Se compounds to the grain. This hypothesis was supported by speciation data of the foliar-treated leaves at GS2, which showed that the SeMet fraction in the leaves treated with F.Se+N was significantly higher than those treated with F.Se only (Fig. 4.4). For example, 3 d after F.Se+N application at GS2, 14% of the applied Se was detected as SeMet, which compared to only 4% SeMet in F.Se-treated leaves. (Fig. 4.5). This could be possible because N and Se share a common metabolic pathway in plants (Schiavon, 2017), and therefore, co-application of foliar Se with N at a stage where plants have a high metabolic activity (GS2) most likely affected the rate of Se assimilation and translocation within the plant. Even though N did not significantly affect the chemical speciation of Se in the grain (> 90% measured as SeMet) (Table 4.5), it was highly beneficial in improving the Se status of the grain (Fig. 4.3), These findings were in agreement with those from experimental Chapter 2, where N co-application with foliar Se led to significantly higher Se concentration in wheat grains compared to the application of foliar Se in pure form, but had no effect on the speciation of Se in the grain.

The target grain Se concentration range desired for biofortification, without running the risk of toxic effects is within the range of 0.1 – 1.0 mg kg^{-1} (Hartikainen, 2005). The application of foliar Se (±N) in this study increased grain Se concentrations to > 0.25 mg kg^{-1} (Fig. 4.3), which is optimal for biofortification, based on an RDI of 55-65 µg day^{-1} (Lyons, 2018; Smoleń et al., 2016). More interestingly, the application of foliar Se with a N source at the heading stage (GS2) was more efficient in accumulating Se in the grain than the application of foliar Se in pure form. The different foliar Se formulations had no significant effect on the chemical speciation in the grain as both

were equally effective in transforming most of the total Se in the grain into bioavailable SeMet fraction. From a biofortification perspective, the application of foliar Se with N can be recommended as a fertilisation strategy to improve the overall status of wheat grains, which would lead to an improved consumption of bioavailable Se in the human diet.

4.5 Conclusions

In this study, the application of Se by the foliar method was much more effective in raising Se concentrations in wheat grain than direct Se application to soil. Given that the soil pots were free-draining, care was taken during watering so that the risk of leaching was kept to a minimum throughout the plant growth. However, unforeseen leakage through the glasshouse (following rainfall) may have caused inadvertent leaching of the soil-applied Se^{VI}. Leachates were not collected on this occasion but it seems likely that such poor recovery was due to more than just immobilisation of Se in the soil, especially since Se^{VI} was likely to be the predominant form of Se in the soil. A rainfall simulation study whereby plants grown in a comparable soil and fertilised with soil-applied Se^{VI} would be exposed to a controlled rainfall event, could be carried out to verify this hypothesis. Collection and analysis of the leachate for Se^{VI} should provide more precise information about the leaching potential of Se^{VI}.

Applying foliar Se, irrespective of the formulation, at 10 g ha^{-1} brought grain Se concentration to a level high enough to be considered adequate for biofortification. The fertilisation of plants with foliar Se at an early growth stage was made more efficient (greater uptake and recovery) by its co-application with urea, potentially due to the improved absorption of Se through the cuticular membrane of the leaf. At a later growth stage, this absorption mechanism seemed to matter less. Nevertheless, the inclusion of N in foliar solutions led to significantly higher Se concentration in the grain,

potentially by aiding Se assimilation into organic compounds in the leaves and subsequently, their translocation to the grain.

Although the results about the effectiveness of foliar fertilisers in comparison to soil-applied ones in this study are in agreement with others (Ros et al., 2016), the recovery of foliar-applied Se fertilisers in the aboveground biomass of crops (> 60% at GS1 and > 96% at GS2) was considerably higher than those in previous studies (< 30%) (Ros et al., 2016). This higher fertiliser use efficiency could be attributed to two main reasons: (1) Se fertilisers were applied in a careful controlled manner, through the application of discrete droplets to the surface of the leaves and (2) plants were grown under controlled environmental conditions, relatively to field trials, which obviated potential losses by runoff after rainfall or irrigation. Nevertheless, this study provided practical information about the time-dependent changes in the distribution of Se fertilisers in the crop, which could be useful in the field. For example, when foliar Se was applied on its own at an early growth stage, up to 89% of the applied Se_{fert} was recovered unassimilated (89% Se^{VI}) in the treated leaf 3 d after application. This increases the risk of fertiliser loss to the environment should runoff of foliar-applied Se occur within that time. Such practical knowledge is critical to making informed agronomic decisions in biofortification programmes.

4.6 Supplementary Information

Table 4.6: The relative contributions of native Se (Se_N) and fertiliser ^{77}Se (Se_{fert}) to the total Se measured in the aboveground biomass of plants fertilised by either soil Se application, foliar Se-only or foliar Se with 2% w/v urea.

Growth stage	Treatment	Total Se	Proportion of total Se originating from	
			Se_N	Se_{fert}
		µg pot^{-1}	%	%
GS1 H3	Soil Se	0.06	78	22
	F.Se	3.51	1.4	99
	F.Se+N	5.53	0.8	99
GS1 H10	Soil Se	0.09	70	30
	F.Se	4.68	1.7	98
	F.Se+N	5.76	1.1	99
GS1 H17	Soil Se	0.15	83	17
	F.Se	4.42	2.0	98
	F.Se+N	5.69	1.2	99
GS2 H3	Soil Se	0.35	66	34
	F.Se	6.03	4.1	96
	F.Se+N	5.83	4.1	96
GS2 H10	Soil Se	0.33	73	27
	F.Se	6.45	3.6	96
	F.Se+N	7.29	3.5	96
GS2 H34	Soil Se	0.35	52	48
	F.Se	7.89	3.7	96
	F.Se+N	5.54	5.7	94

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3124

5 General discussion and conclusions

Agronomic biofortification of Se provides an efficient way of linking nutritious agriculture to human health (Lyons, 2018). Selenium application not does improve crop yield, which means its application is of no economic benefit to farmers (Bouis and Saltzman, 2017). Incentives are therefore required by farmers to adopt Se fertiliser strategies; this could be simple legislation, whereby governments compel fertiliser companies to add Se to all multi-nutrient fertilisers aimed at application to agricultural produce (for example in Finland), or it could be a premium on Se-enriched cereals and cereal products. The present study provides helpful insights about ways in which Se application can be optimised to adapt to site-specific properties and existing agronomic practices, in order to maximise uptake by wheat. Improving Se fertiliser use efficiency would imply lowering the rates of application and hence, reducing any associated costs, which would be incentives for stakeholders – from farmers to commercial producers – to adopt the proposed strategies.

