The bioactive properties of selenium enriched *Allium sativum* (garlic)

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Abstract

Selenium (Se) is an essential micronutrient for human health, low Se status has been found to predispose people to multiple health conditions. Se is obtained through dietary sources, however intake of Se is decreasing within the UK population, due to multiple factors including low Se soils and shifts in dietary patterns. Crops are generally low in Se, biofortification methods aim to increase the Se content. However, there is a narrow therapeutic window of Se supplementation and the toxicity of Se is poorly understood therefore there is concern that enriching food crops with Se without further understanding of toxicity could have adverse effects. The aim of this work was to enrich garlic, a widely consumed food crop with Se, and assess the bioactivity of the plant tissues and further the understanding of Se toxicity mechanisms.

Results found that Se foliar application of 25µM or 50µM of sodium selenate had no significant effect on the majority of growth measures ($p<0.05$). Although significant differences in clove weight were seen with increasing Se concentration of the cultivars Lyubasha, Bulbils and Solent ($p<0.05$) as well as increased pseudostem diameter in the cultivar Solent at 25µM Se application ($p=0.034$), however none of these differences impacted significantly on overall bulb weight. Foliar application was found to not significantly impact on Se accumulation of any of the cultivars as compared to control ($p>0.05$). Significant decreases in antioxidant capacity of clove tissues was also seen with increasing concentration of Se treatments in all cultivars ($p>0.05$) using the ABTS assay.

In a second series of experiments hydroponic systems were used to enrich the cultivars with Se and this approach resulted in a significant accumulation of Se within tissues of up to 230mg Se/kg DW as compared to 0.16mg Se/kg DW in control tissues ($p<0.05$). The highest levels of Se accumulated in the roots > shoot> clove with levels differing between cultivars. Chemical speciation analysis as determined using HPLC-ICP-MS analysis showed that Se application promoted the accumulation of organic and inorganic forms namely Selenomethionine, Selenocystine, Selenate and Selenomethylselenocysteine. Interestingly, Se accumulation did not significantly influence the cytotoxicity of the majority of cultivar extracts ($p>0.05$). This finding indicating that other factors in addition to Se levels and speciation are responsible for cytotoxicity. However, significant differences in IC_{50} values between control and enriched clove tissues of the cultivars Mersley and Solent ($p<0.001$) were found. Se speciation results suggest that difference in cytotoxicity is not solely due to presence of Se species.

Further, analysis of synthetic Se species was conducted to better define the cytotoxic effects of Se species found in Se enriched crops. Using a human hepatoma HepG2 model, $SeCys_2$ and Selenite were found to be the most cytotoxic Se species, however, the cytotoxicity of selenite was found to be enhanced by the presence of 500µM of cellular antioxidant glutathione ($p<0.05$). We also confirm that the cytotoxicity of selenite + GSH is due to the presence of a short-lived reaction intermediate (<10 minutes) previously proposed to be the superoxide anion. However, despite increasing ROS production in mammalian cells caused by this reaction mix over time ($p<0.05$), incubation with 500µM of radical scavengers (Ascorbic acid, N-acetyl cysteine, Uric acid, Sodium hydrosulphide, Cytochrome C,

Trolox and Catalase) failed to prevent toxicity ($p>0.05$). Therefore, we postulate that H_2Se, a central metabolite in Se metabolism and generated in reaction between selenite + GSH, plays an important role in mediating cell death when exposed to selenite + GSH.

To further explore this possibility, we synthesised a novel slow release H_2Se donor, TDN1042 (SeGYY). HepG2 exposure to SeGYY resulted in a concentration dependent decrease in cell viability as well as LDH leakage ($p=0.032$), DNA damage, ROS production ($p<0.05$), caspase activation ($p<0.001$) and PARP cleavage ($p<0.001$). However, its toxicity was not as dramatic as Selenite likely due to the slow release of H_2Se in cell culture media. Therefore, we postulate that H_2Se could be a fourth gaseous mediator alongside CO, NO and H_2S, however, we acknowledge additional research is needed to confirm this.

In conclusion, further work is needed to assess the bioactivity and cytotoxicity of Se enriched crops intended for human consumption, further work is also needed to understand the role of H_2Se in Se toxicity.

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Se – Selenium

SeCys – Selenocysteine

$SeCys_2$ – Selenocystine

SeMet – Selenomethionine

HSe^- - Hydrogen selenide ion

GSH – Glutathione

DW – Dry weight

H_2Se – Hydrogen selenide

H_2S – Hydrogen sulphide

SeGYY- TDN1042 slow release Hydrogen selenide donor

1 Introduction

1.1 Overview

The global population is expected to rise to 9.7 billion by 2050 according to the United Nations (UN, 2019), and it is currently estimated that by 2050 we will need to have increased food production by 60% from 2005 levels (Alexandratos and Bruinsma, 2012). Climate change and other pressures on land use are putting increasing stress on an already struggling food production system. However, it is not just the quantity of crop production that is becoming a concern, but also the impacts of climate change on the micronutrient quality of crops (Jones et al., 2017). In 2006 it was estimated that more than 2 billion people worldwide were deficient in key vitamins and minerals (WHO and UNICEF, 2006). There has not been a more recent estimate although the prevalence of undernourishment in general has increased sharply over the last 2 years (UN, 2022) therefore the number of people deficient in vitamins and minerals is likely to have increased. This global challenge is often referred to as 'hidden hunger'. Studies have suggested that in order to mitigate the impacts of climate change, the supply of nutritionally rich foods needs to be increased. One method being investigated is biofortification (Semba et al., 2022).

Selenium (Se) is an essential micronutrient needed for human health, and deficiency is estimated to affect up to 1 billion people worldwide (Jones et al., 2017). Chronic Se deficiency can result in multiple conditions such as Keshan disease (Shi et al., 2021) and Kashin-Beck disease (Yao et al., 2011) but Se deficiency also has subtle impacts

on redox systems involved in several diseases such as cardiovascular disease (Dabravolski et al., 2023), immune system (Nkengfack et al., 2019) and various cancer (Stolwijk et al., 2020). In the UK suboptimal Se status is widespread (Stoffaneller and Morse, 2015), it is estimated that 55.1% of adult women and 25.4% adult men consume less than the lower reference nutrient intake (LRNI) of 40µg/day (Mensink et al., 2013). This is primarily driven by low Se soil conditions (Broadley et al., 2006). Biofortification can be used to increase the uptake of nutrients into plants and subsequent intake of nutrients into consumers, there are 3 broad approaches of biofortification including biotechnology, breeding and agronomic practises (Garg et al., 2018). Biofortification to increase levels of Se has been used in multiple crop species such as broccoli (Muñoz et al., 2021), potatoes (de Lima Lessa et al., 2020), beans (Wang et al., 2022a) as well as fruits such as grapes (Fontanella et al., 2017) and peaches (Pezzarossa et al., 2012) and microgreens such as kale, kohlrabi and wheat sprouts (Viltres-Portales et al., 2024) but little is known about the bioavailability, metabolism or possible impacts on plant bioactivity. Due to the narrow therapeutic range for Se (Recommended nutrient intake of 60-75µg/day depending on sex (SACN, 2013) and upper tolerable level 255µg/day (EFSA Panel on Nutrition et al., 2023), it is possible that changes in the levels of Se could alter the bioactive nature of plant tissues and associated extracts with this partly explaining some of the influence that Se has on the immune system, and anti-cancer properties of certain food plants. The following review addresses current research behind the absorption and metabolism of Se in plants, the use of biofortification of crops with Se, and current knowledge about Se and its impact on human health.

1.2 Selenium

Selenium (Se) was first discovered by Swedish scientist Jacob Berzelius in 1817 (Bodnar et al., 2012, Reilly, 2006). It is located in group 6A (VIA) (Chalcone), positioned below Sulfur (S) and above tellurium (Te) and has both metal and non-metal chemical and physical properties making it known as a semi-metal (Fordyce, 2013). Se has an atomic mass of 78.96 (Cupp-Sutton and Ashby, 2016). There are six stable isotopes of Se; ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se and ^{82}Se (Jacobs, 1989, Neal, 1995). Se can exist in 4 oxidation states, 2^- (selenide (Se^{2-}, HSe^-, H_2Se aq), 0 (elemental selenium (Se^0), 4^+(selenite (SeO_3^{2-}, $HSeO_3^-$, $H_2SeO_{3\ aq}$) and 6^+ (selenate (SeO_4^{2-}, $HSeO_4^{2-}$, $H_2SeO_{4\ aq}$). Selenium resembles Sulphur in terms of atomic size, bond energies, ionization potentials and main oxidation states (Tinggi, 2003). These physio-chemical similarities allow selenium to replace sulphur in some compounds (Bodnar et al., 2012). However, the Se atom does have a marginally larger atomic radius as compared to S of 0.5Å compared to 0.37Å, this results in the diselenium bond being one seventh longer and one fifth weaker than a disulphide bond (Sors et al., 2005).