Two experiments were undertaken, in the UK and Australia, to test the hypotheses that the current Se biofortification methods could be made more effective by (1) the application to soil or foliage with macronutrient carriers such as N, P, K and S compounds and (2) the optimisation of fertiliser formulations, application methods and timing. Pot trials were carried out with wheat (*Triticum aestivum*), which has proven to be very responsive to Se biofortification strategies in earlier studies (Gupta and Gupta, 2017).

The first hypothesis was rejected because the application of Se with macronutrient carriers, especially P, K and S, in the form of Se-enriched DAP, MOP and SOA fertilisers, was not effective in raising wheat grain Se levels above controls (where no Se was added). The recovery of Se in these treatments was lower than the 12-27% reported by previous studies (Stroud et al., 2010a), which, given the controlled conditions and lined pots (no leaching) used, suggested the use of P, K and S for wheat

biofortification were not the most effective carriers for Se. It was speculated that the locally reduced environment created around the fertiliser granule as it dissolves in the soil may have prompted the formation of Se^{IV}, which is more strongly retained on soil particles and less bioavailable than Se^{VI} (Hettiarachchi et al., 2006; Sors et al., 2005). Moreover, the close proximity of Se with competing ions such as SO_4^{2-} and PO_4^{3-} in Se-enriched S and P granules may have hampered Se uptake into plant roots (Lee et al., 2011). Nitrogen was more effective than P, K and S as a carrier in elevating grain Se levels to 0.10-0.26 mg kg^{-1}.

The effect of fertiliser application timing (stem elongation vs. heading) on the uptake efficiency of soil-applied Se into plants could not be reliably determined in this study possibly due to inadvertent losses of the soil-applied Se leaching from the free draining pots. The experiment did, however, highlight the mobility and leaching potential of Se^{VI} from the soil, which can lead to significant losses in the field as a result of intense rainfall soon after fertiliser application.

Although the efficiency of foliar fertilisation compared to soil fertilisation has been appraised before (Ros et al., 2016), this study is perhaps the first to demonstrate that the efficacy of foliar Se fertiliser can be improved by its co-application with 2% w/v or v/v N source such as urea or UAN. The application of foliar Se with a N source was observed to almost double the uptake of Se in wheat grain and the majority (> 90%) was in the highly bioavailable seMet fraction. This was attributed to promotion in the formation in organic Se species and an increased rate of transfer of Se from the point of application to the rest of the plant, including grain, when N was co-applied with foliar Se. The grain Se concentration when foliar Se (±N) was applied was on average 0.37 ± 0.03 mg kg^{-1}, which, assuming the average intake of cereal and cereal products in the diet to be 110 g per day (Mathers et al., 2017), would provide approximately 40 µg Se $person^{-1}$ day^{-1}. The consumption of such Se-biofortified wheat grain would

contribute to 67% and 53% of the recommended nutrient intake for adult women (60 µg day^{-1}) and men (75 µg day^{-1}), respectively (British Nutrition Foundation, 2001) . Bearing in mind that the upper threshold limit of Se intake by humans is 400 µg Se day^{-1} (Institute of Medicine, 2007), even for individuals with a diverse diet (Chilimba et al., 2012b), it is unlikely that this level of biofortification could pose a toxicity risk.

The influence of N on the efficiency of foliar Se fertilisers was dependent on the stage at which fertilisers were applied. At stem elongation, higher recovery (> 90%) of applied Se was observed when foliar Se was co-applied with N compared to foliar Se application on its own (< 70%). In contrast, when applied at the heading stage, the presence of N did not significantly influence the overall recovery of Se in the plants. However, it did improve the assimilation of Se into bioavailable SeMet species in the leaves, which was then efficiently translocated to the grain The inclusion of N with foliar Se solutions was therefore generally beneficial in improving the Se status of the plant, either by enhancing the absorption through the leaf or by improving its rate of assimilation and translocation from the point of application.. In the field, this has important implications because N is usually added as split applications throughout plant growth. Foliar urea gives the best response in N accumulation in grain when applied at the heading stage (early anthesis (McDonald and Hooper, 2013)). It is perhaps worth combining foliar Se with foliar urea application in the field, to improve grain Se concentration, maintain plant yield as well as reduce application costs. From a nutritional perspective, co-applying foliar Se with N is highly effective as an agronomic strategy for Se biofortification has it improves the accumulation of Se in its most bioavailable form in the grain.

It should be noted that the experiments in this study were carried out in a growth chamber or glasshouse. As a result of their controlled conditions, pot trials suffer from the criticism that their results might not translate back to field situations (de Vries,

3203 1980). For example, precise volumes of aqueous Se were applied to the surface of the
3204 pots or leaves using a pipette in this study, and care was taken not to irrigate plants
3205 immediately after application to prevent any fertiliser runoff from leaves. In field trials,
3206 the application of Se fertilisers is less controlled; soil and foliar applications of aqueous
3207 Se have used a knapsack sprayer (16 L Vermorel 2000 pro, Berthoud Jadin) (Chilimba
3208 et al., 2012b; Mathers et al., 2017) and fertiliser granules have been applied *via*
3209 calibrated cups to the base of individual plants by 'hand-placement' (Chilimba et al.,
3210 2012b; Gupta and MacLeod, 1994). It is therefore not surprising that a higher than
3211 average (< 30%) recovery of applied Se in wheat grain from foliar Se treatments in this
3212 study (20-44% for the foliar Se-only treatment and 42-54% for the foliar Se plus N
3213 treatment) was observed (Curtin et al., 2006; Ros et al., 2016). However, the controlled
3214 conditions were necessary to single out treatment effects from environmental variables
3215 such as weather and soil conditions that could complicate the interpretation of the
3216 results. Moreover, novel techniques such as isotope labelling employed in this study
3217 provided precise information about the mechanisms underlying the higher efficiency of
3218 Se uptake into plants when applied as foliar Se with N rather than foliar Se on its own.
3219 It is therefore highly probable that similar results, albeit with higher losses to the
3220 environment, would be observed in the field as well.

3221 This study also investigated how the solubility of selenate added to eight soils
3222 varying in physiochemical properties changed over a 300 d period, using both chemical
3223 and biological assays of Se availability. The impact of ageing on Se, especially Se^{VI},
3224 had not been extensively researched before. Calcareous soils showed more
3225 pronounced ageing, in terms of the overall rate and extent to which solubility of Se
3226 declined, compared to non-calcareous soils. In soils with > 10% $CaCO_3$, Se solubility
3227 declined to almost 0 within the first 30 d after application of soluble Se (ageing).
3228 Similarly, concentrations of Se in wheat plants grown on the same calcareous soils

decreased from 37 mg kg^{-1} at the beginning of the experiment to < 5 mg kg^{-1} after 30 d of ageing. The rate of decline in Se concentration of plants grown in non-calcareous soils was much slower, with > 100 d required to reach < 5 mg kg^{-1}. This highlights the strength of the retention mechanism of Se in calcareous soils, which most probably occurred by Se substituting for CO_3^{2-} ions in calcite.

The progressive reduction in solubility of Se in most soils was predicted reasonably well by a reversible first order (RFO) model. This may have some practical applications for biofortification programs in which soils are amended with selenate. Using only (i) the Se concentration added to the soil and (ii) the length of time for which soils were aged, as inputs, the model predicted (i) the proportion of Se that appeared to be instantly removed from the soluble pool, (ii) the fraction of the added Se that remained soluble at equilibrium and (iii) the forward and reverse fate constants for the ageing process. Although it was useful to describe the dynamics of Se in soil, the model had limitations as it only described the ageing process of Se in a closed system. For more comprehensive prediction, the model would need to be modified to include other source terms describing the fate of Se in a realistic soil-plant system, for example plant uptake and leaching in response to rainfall and irrigation.

Agronomic biofortification is a proven and effective way of improving dietary intake of Se in a sustainable way (Lyons, 2018). When working with fertilisers, strong emphasis is placed on improving their use efficiency through the 4R Nutrient Stewardship principle, i.e. the use of fertiliser from the right source, at the right rate and at the right time, with the right placement (Bindraban et al., 2015). This stewardship applies for Se as well; as this study successfully demonstrated, the common practices for Se biofortification can be further optimised by adapting application methods, formulations and timing to maximise plant uptake immediately after application. Characterisation of soils specific to areas where biofortification programs are

established is also crucial to the optimisation of Se uptake by plants. Furthermore, the study addressed a key gap in the literature; the residual fate of Se, especially Se^{VI}, fertilisers in the soil. The knowledge gained in this thesis on the most effective Se fertilisation strategies for wheat fortification along with an enhanced understanding of the fate of soluble Se^{VI} in agricultural soils should assist biofortification programs manage fertiliser Se in the most effective way in the future.

5.1 Future research

From the current study, the following areas could potentially be further explored to better understand Se dynamics in arable systems.

5.1.1 Biofortification

The present study showed promising results of applying foliar Se with an N source in accumulating Se in the grain. As discussed above, although lower recovery of applied Se in plants can be expected in field trials compared to pot experiments, the effect of N in enhancing uptake of foliar Se is likely to be expressed in the field as well. This hypothesis could be tested by applying similar foliar Se treatments (± urea-N) to wheat at the heading stage in the field, with the difference being in the application method (less controlled than glasshouse experiments). Results from such a trial would validate the efficiency of the current proposed Se fertiliser strategies.

Different varieties of the same crop species can accumulate Se to different extents (White, 2016) and a clear interaction between Se and N was observed in this study. Perhaps, supplying Se to cereal crops with high protein or high N use efficiency traits, brought about either by natural selection or genetic engineering (Hawkesford and Griffiths, 2019), could be a potential way of further improving the uptake efficiency of Se. This would be possible because, as discussed earlier, Se and N share a metabolic pathway. Having a greater protein content or ‘sink’ in the grain could stimulate uptake and assimilation of Se. Greater recovery of the applied Se fertiliser in

cereal grain would imply lower application rates required to achieve the desired Se levels, lower costs associated with fertiliser application and potentially less concern over possible environmental damage form Se leaching.

5.1.2 Residual fate of added Se in fertilisers

The power of the model developed to assess solubility of Se in soil could be improved by testing with a greater number of soils (> 8) and continued over longer time (> 300 d) to better assess long-term changes in Se solubility, especially in sandy soils such as Monarto.

Moreover, precise techniques such as isotopic dilution could be employed to distinguish between adsorption and fixation processes, responsible for Se ageing in soils. Isotopic dilution can measure the pool of added Se that is soluble as well as the pool of 'exchangeable' Se, which is adsorbed on the solid phase but still in equilibrium with the soil solution (labile fraction). This method allows the determination of the soluble and the exchangeable Se fractions at the same time, from which information about the proportion of Se that is fixed can be inferred. It can be hypothesised that experimental variables such as soil properties (pH, OM and $CaCO_3$ contents) as well as the chemical form in which Se is added to soil, are likely to influence the partitioning of Se between adsorbed and fixed pools. Using this technique, time-dependent changes in Se fixation can also be determined to assess whether desorption of fixed Se occurs in soils.

One of the drawbacks of the methodology employed in the study is the lack of selectivity of certain reagents for determining specific Se fractions in the soil. The sequential extraction procedures were also rather destructive in nature and would not allow for the chemical speciation of Se in the fractions to be determined. The information about the how the speciation of Se changes with soil properties and contact time would be vital to understanding how Se is retained in soils. Alternative

methodology to SEPs, such as X-ray absorption spectroscopy (XAS), could therefore be employed to reliably assess time-dependent changes in Se speciation in various soils without any pre-treatment. However, it should be recognised that high Se concentrations of Se would be required for accurate analysis by XAS. Agricultural soils with relatively low Se concentrations may have to be spiked prior to analysis, which might interfere with the processes responsible for Se retention in the soil.

5.2 References

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Appendix

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Improving the efficacy of selenium fertilizers for wheat biofortification

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Increasing the selenium (Se) concentration of staple crops by fertilization is a valuable pathway to increase Se in the human diet, thus preventing Se deficiency. A pot trial was set up to investigate whether the application of 3.33 µg kg^{-1} of Se (equivalent to 10 g ha^{-1}) to wheat can be made more efficient by its co-application with macronutrient carriers, either to the soil or to the leaves. In the soil, Se was applied either on its own (selenate only) or as a granular, Se-enriched macronutrient fertilizer supplying nitrogen, phosphorus, potassium or sulfur. Selenium was also applied to leaves at head emergence with, or without, 2% w/v N fertilizers. With grain Se concentrations varying from 0.13–0.84 mg kg^{-1}, soil application of selenate-only was 2–15 times more effective than granular Se-enriched macronutrient fertilizers in raising grain Se concentrations. Co-application of foliar Se with an N carrier doubled the Se concentration in wheat grains compared to the application of foliar Se on its own, the majority of which was in the highly bioavailable selenomethionine fraction. Results from this study demonstrate the possibility of improving the efficacy of Se fertilizers, which could enrich crops with Se without additional application costs in the field.

The essentiality of selenium (Se) as a nutrient for humans and animals was first established in the 1950s by Schwarz and Foltz[1]. Since then, its active role as an antioxidant, thyroid hormone and general immune function regulator has been highlighted, such that a low intake of Se in the diet would result in poor health and in extreme cases, deficiency diseases[2]. Although less common, an excess of Se can also be detrimental to human health[3]. There is a narrow margin between Se deficiency and toxicity and so it is essential that the daily dietary Se intake for humans falls within a restricted range. Currently, the recommended dietary intake is 50–55 µg day^{-1}[4], but it is estimated that 0.5–1 billion people around the world do not consume sufficient Se and are at risk of disease[5,6].