In nature, Se is present in inorganic or organic chemical forms (Mehdi et al., 2013). Selenium is typically found in soils ranging between 0.005 to 1200 mg/kg (Alexander, 2015), with concentrations governed by geochemical conditions. In the UK, total Se in soil ranges from 0.1-4mg Se/kg, however it is estimated that more than 95% of UK soils contain less than 1mg Se/kg (Broadley et al., 2006). The form in which Se is found in the soil is influenced heavily by pH, with selenate being the major form of Se in alkaline soils and selenite in acidic soils, although selenite can be bound by

clays and iron and become unavailable for plant uptake (Alexander, 2015). Selenium can also be present in organic forms such as SeCys and SeMet in soil due to the presence of decomposing organic matter such as plants that have accumulated Se (Mehdi et al., 2013). The presence and availability of Se in soils for plant uptake leads to global variation in soil selenium concentration, causing some areas to be seleniferous, >2-5000mg/kg (Hartikainen, 2005) and others to be deplete/deficient of Se, 0.1-0.6mg/kg of Se (Gupta and Gupta, 2000). About 80% of global Se reserves are located in 10 countries: Peru, Chile, US, Canada, China, Zambia, Democratic Republic of the Congo, Philippines, New Guinea and Australia (Gupta and Gupta, 2017, Liu et al., 2011). This variation has lead to some countries introducing selenium fertiliser (reviewed in Alfthan et al. (2011)) to increase the amount of Se within crops as Se content in soil is directly related to the level of Se accumulated in plants (White, 2018).

1.3 Selenium utilisation in plants

Although Se is not essential for plants, it is considered a beneficial element as it stimulates plant growth, protects plants from abiotic stresses such as cold (Chu et al., 2010), drought (Hasanuzzaman and Fujita, 2011, Pukacka et al., 2011), desiccation (Pukacka et al., 2011), other metal stresses (Kumar et al., 2012, Pandey and Gupta, 2015), and more recently has been shown to provide resistance to pathogens and herbivory (White, 2015).

Efficiency of uptake, translocation and distribution of organic and inorganic Se compounds by plants differs between plant species, growth stages, nutritional status, root morphology and activity of membrane transporters (Gupta and Gupta

2017). Uptake is also affected by the form and concentration of Se in the soil and by the presence of other inhibitory substances such as iron and clay (Fordyce, 2005).

Selenate (SeO_4^{2-}) is the most prevalent bioavailable form of Se in soil and is taken up into roots via high affinity sulphate transporters (HASTs) located on the root membrane (Li 2008). In *Arabidopsis thaliana* sulphate transporter SULTR1;2 has been identified as the predominant Se uptake transporter (Shibagaki 2002). Due to the shared use of SULTR transporters between sulfate and selenate previous studies have noted a competitive relationship between S/Se uptake (Zhou 2020). Some studies in *Triticum aestivum* (Li et al 2008) and *Allium sativum* (Tsyneshi 2006) have shown that S starvation increases Se uptake.

The uptake of selenite (SeO_3^{2-}) is less well understood and was originally thought to occur by passive diffusion (Ellis and Salt, 2003, Terry et al., 2000). However, more recently selenite has been shown to be taken up by phosphate transporters located in roots in both rice and tobacco plants (Zhang et al., 2014, Song et al., 2017) as well as aquaporin NIP2;1 in rice plants (Zhao et al., 2010). More interestingly a competitive relationship between P and Se has also been found where phosphate deficiency stimulates selenite absorption (Wang et al., 2020c).

Little is understood about the uptake of organic forms such as selenocysteine (SeCys) and selenomethionine (SeMet) from the soil into plants. Some literature suggests the involvement of broad specificity amino acid transporters which have been shown to uptake S analogue cysteine (Cys) and methionine (Met) (Trippe and Pilon-Smits, 2021). Other research conducted in rice suggests that SeMet is absorbed primarily

via aquaporins (Wang 2022), however further research is needed to understand the uptake of organic forms of Se from soil into plants.

Once inside the roots Se species such as selenate and organic Se can be translocated to above ground tissues via the plant vasculature system. Selenate is loaded into the xylem via SULTR2;1 (El Mehdawi et al., 2018), and aluminium-activated malate transporters known as ALMT (White, 2018). Whereas organic forms are loaded into the xylem via amino acid permeases (Zhou et al., 2020). Organic forms of Se can also be translocated to specific organs of the plant via the phloem, this has been found to occur in rice via the peptide transporter NRT1.1B (Zhang et al., 2019b) and amino acid transporters (Zhou et al., 2020). In general, selenite is not translocated above ground, but is rapidly converted into organic forms of Se via the S assimilation pathway (Winkel et al., 2015). Translocation rates of Se species depend upon the xylem loading rate as well as the transpiration rate (Renkema et al., 2012). Inside plant cells, Se is mostly accumulated in vacuoles, due to vacuoles being less biologically active compared to other areas of a plant cells thus reducing the potential for interference and oxidative stress (van der Ent et al., 2023). Distribution of Se between plant organs is also influenced by the age of the tissues. In general, the Se concentration in younger leaves is often higher and in the form of organic Se as compared to older leaves in which Se mainly accumulated in the form of selenate (Gupta and Gupta, 2000, Pickering et al., 2000, Sors et al., 2005). This patterning of Se distribution also confers an advantage to deter the plant from herbivore and pathogen attack which more often target younger developing leaves (van der Ent et al., 2023). The distribution of Se in plants also depends on whether the plant is a

hyperaccumulator (>1000µg Se/kg DW) or non-accumulator of Se (<100µg Se/kg DW) (Lima et al., 2018), non-accumulators accumulate Se mainly in roots and seeds, with small proportions in stems and leaves (Sors et al., 2005). Whereas hyperaccumulators such as *Stanleya pinnata* and *Astragalus bisulcatus* (Milk vetch) sequester Se in leaves and petioles (Freeman et al., 2006, van der Ent et al., 2023).

Se assimilation occurs in plastids and involves the conversion of inorganic Se to organic Se species which can either be incorporated into proteins or further methylated and removed from the plant via volatilization (Gupta and Gupta, 2017)(summarised in Figure 1.1). Selenate is converted to selenite in plants via a two-step process involving the conversion of selenate into adenosine phosphoselenate (APS), a short lived intermediate via ATP sulfurylase, and subsequently to selenite via APS reductase (Gupta and Gupta, 2017, Sors et al., 2005). Selenite then can be reduced to hydrogen selenide via either sulfite reductase (SR) or glutathione (GSH)/Glutaredoxins (a group of thioltransferases that reduce disulphide bonds)(Herrero and de la Torre-Ruiz, 2007, Gupta and Gupta, 2017, Wallenberg et al., 2010). SeCys can then be formed via the addition HSe^- to amino acid O-acetyl serine (Gupta and Gupta, 2017). Plants can then further reduce SeCys to elemental Se (Se^0) (Pilon-Smits and Quinn, 2010), methylate SeCys to methylselenocysteine (Neuhierl et al., 1999) or convert SeCys to SeMet via a multi-step process (Gupta and Gupta, 2017). SeMet can be used to form selenoproteins or further methylated to produce dimethylselenide (DMSe) or dimethyldiselenide (DMDSe) depending on accumulator status which can be removed from plants via volatilization (Gupta and Gupta, 2017). Volatilisation of Se compounds can occur in

both aerial and root tissues, volatile forms of Se are thought to be ~600 times less toxic than inorganic Se, therefore volatilization may act as a method of detoxification of Se in plants (Qu et al., 2023).

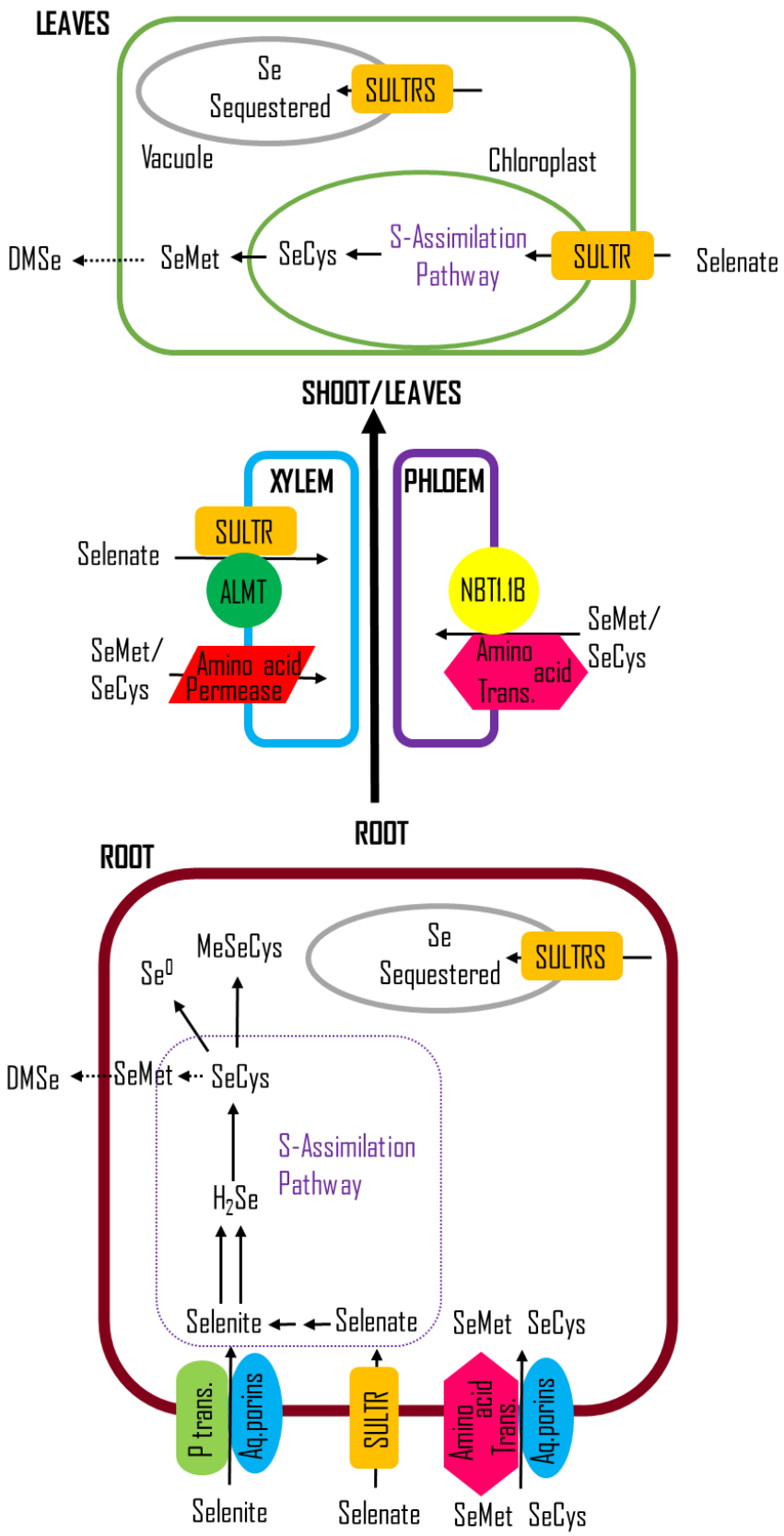

Figure 1.1-Uptake, Metabolism, Translocation and Utilization of Se species in plants. Selenomethionine (SeMet), Selenocysteine (SeCys), Dimethylselenide (DMSe),

Elemental Se (Se^0), Methylselenocysteine (MeSeCys), Hydrogen selenide (H_2Se), Phosphate transporters (P trans.), Aquaporins (Aq.porins), Sulphur transporters (SULTR), Amino acid transporters (Amino acid Trans.), peptide transporter NRT1.1B, aluminium activated malate transporte (ALMT).

1.4 Selenium in Nutrition

Humans and animals mainly obtain Se from their diet, Se can occur in both organic (SeMet, SeCys and SeMeSeCys) and inorganic forms (Selenate and Selenite) within food. Meat, fish and eggs are generally rich sources of Se, as are brazil nuts (Thorn et al., 1978). Crops such as cereals, grains, fruits and vegetables are commonly poor sources of Se (Table 1.1). Low Se in feed crops also has impacts on livestock production with several conditions such as white muscle disease (Delesalle et al., 2017) as a result many livestock feeds are supplemented with Se (Pecoraro et al., 2022). Sources of Se supplementation typically include sodium selenate, sodium selenite and or selenium-enriched yeast (Puccinelli et al., 2017).

Table 1-1. Se content of foods ranging from Se rich sources to poor Se sources.

Food	Se content of food (µg Se/per 100g fresh weight)	Percentage of RNI (%)		Reference
		Female (60 µg Se/day)	Male (75 µg Se/day)	
Brazil nuts	1918	3196.7	2557.33	ODS (2020)
Tuna	107.3	178.83	143.07	ODS (2020)
Beef	42	70	56	Barclay et al. (1995)
Lamb	38	63.3	50.7	Barclay et al. (1995)
Chicken	25.7	42.8	34.3	ODS (2020)
Eggs	19	31.7	25.3	FSA (2009)
Brown Rice	14.3	23.83	19.1	ODS (2020)
Mushrooms	14	23.3	18.7	Holland et al. (1991)
Cashew Nuts	10.6	17.7	14.1	ODS (2020)
Turkey	10	16.7	13.3	Barclay et al. (1995)
Pork	7.6	12.7	10.1	Barclay et al. (1995)
Bread	6	10	8	FSA (2009)
Parsnip	2	3.3	2.7	Holland et al. (1991)
Full Fat Milk	1.5	2.5	2	Barclay et al. (1995)
Carrot	1	1.7	1.3	Holland et al. (1991)
Wheat	1-55	1.7-91.7	1.3-73.3	Hawkesford and Zhao (2007)
Potatoes	<1	<1.7	<1.3	FSA (2009)

The main species of Se in food is SeMet (Weekley and Harris, 2013) which can be incorporated into proteins and can replace the sulfur amino acid methionine (Schubert et al., 1987) (Figure 1.2). Brazil nuts contain Se mostly in the form SeMet (Thomson et al., 2008), sesame seeds are also relatively high in Se and contain 80% in the form of SeMet (Kápolna et al., 2007a). Cereals contain between 80-96% of Se in the form of SeMet (Thiry et al., 2012). Vegetables from the allium and Brassicaceae family are better sources of Se-methylselenocysteine (SeMeSeCys)(Pyrzynska, 2009). Selenocysteine (SeCys) is also present within foods and appears to be a predominant species alongside SeMet in meat (Filippini et al., 2018). Inorganic Se (selenite and selenate) are found in various cereals (Cubadda et

al., 2010), mushrooms (Stefánka et al., 2001) and certain vegetables (Pedrero et al., 2006) exposed to high levels of selenite and selenate during growth. The quantity of Se in fish varies on a fish by fish basis (Cappon and Smith, 1981) some species can contain 14-36% of Se in the form of selenite in the muscle.