Agronomic biofortification is the practice of increasing the nutrient concentration of the edible parts of staple crops through fertilization practices[7]. In recent decades it has been identified as an effective long-term strategy to alleviate micronutrient deficiency because it is relatively easy, efficient and affordable[8]. Cereals, such as wheat and rice, are ideal for Se biofortification because they are widely consumed by the general population and they can act as effective buffers for humans since they accumulate no more than 1.0 mg Se kg^{-1} of dry matter[9].

The form in which Se is applied affects its effectiveness for biofortification. Both selenate (Se^{VI}) and selenite (Se^{IV}) are bioavailable species but the uptake rate of Se^{VI} may be up to 33 times higher than that of Se^{IV} [10]. This is because Se^{IV} is adsorbed more strongly by inner-sphere complexation onto soil mineral oxides/hydroxides surfaces, which limits its mobility and hence plant uptake[11]. Moreover, Se^{IV} has limited translocation through plants and tends to accumulate in roots, compared to Se^{VI} which is highly mobile in the xylem[12]. The predominance of the different species in soils in turn depends on *in-situ* factors such as the soil geocolloidal phases present, pH and redox potential. Under high pH and well aerated conditions, such as arable soils, Se^{VI} is expected to be the dominant inorganic Se species while in more acidic well-drained soils or under anaerobic conditions, Se^{IV} concentrations are expected to be greater[13].

Selenium fertilizers are typically applied at low rates of 10–20 g Se ha^{-1} in biofortification studies[14]. To ease the application of such a small amount of Se in the field, it is usually added to other fertilizer matrices, supplying either a mix of nutrients, for example Selcote Ultra and Top Stock[7], or predominantly macronutrients, such as urea and calcium nitrate[15]. These fertilizer matrices are referred to as "carriers" of Se. In 1993, Gupta *et al.*[16] investigated the application of nitrogen (N) fertilizers ammonium nitrate (NH_4NO_3) and urea doped with either Se^{IV} and Se^{VI} to improve the Se levels of livestock. While their main findings focused on the superiority of Se^{VI} compared to Se^{IV} in increasing plant Se levels, they also pointed out that both N fertilizers were effective as carriers

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for Se. Additionally, Premarathna *et al.*[8] reported that Se-enriched urea granules were very effective in raising Se concentration of rice, hence highlighting the potential of N as a carrier for Se. Rice however has different growth conditions to cereals crops such as wheat, such that findings from such an experiment may or may not be transposed onto other crops. To our knowledge, no study had either investigated this carrier effect with wheat or compared the efficiency of different macronutrients as Se carriers.

A few studies have compared the efficiency of applying Se by different methods – to the soil or to the leaves (foliar). Results showed that, while both are effective in raising plant Se concentrations, foliar fertilization is up to 8 times more efficient than soil Se application[10]. This greater efficiency of foliar-applied fertilizers may be ascribed to (1) rapid uptake and assimilation due to application at a later growth stage, (2) less influence of root-to-shoot ratio on translocation to the edible parts of crops and (3) the avoidance of losses through fixation in soils. On average, only 12% of soil-applied Se fertilizers is taken up by plants; most Se applied is retained and immobilized in the soil[7], with very little residual value for subsequent crops[17]. This means that repeated applications of Se fertilizers are required for each growth period, unless the efficacy of Se fertilizers can be improved.

In this study, we investigated the potential for enriching commonly used fertilizers supplying macronutrients nitrogen, phosphorus (P), potassium (K) and sulfur (S), with Se to biofortify crops. We hypothesized that macronutrients can act as effective carriers for Se and help improve fertilizer use efficiency in the field. We believe this is the first study investigating the efficiency of N, P, K and S as well as water as carriers for Se, applied either to the soil or to the leaves, with the aim of increasing Se levels in wheat grains. In addition, we did Se speciation analysis of the wheat grains to determine whether the different fertilizer formulations had an effect on the bioavailable Se content of the wheat grain.

Results

Macronutrient concentration. Despite the application of macronutrient fertilizers in different ways (either as granules or as a basal solution) in this experiment, all treatments received the same rate of macronutrient N, P, K and S application. Hence, no significant differences were observed in the macronutrient content of the grain, except for the granular DAP-Se treatment in the KI soil, which showed a higher grain P concentration ($3.51 \pm 0.17\,g\,kg^{-1}$) than when P was applied in the basal solution ($2.70 \pm 0.07\,g\,kg^{-1}$) (Supplementary Fig. S1). Slight, although statistically significant, differences in grain K concentration were observed between some treatments in KI and Mallala soils, whereby foliar treatments seemed generally higher than soil-applied ones. However, in all these treatments, a similar rate and method of K fertilizer (MOP in basal solution) was applied; any differences observed were therefore attributed to random effects.

Yield and Se concentration. Irrespective of their formulation and method of application, the different Se fertilizers employed in the study did not significantly affect grain yield, which ranged from 3.5–4.2 g pot^{-1} for the three soils (Supplementary Fig. S2), but significantly increased grain Se concentrations above control levels (Fig. 1). A similar pattern in Se accumulation across the treatments was observed in the three soils, although plants grown in the KI soil generally had higher Se concentrations than Mallala- and Black Point-grown ones. For the soil-applied treatments, the application of Se on its own was the most effective ($0.84 \pm 0.01\,mg\,kg^{-1}$ in KI; $0.46 \pm 0.04\,mg\,kg^{-1}$ in Mallala and $0.13 \pm 0.02\,mg\,kg^{-1}$ in Black Point) followed by granular Se + urea treatments ($0.26 \pm 0.11\,mg\,kg^{-1}$ in KI; $0.10 \pm 0.01\,mg\,kg^{-1}$ in Mallala and $0.11 \pm 0.02\,mg\,kg^{-1}$ in Black Point) (Fig. 1). In comparison, soil application of Se with the other macronutrients P, K and S had a much smaller effect on Se accumulation in the plants. Grain accumulation of Se following foliar fertilization was consistently higher when 2% w/v N, in the form of urea or UAN, was added to the foliar Se solutions (Fig. 1): grain Se concentrations under the foliar Se only treatment averaged at $0.20 \pm 0.02\,mg\,kg^{-1}$, which compared to $0.37 \pm 0.02\,mg\,kg^{-1}$ and $0.41 \pm 0.07\,mg\,kg^{-1}$ when foliar Se was co-applied with urea and UAN, respectively. The use of either liquid urea or UAN were equally effective in enhancing grain Se accumulation. No Se was measured in the foliar rinses of the treated leaves, suggesting that the surface-applied Se had been absorbed into the leaves.