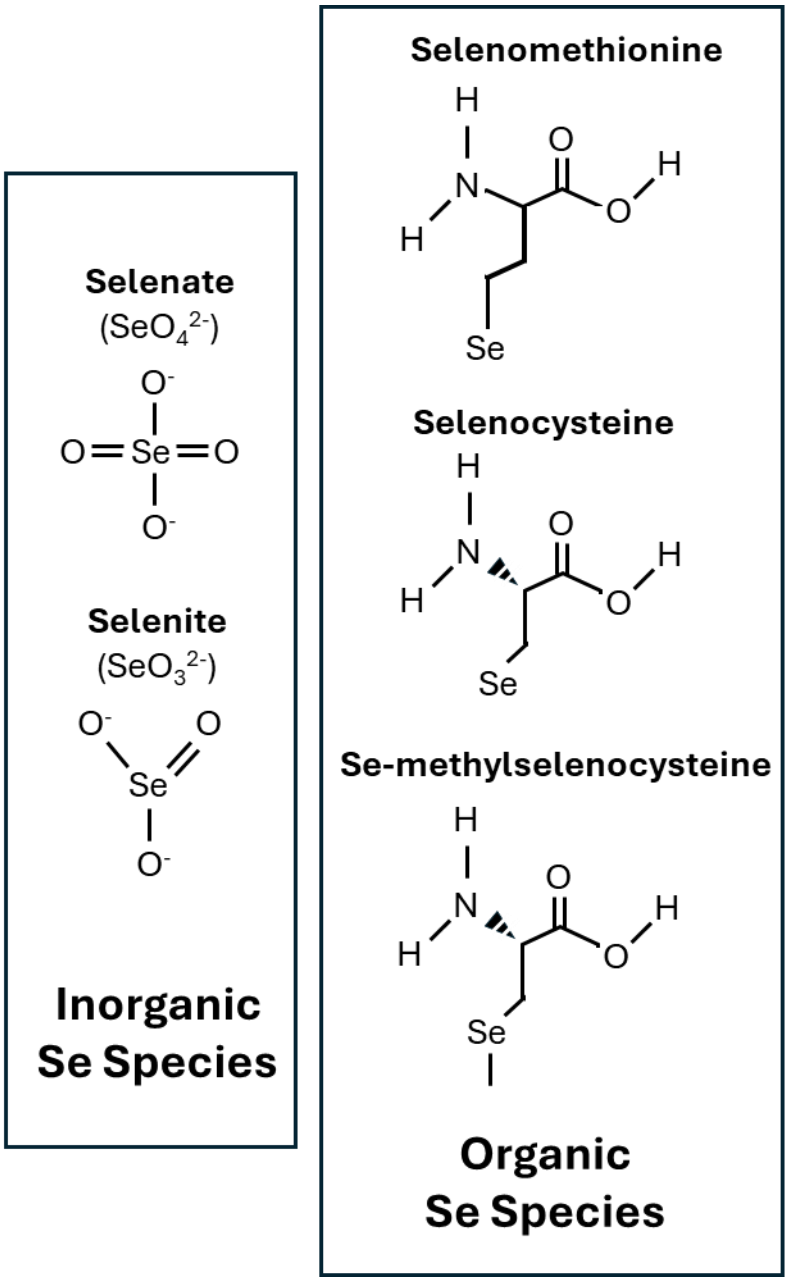

Figure 1.2- Common Se Species found in food.

Currently, daily Se intakes as described by the World Health Organisation recommend intakes of 55µg/day (WHO, 2009) with requirements being influenced by age, sex, and stage of life of a person (Table 1.2). The UK government advises a higher daily intake of 60 µg/day for women and 75 µg/day for men (SACN, 2013). Se deficiency occurs due to suboptimal levels of Se viz. <40µg/day (Winkel et al., 2012), multiple conditions have been associated with Se deficiency such as thyroid autoimmune disease (Wu et al., 2015) and cardiovascular diseases (Shimada et al., 2021) amongst others. A large body of literature suggests that Se deficiency predisposes people to Keshan disease (associated with heart enlargement and cardiac arrhythmia) (Johnson et al., 2010) and Kashin-Beck disease (enlarged joints and osteoarthritis)(Xiong et al., 2010). Similarly, chronic toxicity Se occurs at >400µg/day (Winkel et al., 2012), with early signs of acute toxicity including hypotension and tachycardia. More recently the upper tolerable intake has been reduced from 300 µg/day to 255 µg/day by EFSA after the results of large population based randomised control trials (EFSA Panel on Nutrition et al., 2023). Other symptoms of Se toxicity include diarrhoea, fatigue, hair loss, joint pain, nail discoloration or brittleness, nausea, headaches and a distinct garlic odour to the breath (MacFarquhar et al., 2010).

Table 1-2- Reference nutrient intake (µg Se/day) depending on age based on information from SACN (2013), Winkel et al. (2012) and EFSA Panel on Nutrition et al. (2023).

	Reference Nutrient Intake (µg Se/day)	
Age	**Female**	**Male**
0-12 months	10	10
1-3 years	15	15
4-6 years	20	20
7-10 years	30	30
11-14 years	45	45
15-18 years	60	70
Adults	60	75
Pregnant and Lactating	60	-
Deficiency	<40	
Upper tolerable level	255	

In recent times concerns have been raised that Se intakes in the general population are on the decline due to changes in sourcing of foods from seleniferous areas to low Se areas (Broadley et al., 2006), as well as shifts in dietary patterns reducing meat consumption (Steenson and Buttriss, 2021). Indeed, Se intakes and status have declined from >60µg Se/d in 1974 to 29-39µg Se/d in just 30 years (Rayman, 1997, Rayman, 2000, Rayman, 2002, Rayman, 2004). This means that the dietary intake of the UK is less than half the RNI of 60µg/day for women and 75 µg/day for men. The decline in Se intake of the UK population has been attributed to multiple factors, the most impactful being the change in sourcing of wheat from high Se wheat from the seleniferous soils of North America to the 'homegrown' low Se containing wheat from poor selenium soils of the UK (Broadley et al., 2006). Cereals such as wheat are a major source of Se in UK diets, and whilst worldwide Se content of wheat ranges from 0.001-30mg/kg, most wheats contain between 0.02-0.6 Se/kg (Lyons et al.,

2005). In 1982, 1992 and 1998 UK bread wheat averaged between 0.025-0.033mg Se/kg compared to 0.37-0.457 mg Se/kg in the high Se content North American bread wheat (Adams et al., 2002). Other factors impacting on Se content in food and subsequent human intake in the UK include the 'Clean Air Act 1956' (Broadley et al., 2006), the changing use of fertilisers (White et al., 2004) and changes to crop growing habits (Adams et al., 2002) all of which have reduced the input of Se into the soil. Given the declines in Se entering the human food chain efforts have been focused on improving Se status of foods. One such approach is that of plant biofortification.

1.5 Strategies to improve Se status in foods - Plant Biofortification

Biofortification is the process of increasing the concentration of micronutrient in the plant during the growth period rather than during post harvest crop processing in order to achieve improved nutritional quality (Puccinelli et al., 2017). The goal of biofortification is to reach a balance of increased Se content within the plant without causing detrimental effects to plant growth and yields (Puccinelli et al., 2017). Biofortification is advantageous as compared to direct supplementation as plants can metabolize more bioavailable organic forms of Se from applied inorganic forms (D'Amato 2020).

There are multiple methods of biofortifying crops with Se, including Se fertilisation of soil, Se-seed treatments, Se foliar (or fruit) spraying and hydroponic cultivation (Puccinelli et al., 2017).

Biofortification via the addition of Se to soil, mainly in the form of selenate or selenite or a combination of both has been shown to be an effective method of increasing Se content in crops (Alfthan et al., 2015). The application of sodium selenate as fertilisers have shown to be an effective method of increasing Se intake in crops such as leeks (Lavu et al., 2012), wheat (Ducsay and Lozek, 2006) and maize (Wang et al., 2013) amongst others and has been nationally rolled out in Finland (Newman et al., 2019). Soil fertilisation is generally regarded as a simple and successful method of Se enrichment (Wang 2020). However, the addition of Se fertilizer directly to soil has some drawbacks. Presence of clay and iron can bind Se rendering it not bioavailable for uptake into plants (Zhao et al., 2005). There are also now growing concerns surrounding the impact of long-term Se fertilizer use on soil microorganisms (Liu et al., 2021) and potential for drinking water contamination as Se species such as selenite and selenate are highly water soluble (Hasanuzzaman et al., 2020). Foliar application is an alternative to application of Se fertilizers to soil, as fertiliser is applied directly to leaves. Foliar application has been shown to be a successful method of Se enrichment in several crop species such as potatoes (Zhang et al., 2019a), rice (Xu and Hu, 2004) and cabbage (Mechora et al., 2014) amongst others. Again, foliar application is regarded as a practical method of Se enrichment but avoids soil-Se interactions and potentially reduces some of the pollution concerns, studies have also suggested that for some crops foliar application is more efficient than soil application (Galić et al., 2021).

However, more recently the use of Se fertiliser in hydroponic growing systems has also been trialled as a mechanism of biofortifying food without the concern for

environmental contamination (Saha, 2017). Hydroponics is a method of growing plants in a soil free water-based system where minerals and nutrients are supplied directly to the roots via an aqueous growing solution such as Hoaglands solution (Gaikwad and Maitra, 2020). Studies have applied this method successfully to enrich garlic (Tsuneyoshi et al., 2006), onion (Wróbel et al., 2004), sprout (young plant/seedling) brassica species (Ávila et al., 2014), lettuce (Smoleń et al., 2014) and tomatoes (Pezzarossa et al., 2014).

Some crops have a natural ability to uptake and accumulate Se more easily and to higher levels as compared to other crops. Brassicacae, Fabaceae, Asteraceae and Alliaceae accumulate higher levels of Se (González-Morales et al., 2017). In particular Alliums are known to accumulate large quantities of S and therefore lend themselves to accumulating and assimilating large quantities of Se due to the shared use of transporters (González-Morales et al., 2017). Se enrichment of allium species such as in *Allium fistulosum* (chives)(Kápolna et al., 2007b), *Allium cepa* (onion)(Wróbel et al., 2004), *Allium tricoccum* (ramp)(Whanger et al., 2000) and *Allium sativum* (Yang et al., 2021a) have proved very successful, with levels accumulated reaching 784 mg Se/kg in ramp bulbs (Whanger et al., 2000). Garlic is a particularly interesting member of the Allium family as the use of garlic has been traced through ancient Egyptian, Greek and Roman cultures (Ekşi et al., 2020) and has been used (intentionally or not) to reduce blood pressure and cholesterol and improve the function of the immune system (Londhe et al., 2011). Studies by Ip and Lisk (1994) have shown Se enriched garlic to have a reducing effect on mammary tumour yield in rats, more so than Se enriched onions (Ip and Lisk, 1994). In terms of garlic

cultivation, the Jinxiang region in the province of Shandong is China's largest and oldest producer of garlic- exporting 70% of what is produced there (Nie et al., 2021). Generally, garlic is accepted within the diet in the raw form or as dried tissues to enhance the flavour of food and thus provides an interesting model for biofortification for increasing Se status in humans.

1.6 Se metabolism in humans

In humans, both inorganic and organic Se species absorption occurs in the lower part of the small intestine (Roman et al., 2014), with an overall absorption efficiency of 70-90% (Fairweather-Tait et al., 2010).

Similarly to Se uptake from soil the different forms of selenium are taken up from the gastrointestinal tract into the bloodstream via different mechanisms (Roman et al., 2014) (Figure 1.3). Selenite is taken up into enterocyte cells via passive diffusion (paracellular transport), some selenite is thought to enter the bloodstream unchanged however selenite is also converted here by glutathione (GSH) and glutathione reductase to form HSe^- (Ferreira et al., 2021, Roman et al., 2014). Selenate, however, is co-transported into cells via $Na^+/K^+/Cl^-$ and OH^- transporters (transcellular transport) ((Kato et al., 1992, Minich, 2022) utilizing the same system as S analogue sulphate (Thiry et al., 2013, Wolffram et al., 1988) (Figure 1.3). A much greater percentage of selenate is absorbed compared to selenite, 90% and 50% respectively (Mangels et al., 1990). Organic Se SeMet is taken up via the same Na^+ dependent transporters as the S analogue methionine which is also postulated to occur for SeCys the Se analogue of cysteine (Thiry et al., 2013). Very little is understood about the transfer of Se species from the enterocytes to the

bloodstream (Whanger et al., 1996). Once in the bloodstream, Se species, selenate and organic forms are delivered to the liver unchanged, however selenite that entered the bloodstream unchanged is taken up by red blood cells and reduced to selenide (Suzuki, 2005, Thiry et al., 2012) (Figure 1.3). The liver is the organ with the greatest Se concentration (Thiry 2012), and is considered to be the central hub of Se metabolism, where the majority of selenoproteins and Se metabolites for excretion are synthesized (Roman et al., 2014, Suzuki et al., 2010).

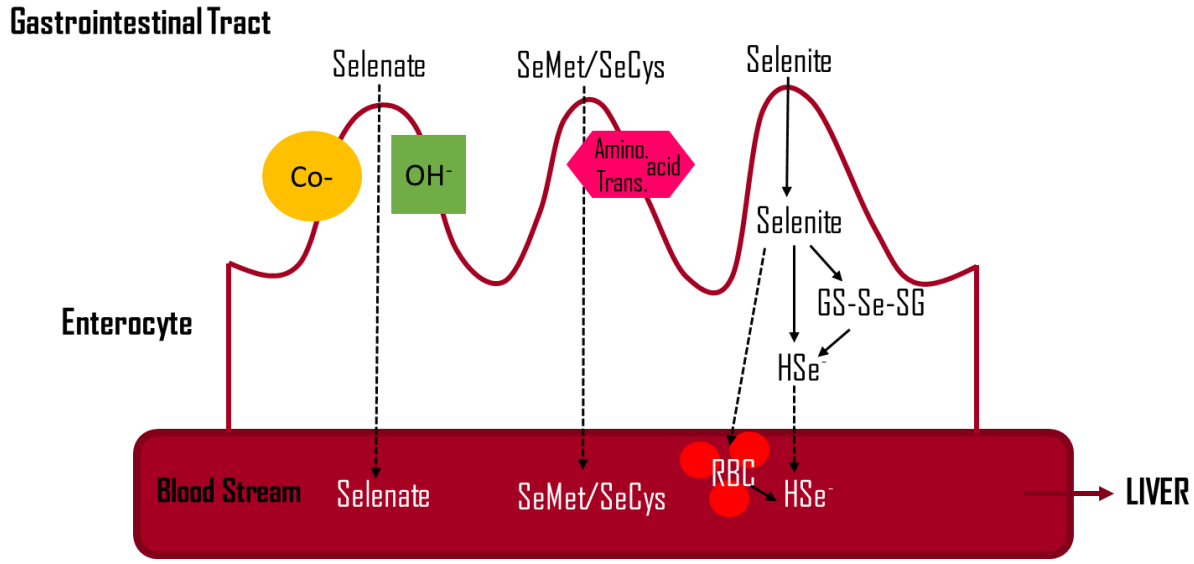

Figure 1.3- Uptake of Se species from the gut into the enterocyte cells by Co-transporters (Na^+, K^+ and Cl^-), OH- transporters, Amino acid transporters or Na+ dependent amino acid transporters depending on Se species. Se species subsequently enter the bloodstream and are delivered to the liver. Red blood cells (RBC).

In the liver, Se species enter a convergent metabolic pathway that leads to the formation of hydrogen selenide (HSe^- at physiological pH)- a key compound for the incorporation into organic Se containing species (Roman et al., 2014) (Figure 1.4).

Selenite is reduced via GSH to produce selenodiglutathione (GS-Se-SG) and further reduced by the enzyme glutathione reductase in the presence of NADPH to produce HSe^- (Hsieh and Ganther, 1975). Alternatively selenite can be directly reduced by thrioredoxin reductase in the presence of NADPH to produce HSe^- (Roman et al., 2014). Both of these pathways are comprehensively reviewed in (Roman et al., 2014, Weekley et al., 2011)(Figure 1.4).

In contrast, organic Se species like SeMet can be directly incorporated into proteins or metabolised to produce HSe^- to facilitate the formation of SeCys (Suzuki, 2005). Other organic forms like SeCys can undergo hydrolysis by the enzyme selenocysteine lyase to liberate HSe^- (Figure 1.4). This pathway shows similarities to the widely characterised trans-sulfuration pathway (Suzuki, 2005). Ultimately, the formation of HSe^- drives the biosynthesis of SeCys in cells and tissues with this metabolite in turn being used to form various selenoproteins (Suzuki, 2005).