Nitrogen content and speciation. Grain N was around 2.1% of the total weight across the different treatments where N was analyzed, except when Se-enriched urea granules were soil-applied in KI soil, which resulted in higher grain N content (3.53%) (Supplementary Fig. S3). Protease hydrolysis of the grains measured $104 \pm 4.39\%$ of the total Se, suggesting that it was a reliable way of releasing Se from the grains (Fig. 2), the majority of which was in SeMet form (average $97 \pm 6\%$). The distribution of SeMet therefore followed that of the total Se (Supplementary Fig. S4), suggesting that the use of different carriers and methods of application did not affect speciation of Se in the grains. Other Se species such as selenocysteine (SeCys) and Se-methyl-selenocysteine (MeSeCys) generally found in wheat grains were not quantified in this study, but it is likely that that the small percentage of unidentified Se species in the grains was in organic form[18].

Selenium recovery and translocation to grains. Generally, the recovery of fertilizer in the aboveground biomass was less than 50% when Se fertilizers were applied to the soil, except for soil-applied selenate-only in KI and Mallala soils (100% and 56% respectively; Fig. 3). Although the roots or the soils were not analyzed for Se concentrations in this study, we believe that the rest of the applied Se might either be stored in the roots or lost to the environment either through a retention mechanism onto soil particles or volatilization from the plants[7,19]. Crop Se recovery was especially low (2–38%) when Se was applied to the soil with macronutrient fertilizers, with the highest recovery recorded for the soil-applied Se + urea treatment in KI. The foliar Se fertilizers were more efficient in accumulating Se in crops with 19–30% and 46–61% Se recovered in the harvested biomass when Se was applied on its own and with an N carrier, respectively.

To examine translocation of Se into grain, the uptake (Se concentration x grain dry weight) of Se by wheat grains was expressed as a percentage of the total amount of Se accumulated in the aboveground biomass

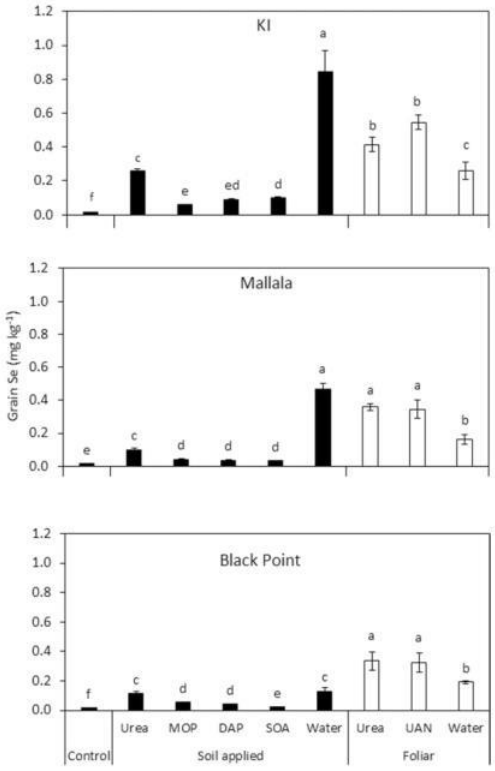

Figure 1. Grain Se concentration across different Se fertilization treatments used in the three soils. Under soil-application, Se was applied with N, K, P and S fertilizer carriers as Se-enriched urea, MOP, DAP and SOA granules respectively. Also a treatment with water as carrier (spot-applied sodium selenate solution) was included. Results show means and standard errors (n = 4). Different letters above the bars indicate significant ($p \leq 0.05$) differences between treatments (Duncan multiple range test) at a 5% significance level.

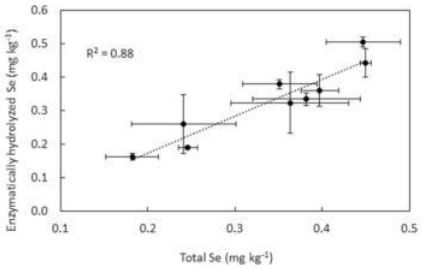

Figure 2. Correlation between total grain Se concentrations measured by two methods: acid digestion and enzymatic hydrolysis. Error bars represent standard errors (n = 4).

(grains + shoots). Our results showed that when Se fertilizer was soil-applied with water or with an N carrier, >75% of the Se fertilizer taken up in the aboveground biomass was translocated to the grains (Fig. 4). On the other hand, limited translocation (<50%) was observed when Se was applied with MOP, DAP and SOA (except in Mallala). The foliar applications, both with and without N, showed a large translocation to the grain.

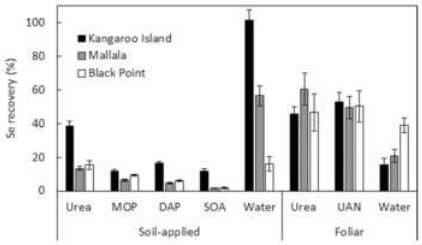

Figure 3. Percentage of applied Se fertilizer recovered in aboveground biomass. Error bars show standard errors (n = 4).

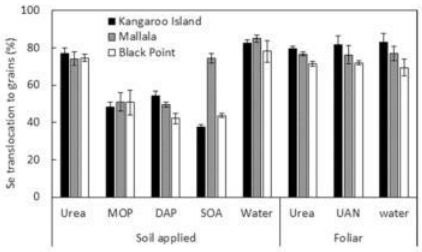

Figure 4. Percentage of Se translocated to the grain across the different fertilizer treatments used in the three soils. Error bars show standard errors (n = 4).

Discussion

Yield did not differ significantly across treatments in this study, in agreement with previous studies when rates of up to 100 g ha^{-1} of Se have been applied[7,20,21] (Supplementary Fig. S2). In other, albeit fewer, instances where a positive relationship between Se application and plant yield was observed, the response was attributed to a stimulation of antioxidant activity and subsequent plant protection from abiotic stresses such as cold, desiccation and the presence of toxic metals[22]. The essentiality of Se for higher plants is still unconfirmed; it is generally thought to be beneficial for several physiological processes but is not a limiting factor for growth[23].