The primary use for dietary Se is in the formation of Se containing amino acids. These amino acids are utilised in the formation of a range of redox sensitive proteins important in the immune system, DNA repair pathways and cell cytoprotection (Ye et al., 2022). Much is reported on the formation of the main Se metabolite, SeCys. SeCys is unique in its mechanism of synthesis which occurs directly on the tRNA (Turanov et al., 2011). The first step in producing selenocysteinyl transfer RNA $tRNA^{Sec}$ is the amino acylation of $tRNA^{Sec}$ via seryl-$tRNA^{Sec}$ synthetase (SerRS) to form Ser-$tRNA^{Sec}$ (Roman et al., 2014). tRNA-linked Ser is then converted to Sec via a two step process, the first of which is the phosphorylation of the hydroxyl group in the presence of ATP and Mg^{2+} by L-seryl-$tRNA^{Sec}$ kinase to form Sep-$tRNA^{Sec}$ (known as

phosphoserine) (Kang et al., 2020). The second step requires the generation of a selenium donor molecule called selenophosphate (SePhp) produced from the dietary intermediate HSe^- by the enzyme selenophosphate synthase 2 (SPS2) located in the cytosol using ATP as a phosphate donor (Tamura et al., 2011). The enzyme O-phosphoseryl-$tRNA^{Sec}$ selenium transferase (SEPSECS) then replaces the phosphoryl group on Sep-$tRNA^{Sec}$ with a selenol moiety from the selenophosphate donor (SePhp) to form Sec-$tRNA^{Sec}$ (SeCys)(Simonović and Puppala, 2018)(Figure 1.4).

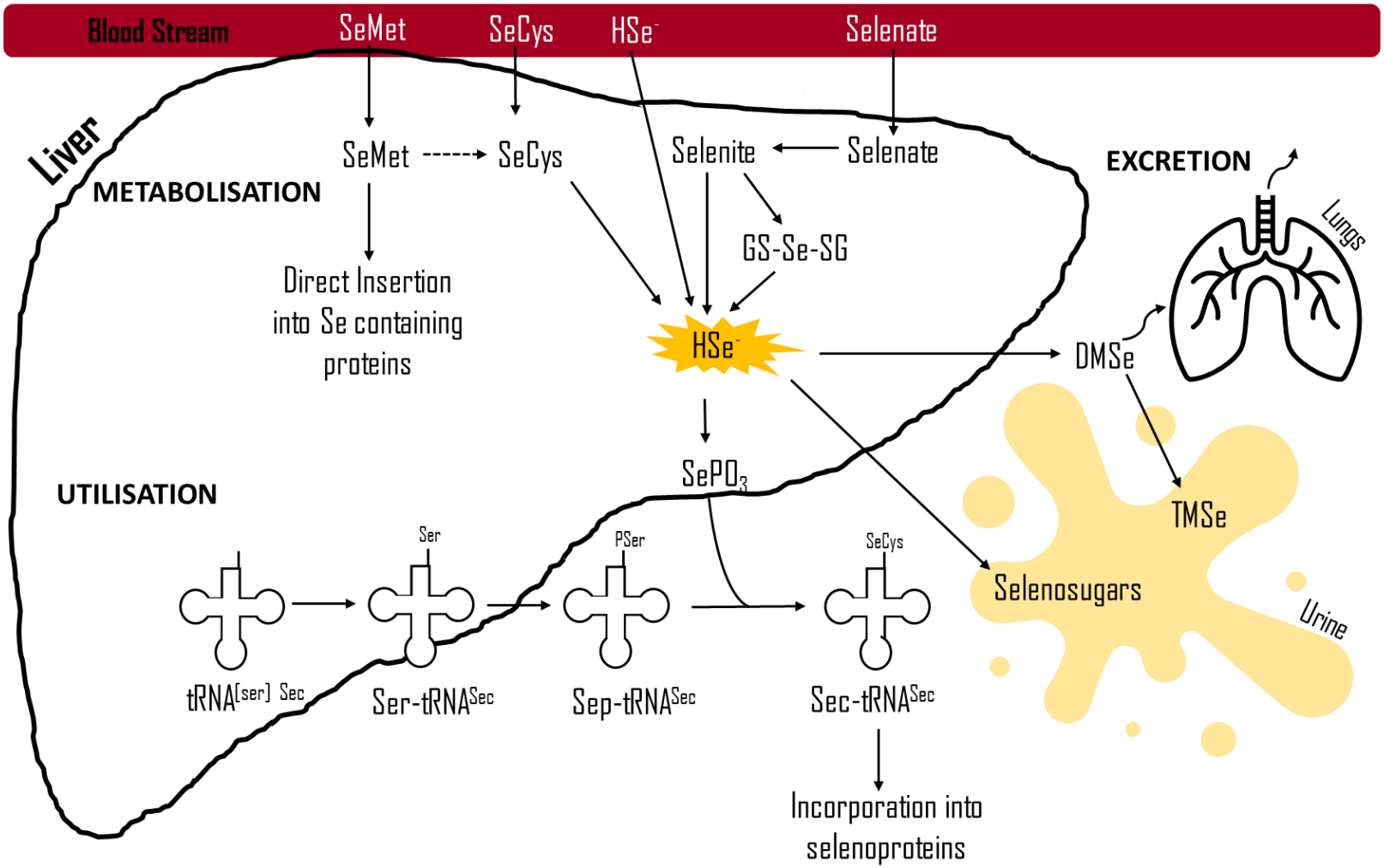

Figure 1.4- Summary of metabolism, utilisation and excretion of Se species regulated by the liver. Se species from the blood are taken up into the liver where they are metabolised by different mechanisms to form the hydrogen selenide anion (HSe^-). The liver regulates the utilisation and excretion of Se either by converting HSe^- into metabolites such as dimethylselenide (DMSe), trimethylselenide (TMSe) and Selenosugars which can be removed from the body or by forming selenophosphate ($SePO_3$) which acts as a Se donor for SeCys formation which happens directly on the t-RNA.

In order for the Sec insertion codon UGA to be read efficiently as Sec insertion and not STOP codon at least two trans-acting factors are needed, SECIS binding protein 2 (SBP2) and Sec-specific translation elongation factor (eEFSec) (Labunskyy et al., 2014). SBP2 binds to SECIS (selenocysteine insertion sequence) loop located in the 3' UTR region of selenoprotein mRNA, SBP2 also interacts with eEFSec which mediates the delivery of Sec-tRNA^{Sec} to ribosomal A site (Kang et al., 2020, Labunskyy et al., 2014). The UGA codon is recognised as the Sec integration codon and Sec is inserted into the nascent polypeptide chain (Kang et al., 2020) (Figure 1.5). Ribosomal protein 30 (L30) is also thought to be a part of basal Sec insertion machinery and proteins such as nucleolin and eukaryotic initiation factor 4a3 (eIF4a3) act as regulatory proteins for selenoprotein synthesis (Labunskyy et al., 2014). Selenoprotein P (SelP) produced in the liver, re-enters the bloodstream and is used to transport Se to other organs and tissues, where further selenoproteins can be synthesized (Roman et al., 2014).

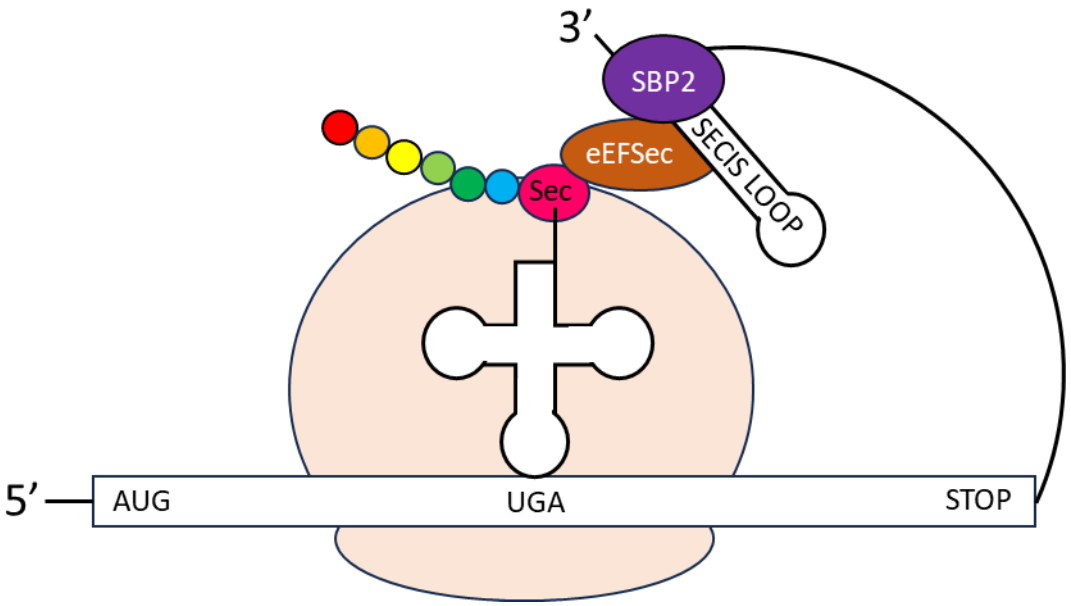

Figure 1.5-Cellular machinery required to insert Sec into a polypeptide chain. Selenocysteine insertion sequence (SECIS), SECIS binding protein 2 (SBP2) and Sec-specific translation elongation factor (eEFSec) are all required for Sec (SeCys) insertion into polypeptide chain. Adapted from Kang et al. (2020).

1.7 Selenium in health and disease

Selenium is critically important for the production of Se containing proteins involved in cellular redox systems. Importantly, selenium deficiency is reported to predispose people to diseases such as cancer (Clark et al., 1996), cardiovascular disease (Flores-Mateo et al., 2006), thyroid issues (Rasmussen et al., 2011), fertility issues (Lima et al., 2022) as well as reducing the immune system efficiency (Avery and Hoffmann, 2018) and depressing mood (Sajjadi et al., 2022). It is however, important to note that a 'U-shaped relationship' has been found where both deficient and excessive Se intakes can cause increased risk of certain diseases such as type-2 diabetes (Rayman and Stranges, 2013), cardiovascular mortality (in patients with hypertension) (Tan et al., 2021) and increased risk of prostate cancer (Duffield-Lillico et al., 2003) and really highlights the need to understand an individuals Se status before supplementing with Se (Reviewed in Rayman 2020).

1.7.1 Selenoproteins

Up to now 25 selenoproteins have been identified in the human genome (Tsuji et al., 2022). Selenoproteins have key roles ranging from antioxidant properties, energy metabolism and transport of Se round the body to specific tissues (reviewed in Zhang et al. (2023))(Table 1.3). There are three major families of selenoproteins: glutathione peroxidases (GPx), thioredoxin reductases (TrxRs) and Iodothyronine deiodinases (DIO). GPx are a large family of selenoproteins with antioxidant functions, mainly involving the reduction of H_2O_2 and hydroperoxides in order to

prevent oxidative damage to cells (reviewed in Roman et al. (2014)). TrxRs are a smaller family of selenoproteins and play a role in cell growth, recycling of ascorbate as well as preventing oxidative damage (Mustacich and Powis, 2000). Meanwhile DIO consists of 3 selenoproteins (DIO1, DIO2 and DIO3) and are involved in the metabolism, activation (DIO1 and DIO2) and deactivation (DIO3) of thyroid hormones (Tsuji et al., 2022). Other selenoproteins, such as Selenoprotein synthase 2 (SEPHS2) and Selenoprotein P play roles in human health by providing the active Se donor for Sec synthesis and transport of Se to tissues respectively (Tsuji et al., 2022). However, as detailed below other selenoproteins have been found to be implicated in cardiovascular disease, cancer and inflammation. In particular single nucleotide polymorphisms in alleles of selenoproteins have been found to associated with increased risk of disease (Rayman, 2009). Genetic polymorphisms in selenoprotein S (SEPS1) have been found to be significantly associated with increased risk of cardiovascular disease and ischemic stroke in females (Alanne et al., 2007). Polymorphisms in GPx1 have also been associated with increased risk of several cancers such as bladder, breast and lung (reviewed in Rayman (2009)). Although polymorphisms in the selenoprotein GPx4 have not been associated with increased risk of breast cancer, the presence of GPx4 polymorphism has been associated with a poor prognosis after diagnosis (Udler et al., 2007). This suggests that selenoproteins could be key in potentially pre-disposing people to disease and therefore optimum levels of selenoproteins need to be maintained within the body.

Table 1-3- Selenoproteins and their function by group.

Selenoprotein Group	Abbreviations of selenoproteins within group	Function	Reference
Glutathione Peroxidase	GPx1, GPx2, GPx3, GPx4 and GPx6	Defence against oxidative stress, has roles within multiple systems of the body and organelles of the cell	(Brigelius-Flohé and Maiorino, 2013)
Iodothyronine deiodinase	DIO1, DIO2 and DIO3	Activation and deactivation of thyroid hormones	(Labunskyy et al., 2014)
Thioredoxin reductases	TrxR1, TrxR2 and TrxR3	Defence against oxidative stress as well as roles in DNA synthesis, apoptosis and redox signalling	(Holmgren and Lu, 2010)
Methionine-R-Sulfoxide reductase	MSRB1	Reduction of methionine sulfoxide to methionine	(Sreekumar et al., 2011)
Selenophosphate synthase 2	SPS2	Synthesis of selenoproteins	(Tamura et al., 2011)
Selenoprotein P	SelP	Selenium homeostasis and transport	(Burk and Hill, 2009)
Selenoprotein K	SelK	Role in quality control of folded proteins to avoid endoplasmic reticulum stress and calcium dependent signalling	(Marciel and Hoffmann, 2019)
Selenoprotein S	SelS	Role in response to stress in endoplasmic reticulum and inflammation control	(Curran et al., 2005)
Selenoprotein N	SelN	Defence against oxidative stress and calcium homeostasis	(Arbogast and Ferreiro, 2009)
Selenoproteins W, T, H, V, I, M, O	SelW, SelT, SelH, SelV, SelI, SelM, SelO	Role unknown	(Labunskyy et al., 2014)

1.7.2 Se and cell signalling

Se compounds have been found to interact with cell signalling systems in mammalian cells and tissues. These cell signalling cascades are important in cell cycle progression, apoptosis and inflammation and have been shown to reduce the risk of certain cancers, vascular ageing and conditions such as liver fibrosis (Liu et al., 2018, Qiao et al., 2022, Dabravolski et al., 2023, Zhu et al., 2023). To date the spectrum of signalling systems that are influenced by Se compounds either from dietary sources or metabolised forms include apoptosis signalling regulating kinase (ASK1)(Zhou et al., 2015), C-Jun NH_2 terminal kinase (Fang et al., 2010), ERK pathway (Sanmartín et al., 2012), apoptosis inducing factor (AIF) (Rudolf et al., 2008a), TNFα (Zhang et al., 2002), NF-κb (Xu et al., 2023a), Nrf2/Keap1 (Wang et al., 2022c), Wnt (Zheng et al., 2017), PI3K/AKT (Wang et al., 2007), p38 MAPK (Hui et al., 2014), cyclin dependent kinases (Chigbrow and Nelson, 2001), protein kinase Cα (Li et al., 2010), AMP-activated protein kinase (AMPK) (Hwang et al., 2006), PPARγ (Finch et al., 2017), SIRT1 (Opstad et al., 2023), AKT/mTOR (Lee et al., 2010). Two areas of particular note that have received considerable attention are the roles of Se in the immune system and in cancer due to reported anti-cancer effects.

1.7.3 Immune system

Se is essential for the correct functioning of the immune system, which relies on the formation and incorporation of Se into selenoproteins, which have an important role in antioxidant defence against oxidative stress (Zoidis et al., 2018). Selenium deficiency impairs the ability of a host to rapidly respond to a pathogen and activate the innate immune system (Avery and Hoffmann, 2018), decreases dendritic cell

differentiation (Sun et al., 2018b) and reduces the number and maturation of T-cells (Peng et al., 2011). Se deficiency can also affect the function of neutrophils, leading to increased adhesion due to enhanced expression of adhesion molecules (Arthur et al., 2003). The inability to lyse ingested pathogens by the neutrophils of mice, rats and cattle under Se deficiency has also been observed (Turner and Finch, 1991). This has been postulated to be due to the decrease in GPx1 activity and the inability to produce the required free radicals needed to lyse the pathogens (Arthur et al., 2003). Macrophages are sensitive to Se levels which can impact inflammation, signalling capacity and anti-pathogen activities (Avery and Hoffmann, 2018). Under Se deficiency macrophages create a severe inflammation response via the NF-kB signalling pathway due to the accumulation of reactive oxygen species and reduced expression of TNF-α leading to the inhibition of phagocytosis (Xu et al., 2020b). One key role of Se in ensuring the function of macrophages is to induce the switch from pro-inflammatory phenotype (M1) to anti-inflammatory phenoptype (M2), which is thought to be dependent on adequate Se levels (Nelson et al., 2011). Studies investigating chronic infectious diseases such as HIV and TB have shown that serum Se concentration decreases significantly as the disease progresses which correlates with an increased transcription and production of oxygen free radicals, hydrogen peroxide and pro-inflammatory cytokines which go on to induce inflammation of organs due to the intracellular redox imbalance (Look et al., 1997, Liu et al., 2016, Liu et al., 2017, Avery and Hoffmann, 2018, Carlson et al., 2009). Se is known to regulate inflammation by altering expression of different cytokines (Mattmiller et al., 2013), therefore under deficiency conditions there is significantly increased expression of

inflammation factors including iNOS, IL-1β, IL-12, IL-10, PTGE and NF-KB (Xu et al., 2020b). The functional properties of the innate immune system, in particular neutrophils have been shown to be rapidly restored with Se supplementation of sodium selenite (20-200µgSe/ml) (Aziz and Klesius, 1985, Urban and Jarstrand, 1986). Increased Se status has also been shown to increased immune response to vaccination and bolster immunity (Broome et al., 2004).