Grain Se concentration of control plants in this study was very low, averaging 0.015 ± 0.00 mg Se kg^{-1}, which is below the target Se concentration of 0.1 mg kg^{-1},suggested to be adequate for human consumption[24] (Fig. 1). Under soil application treatments, the effectiveness of the Se fertilizers depended on the macronutrient carrier as well as the soil characteristics. When Se was co-applied with macronutrient fertilizers such as MOP, DAP and SOA as granules to the soil, most (>90%) of it remained unutilized by the crop. Recovery rates of Se in those soil-applied treatments were lower than the average 12–27% reported by Stroud *et al.*[25] and Broadley *et al.*[7] but compared favorably with rates in the field trial by Stephen *et al.*[26] who reported 6.9% to 4.9% recovery in autumn-grown wheat (Fig. 3). However, unlike their autumn field trial, where considerable amounts of the applied Se fertilizer might have been lost by leaching[26], ours was a pot trial conducted under controlled conditions. This suggests that mechanisms other than leaching, for example, sorption by soil, were responsible for the poor efficiency of Se-enriched macronutrient fertilizers. The exact mechanism explaining their poor efficiency compared to the application of selenate on its own to the soil is not known yet, but a possible explanation might be that the reduction of Se^{VI} to Se^{IV} was faster for the granular treatments. Since Se^{IV} is more strongly sorbed to soil hydrous oxides and organic matter and has a relatively low root-to-shoot translocation compared to Se^{VI} [27,28], its predominance in the soil would explain the low Se uptake in the plants. A positive relationship between Se translocation and Se recovery was observed (Fig. 5), which supports this hypothesis. The low Se translocation for the treatments with low recovery (with the exception of Se-enriched SOA in Mallala soil) suggests that Se^{IV} was the predominant species available for plant uptake in these treatments. This change in Se chemical speciation could have been because, as the fertilizer granule dissolved in the soil and salt concentration built up, water would flow towards the granule as a result of the high osmotic pressure[29], and that could create a locally reducing environment. For Se-enriched urea granules, this mechanism might be less relevant because urea is initially uncharged and even though its hydrolysis is rapid[30], the urea would already have started to diffuse away from the application site before hydrolysis, resulting in less osmotically-driven water flow towards the application site. Moreover, the consumption of H^+ ions during urea hydrolysis (NO_3^- assimilation) is usually accompanied by a temporary increase in soil pH[31]. All these conditions would tend to favor the predominance of Se^{VI} ions, which could explain the higher Se uptake when urea was co-applied with Se compared to the other macronutrient fertilizers.

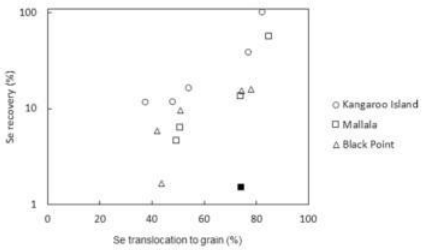

Figure 5. Percentage of Se recovered in the aboveground biomass vs. % of Se translocated to the grain of plants fertilized with soil-applied Se-enriched macronutrient fertilizers (urea, MOP, DAP and SOA). The single filled data point indicates the Se-enriched SOA treatment in Mallala soil.

Se-enriched fertilizer	Water-soluble Se mg kg^{-1}	Acid-soluble Se mg kg^{-1}	[a]Water solubility %
Urea	34.7	32.3	107 ± 0.92
MOP	29.3	31.6	93 ± 0.36
DAP	25.8	26.7	96 ± 0.24
SOA	37.7	36.3	104 ± 0.25

Table 1. Water solubility of Se-enriched macronutrient fertilizers. [a]Water solubility is presented as a percentage of the total Se released by acid digestion.

For the treatment with Se-enriched SOA granules in the Mallala soil, a very low Se recovery (2%) was recorded in the aboveground biomass of these plants despite the high translocation of Se to the grain (Fig. 5). While the high translocation rate suggests that Se^{VI} was the predominant species available for uptake, probably because roots were exposed to alkaline aerobic conditions[13,32], the low Se recovery suggests that the uptake of Se from the soil was restricted. The negative effect of S fertilizer on grain Se uptake has been documented before[33]; the antagonism arises as a result of the competition between chemically similar selenate and sulfate ions for uptake transporters in the root, where sulfate is preferentially taken up to selenate due to its higher affinity for the transporters[32,34]. More recent studies by Tan *et al.*[35], investigating novel mechanisms behind the competitive relationship between sulfate and selenate showed that the reduced plant uptake of selenate in the presence of sulfate ions could also be due to a suppression in microbial ability to assimilate Se^{VI}. In our study, even though sulfate and selenate were applied at the same rate for all treatments, their close proximity in Se-enriched SOA granules potentially enhanced the competition, thus reducing the uptake of Se.

In comparison to the application of Se with macronutrient carriers, the application of Se^{VI} on its own to the soil was far more effective in increasing grain Se concentration (high Se recovery and high translocation to grain), especially in the KI and Mallala soils. We suggest three possible explanations for this phenomenon: (1) there was potentially a lower propensity for Se^{VI} to be reduced to Se^{IV} as a result of the lower osmotic pressure (no granule dissolving); (2) there was a lack of competition between ions since Se^{VI} was applied in pure form and; (3) there was no added physical restriction of Se having to diffuse out of the granule when it was applied in pure fluid form to the soil. Despite the granular fertilizers being highly soluble in water (Table 1), the dissolution of the individual granule in the soil might have been slower than expected, hence restricting Se release.

Under soil-applied Se treatments, plants grown in KI soil accumulated more Se compared to those grown in Black Point or Mallala soils (Fig. 1), indicating that soil properties affected the effectiveness of the fertilizers. Soil properties can affect mobility and availability of Se for plant uptake through their effect on soil conditions (e.g. pH and pe), which in turn affects Se chemical speciation and sorption behavior. Under high soil pH and aerobic conditions, Se^{VI} ions would predominate in the soil, which would favor plant uptake because Se^{VI} is adsorbed to a much lesser extent on geocolloids compared to Se^{IV}, which makes it more mobile and bioavailable[36]. However, in soils with such conditions (good aeration and high pH) for example Mallala, Se uptake was lower than expected, suggesting that other factors, such as $CaCO_3$, might have limited Se bioavailability. Previous studies have shown that Se^{IV} can get adsorbed onto calcite surfaces via an anion exchange mechanism as CO_3^{2-} and SeO_3^{2-} have a similar charge and ionic radius[37]. Soil texture and organic matter content are also factors which can influence Se bioavailability. With only 5% clay content, KI soil is very sandy (Table 2), which, not only makes it more likely to be well aerated, hence promoting the predominance of mobile Se^{VI} ions, but also lowers its adsorption capacity, compared to the Black Point and Mallala soils.

The foliar application of Se fertilizers tended to be more efficient than the soil application, with higher Se uptake and recovery rate in the plants (Fig. 3). In this study, a foliar application equivalent to 10 g Se ha^{-1} led to grain concentrations of 0.1–0.3 mg kg^{-1} when Se was applied on its own and up to 0.5 mg kg^{-1} when Se was applied with an N carrier to the leaves (Fig. 1). These concentrations compare favorably with the average Se concentration of 0.4–0.5 mg kg^{-1} measured in studies by Curtin *et al.*[38] and Ducsay *et al.*[39], where twice the amount of Se (20 g ha^{-1}) was applied to the leaves. Thus there is clearly greater efficiency in co-applying foliar Se with an

Soils	[a]EC dS m^{-1}	pH	$CaCO_3$ %	Clay %	Sand %	Organic C %	Exchangeable cations (cmol$_c$ kg^{-1})				
							[b]ECEC	Ca	Mg	Na	K
Kangaroo Island	0.07	5.5	<0.5	5	94	1.6	2.71	2.09	0.62	<0.1	<0.2
Mallala	0.13	8.5	4.7	11	47	1.6	30.8	26.2	2.58	0.16	1.85
Black Point	0.07	8.3	<0.2	18	73	0.4	17.9	14.2	2.54	0.17	0.97

Table 2. Physicochemical properties of the three soils used in this pot experiment. [a]Electrical conductivity (EC) of soils. [b]Effective cation exchange capacity (ECEC) of soils.

N carrier to enrich wheat grain with Se, although the reasons for this have not yet been established. In studies looking at the effect of co-applying trace elements such as Fe and Zn with N, the N nutritional status of the plants was given as an explanation for improved grain micronutrient uptake because proteins can act as a sink for micronutrients and aid their re-translocation from shoots to the grain[40,41]. However, our study showed that the addition of 2% w/v N in foliar solutions did not significantly alter grain N (protein) content (Supplementary Fig. S3), suggesting that a physiological mechanism may instead be responsible for the improved plant uptake when foliar Se was co-applied with N. The physiological response might have improved Se absorption into the leaf and/or improved translocation into the grains. Nitrogen fertilizers such as urea and UAN are often foliar-applied as they are uncharged molecules which can easily permeate waxy leaf cuticle though a simple diffusion mechanism[42]. Co-applying Se with such N carriers potentially facilitated the Se sorption pathway. Moreover, once absorbed, N and Se have a similar assimilation pathway in plants in the sense that both get metabolized into N organic compounds such as amino acids. Therefore, co-applying Se with a N carrier potentially improved its rate of assimilation into selenoamino acids, which would then be transported into sink organs (grains). Comparatively, when applied without a N carrier, Se may take a longer time to penetrate the cuticular membrane and get assimilated, leaving a greater window of opportunity for losses by (phyto)volatilization[43]. Effectively, losses of Se under foliar Se-only treatment were twice as much as those under foliar Se + urea and Se + UAN treatments (Fig. 3). To the best of our knowledge, this is the first study showing an improved plant Se uptake when Se was foliar-applied with a N source. Similar effects have been observed with other micronutrients, for example, in studies by Aciksoz *et al.*[44], where improved Fe translocation from the foliar-treated leaf to the grain was observed when Fe was co-applied with up to 0.8% w/v urea to wheat plants.

Conclusions

Our study aimed to determine whether fertilization strategies for Se biofortification could be made more cost-effective by co-applying Se with commonly used macronutrient fertilizers. It was observed that the effectiveness of those Se-enriched fertilizers was highly dependent on soil properties and that the co-application of Se with macronutrients in granular form generally led to poor Se uptake and translocation within the plant. In two of the three soils used in this experiment, the application of selenate on its own to the soil was more effective in increasing grain Se concentrations than any other soil-applied fertilizer strategy. Our study also showed that foliar application of Se with 2% w/v N can lead to twice as much Se uptake and recovery in plants, compared to foliar application of Se only. It should be noted that foliar solutions were applied as targeted droplets on specific leaves in this pot trial, and that, in the field where foliar sprays would be used, lower Se recovery rates can be expected. However, it appears likely that foliar co-application of Se with a N carrier would still be more effective in raising grain Se concentrations compared to foliar Se only or soil-applied Se-enriched macronutrient fertilizers.

Materials and Methods

Soils. The experiment used three Australian soils, Kangaroo Island (KI), Mallala and Black Point, air-dried and sieved to < 2 mm. They were chosen to provide a range of physical and chemical characteristics likely to affect Se dynamics (Table 2). Soil pH and electrical conductivity (EC) were measured in a 1:5 soil-to-solution suspension on an automated Skalar pH/EC system. Soil organic carbon (C) content was measured using a dry combustion method[45]. The textural classification of the soils were determined using mid-infrared spectroscopy and R code to generate the classification from the Australian soil textural triangle. To determine the exchangeable cations contents and effective cation exchange capacity (ECEC), the soil samples were shaken with a 1 M ammonium acetate solution at pH 7 in a 1:10 soil-to-solution ratio and the extracts were analyzed for elemental concentrations using inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 8300; PerkinElmer Inc., Waltham, Massachusetts).

Selenium fertilizers. Based on application suggestions from previous biofortification studies[38], Se was applied as sodium selenate (Na_2SeO_4) at a single rate of 3.33 µg Se kg^{-1} (equivalent to 10 g ha^{-1}, based on a 20 cm depth and 1.5 g cm^{-3} bulk density). There were nine treatments for each soil, each replicated four times. Treatments included: (i) a control without added Se, (ii) a treatment with Se added to soil as sodium selenate solution, (iii) four treatments with Se-enriched granular fertilizers and (iv) three treatments with foliar Se fertilizer.

The granular fertilizers used were urea, di-ammonium phosphate (DAP), muriate of potash (MOP) and sulfate of ammonia (SOA), supplying the macronutrients N, P, K and S respectively. To enrich these fertilizers with Se, a sodium selenate solution was added to powdered commercial fertilizer and mixed thoroughly to ensure homogeneity. The paste was then oven-dried overnight at 30 °C and ground to a fine homogenous powder using a pestle and mortar. The Se-enriched fertilizer powder was then pressed into tablets (5 mm diameter, ca. 2 mm height) using a tablet press (TDP-5, Shanghai Develop Machinery Co., China). For the treatment with the soil-applied

selenate only, a Na_2SeO_4 solution containing 0.042 mg Se L^{-1} was applied to the soil as 3 × 26 µL droplets, in the same position as the granular fertilizers.

Foliar treatments included a Se-only solution (water as carrier), Se + N in the form of either 2% w/v urea or 2% v/v urea ammonium nitrate (UAN). All three solutions contained Se as sodium selenate at a concentration of 0.083 g Se L^{-1} (rate equivalent to 3.33 µg Se kg^{-1}) and were mixed with 0.5% "Spreadwet 1000" (SST Australia PTY LTD., Victoria, Australia) surfactant prior to application.

Pot trial. All soils were mixed with the following nutrients (mg kg^{-1} of soil): Ca (10), Mg (10), B (1.0), Cu (2.0), Mn (2.0), Mo (0.1) and Zn (2.0) and left to equilibrate overnight prior to potting into 1 kg pots. Macronutrients were also supplied, including 80 mg kg^{-1} N as a split application, 20 mg kg^{-1} P and S, and 40 mg kg^{-1} K. The application method of the macronutrients depended on the treatment; when enriched with Se, the macronutrient fertilizer was applied as granules (3-4 per pot) in a circle at a distance of 1 cm from the side of the pot halfway through potting. The other macronutrients were then applied as part of the basal solution, such that, regardless of their form of application, all nutrients were balanced in all the soil pots. After fertilization, five pre-germinated wheat seedlings (*Triticum aestivum cv*. Axe) were transplanted into each pot and thinned to two plants after two weeks. The soils were maintained close to field capacity by watering the soil surface regularly with reverse osmosis (RO) water. At heading stage, foliar solutions were applied to the youngest flag leaf as four 5-µL drops per plant using a micropipette. The soil surface was covered with cling film to avoid any contamination during foliar application and care was taken to water the plants at the soil surface only, avoiding irrigation of leaves. Plants were grown to grain maturity under controlled conditions (temperature of 23.2 °C, humidity of 72% and 12 h daylight cycle).

Harvest. At grain maturity, shoots and heads were harvested separately. Marked treated leaves were also separated from the rest of the biomass and washed in dilute hydrochloric acid (HCl; 0.1 M) and then rinsed with reverse osmosis (RO) water; acid rinses were saved and analyzed for Se. All plant biomass was dried at 60 °C for 72 h, after which wheat heads were hand-threshed to separate grains. Prior to analyses, the grains were ground to fine powder using a pestle and mortar, and the rest of the head biomass was combined with the shoots and ground using a laboratory grade grinder.

Analyses. *Fertilizers.* Total Se concentration in the fertilizers was measured following acid digestion. Two mL of concentrated nitric acid (HNO_3) and 0.5 mL of 30% hydrogen peroxide (H_2O_2) was added to 0.25 g of Se-enriched fertilizer and left to stand overnight. The samples were then heated to 80 °C for 45 min followed by 125 °C for 160 min on a block digester. After acid digestion, the samples were cooled for 30 min then made to 10 mL volume using ultrapure Milli-Q water. To measure water-soluble Se in the fertilizer, 0.5 g of granular Se-enriched fertilizer samples was dissolved in 10 mL of Milli-Q water and the mixture was shaken end-over-end for 4 h. The samples were then centrifuged (15 min at 3000 *g*) and filtered through 0.22 µm filters (Sartorius, Göttingen, Germany). All solutions were analyzed for total Se by ICP-OES.

The water solubility test of our Se-enriched fertilizers indicated that they were highly soluble, releasing 100 ± 10% of the added Se in water (Table 1).

Plants. Approximately 0.25 g of plant sample (4 replicates) were weighed into 50 mL digestion tubes (Axygen, Thermo Fisher Scientific, New York) and left overnight in 2 mL of HNO_3 acid and 0.5 mL of H_2O_2 to predigest. The samples were digested using the same method as for the fertilizers, cooled and made to a final volume of 20 mL with Milli-Q water.

The acid digests were analyzed after hydride generation using a Multimode Sample Introduction System (MSIS) (Agilent Technologies, Victoria, Australia) mounted onto conventional ICP-OES[46]. Since only selenite forms hydrides, all samples were pre-reduced to Se^{IV} by heating an aliquot (5 mL) of the acid digest with an equal volume of concentrated HCl at 90 °C for 30 min prior to analysis. Other elements (Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn) were analyzed by conventional ICP-OES, after a 5-fold dilution of the plant acid digests.

Analytical accuracy was verified through the analysis of wheat flour certified reference materials, NIST 8437 and NIST 1567b (National Institute of Standards and Technology, Maryland). The total Se concentration of the reference materials was within the range 90–110% recovery of the certified values.

After initial analysis, grain samples with the highest measured Se concentration (from foliar and soil-applied selenate-only treatments) were analyzed for total N content and Se speciation. Grain nitrogen was determined by the combustion (Dumas) method, as described by Horneck and Miller[47], and analyzed on an N analyzer (Model Leco FP-528L 601-500-100; Leco Corporation, St Joseph, Michigan). For Se speciation, 0.2 g of ground grain was weighed into 15 mL polypropylene tubes with 20 mg of protease XIV enzyme (Sigma-Aldrich, Queensland, Australia) and dissolved in 5 mL of 30 mM TRIS-HCl buffer solution. The solution pH was adjusted to 5.5 using ammonia (NH_3) solution. The samples were shaken end-over-end in an incubator at 37 °C for 24 h, centrifuged at 3000 *g* for 30 min and filtered through 0.22 µm filters. The resulting solutions were analyzed for Se^{IV}, Se^{VI} and SeMet using high-performance liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC-ICPMS, Agilent 7500ce, Agilent Technologies). The operating conditions were adapted from Premarathna *et al.*[8] (Supplementary Table S1). The concentration of Se species in the samples was determined by comparison of their retention times with those of standards, prepared from individual and mixed stock solutions of sodium selenite (Na_2SeO_3), Na_2SeO_4 and selenomethionine (SeMet).

Recovery of the applied Se in the plants ($Se_{recovery}$; µg pot^{-1}) was calculated as the total amount of Se measured in the aboveground biomass as a percentage of the applied Se fertilizer (Eq. 1).

$$Se_{recovery} = \frac{(Se_{shoots} - Se_{ctrl,shoots}) + (Se_{grain} - Se_{ctrl,grain}) \times 100}{Se_{applied}} \quad (1)$$

where Se_{shoots} and Se_{grain} are the amounts of Se (μg pot^{-1}) measured in the shoots and grains respectively (as calculated from the dry weight and tissue Se concentration) and $Se_{ctrl,shoots}$ and $Se_{ctrl,grain}$ are the Se amounts in shoots and grain of the control plants.

Statistical analyses. The effects of different fertilization treatments on grain yield and Se concentrations were determined using the analysis of variance (ANOVA) procedure in SPSS (IBM SPSS Statistics for Windows, Version 24.0., IBM Corp, Armonk, New York), with a significance threshold of 5%. Duncan's and Tukey's post-hoc tests were used to compare treatment means.

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Author contributions

F.D., R.C.d.S., R.B. and M.J.M. conceived the study. C.R. carried out the experiment and analyzed the data. S.D.Y. and E.H.B. helped with the writing and revision of the manuscript. All authors contributed to the construct of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Table 1: The coordinates of the locations at which the eight soils used to investigate Se ageing (Chapter 3) were collected.

Soil	Location	Coordinates
Mt Compass	South Australia	-35.35946, 138.61978
Inman Valley	South Australia	-35.46933, 138.45561
Charleston	South Australia	-34.91666, 138.9
Kingaroy	Queensland	-26.53094, 151.83999
Balaklava	South Australia	-34.14642, 138.4187
Black Point	South Australia	-34.61067, 137.87731
Mallala	South Australia	-34.43846, 138.50518
Monarto	South Australia	-35.08014, 139.09023