In particular the selenoproteins methionine sulfoxide reductase (MSRB1) and Selenoprotein K (SELENOK) are characterised as important for the correct functioning of innate immunity. MSRB1 has been found to control immune responses by promoting anti-inflammatory cytokine expression in macrophages (Lee et al., 2017). Selenoprotein K has been shown to be an important enzyme co-factor alongside enzyme DHHC6 needed for promoting calcium fluxes during immune system activation and post-translational modifications of proteins such as palmitoylation (Verma et al., 2011). SELENOK knock out mice were found to be 50% reduced in most immune cell functions when the immune system was challenged (Verma et al., 2011).

1.7.4 Cancer

High Selenium diets in humans were first linked to decreased risk of cancer in observational experiments in the 1960's (Vinceti et al., 2018). An inverse correlation was found by Schrauzer et al. (1977) between Se dietary intake of 27 countries and cancer mortality in leukaemia as well as cancers of the large intestine, rectum, prostate, breast, ovary, lung. Weaker inverse correlations were found in pancreatic, skin and bladder cancer.

Results from the National prevention of cancer (NPC) trial show that supplementation of patients with a history of basal and squamous cell skin carcinomas with 200 μg/day of Se enriched yeast for a mean of 4.5 years did not significantly reduce the incidence of basal or squamous cell skin cancer. However, Se supplementation did result in a significant reduction in total cancer mortality, total cancer incidence and incidences of lung, colorectal and prostate cancers (Clark et al., 1996). However there have been mixed findings in other human supplementation trials, which is likely due to the varying ages and inclusion criteria as well as source of Se supplementation, form of Se species, dosage and length of time supplemented. The Se and Vitamin E cancer prevention trial (SELECT) trial and PREvention of Cancer by Intervention with Selenium (PRECISE) trial found no significant decreases in cancer incidence or mortality at supplementation of 200μg Se/day (Lippman et al., 2009, Rayman et al., 2018).

Selenoproteins have been shown to have protective roles in oxidative damage within other diseases, however within the field of cancer some selenoproteins such as selenoprotein 15 (Selp15) and glutathione peroxidase 2 (GPx2) have been found to show both anti-cancer and pro-cancer activities (Hatfield 2014). Reduced Selp15 expression has been reported in multiple cancer tissues such as malignant lung, breast, prostate and liver tissues (Wright and Diamond, 2011) as well as in lung cancer patients (Jablonska et al., 2008). However, in colon cancer studies (Irons et al., 2010, Tsuji et al., 2012, Tsuji et al., 2011), Selp15 has been implicated in having effects on cell cycle regulation and/or interferon γ-regulated inflammation leading to tumour progression (Tsuji et al., 2012). GPx2 has been found to upregulate

Nrf2/Keap1 in a bid to protect cells from oxidative damage of carcinogens (Brigelius-Flohé et al., 2012), however this has also been shown to confer an advantage to cancer cells (Dewa et al., 2009).

Selenium can also be metabolised to non-protein anti-tumorigenic metabolites such as benzylselenocyanate (Nayini et al., 1989), 1-4-phenylene-(methyl-lene) selenocyanate (El-Bayoumy et al., 1992, Thompson et al., 1994), selenobetaine (Ip and Ganther, 1990), methylselenocysteine (Ip and Ganther, 1990) and aliphatic selenocyanates (Ip et al., 1994) which disturb tumour cell metabolism and stimulate programmed cell death. These anti-tumorigenic effects have been shown in multiple animal studies, such as the inhibition of pancreatic and liver cancer (Curphey et al., 1988) as well as mammary cancer (Thompson and Becci, 1980) in rats fed on a diet supplemented with Se. More recently Xu et al. (2020a) have shown that glioma cells (malignant tumour cells) exposed to Se-nanoparticles display anti-tumorigenic effects causing glucose metabolism reduction and oxidative stress leading to apoptosis. Other Se compounds available from food have also have also been shown to have anti-cancer effects in multiple cell lines at the cellular level such as selenite, selenocystine ($SeCys_2$), selenomethionine and Se-methylselenocysteine (SeMeSeCys)(reviewed in Radomska et al. (2021)), however the mechanism of action are not fully understood and appear to differ between different Se species and cell lines.

1.7.5 Additional roles of Selenium in humans

In addition to roles in reducing cancer risks and influencing the immune response various Se compounds have also been shown to impact on other biological roles in

mammalian cells and tissues. These roles are diverse and span a number of important organ systems in mammals including the brain, thyroid, cardiovascular and reproductive systems.

1.7.6 Cardiovascular Disease

Multiple selenoproteins have been found to have key roles in the correct functioning of the cardiovascular system such as prevention of ischemia/reperfusion injury and regulating redox balance (Benstoem et al., 2015). These include GPx1 (Yoshida et al., 1996, Brigelius-Flohé et al., 2003), GPx3 (Jin et al., 2011), thioredoxin reductase (Yamamoto et al., 2003), thyroid hormones (Trivieri et al., 2006) and SelenoK (Lu et al., 2006). Low serum Se has been linked to increased risk and incidence of cardiovascular diseases (Flores-Mateo et al., 2006, Navas-Acien et al., 2008, Romero et al., 2001, Salonen et al., 1982). Deficiency in selenium can lead to the oxidisation of polyunsaturated fatty acids known as low density lipoproteins (LDLs) which accumulate and cause irritation (due to cytotoxic products of lipid peroxidation) of arterial walls (Esterbauer et al., 1992). This leads to the inhibition of endothelium derived relaxing factor (EDRF) and chemotactic protein release which stimulates platelet aggregation (Esterbauer et al., 1992) and clot formation which can ultimately result in myocardial infarction (heart attack) or stroke. Studies have found that supplementing Se reduces coronary heart disease risk (Luoma et al., 1984), cardiac related deaths (Korpela et al., 1989) and reduced the markers of cardiovascular stress (Zhu et al., 2019). However other studies have found that Se supplementation did not impact on the incidences of cardiovascular disease (Alfthan et al., 2015, Rees et al., 2013).

1.7.7 Thyroid Disease

Thyroid hormones play a critical role in metabolism (Mullur et al., 2014) including selenoenzymes such as iodothyronine deiodinases, which are responsible for activating and deactivating these thyroid hormones T3 (EC.1.21.99.4) and T4 (EC1.21.99.3). Selenoproteins GPx3 and GPx1 (EC.1.11.1.9) catalyse the reaction of H_2O_2 formed during thyroid hormone synthesis into water (Tsatsoulis, 2018). Dysregulation in these enzymatic systems is associated with autoimmune thyroiditis (Xu et al., 2011), thyrocyte damage, fibrosis of thyroid gland (Ventura et al., 2017) and development of thyroid nodules (Rasmussen et al., 2011). Supplementation has been shown to reverse the development of autoimmune thyroiditis as well as reduced size, number and stiffness of thyroid nodules when used in combination with other novel drugs (Nordio and Basciani, 2018).

1.7.8 Reproduction

Se status has been linked to fertility of both men and women. It has long been understood that Se status is of extreme importance in male fertility for testicular development (Li et al., 2020a), testosterone biosynthesis (Shi et al., 2017), sperm development (Xu et al., 2023b) and motility (Foresta et al., 2002). Animal trials have shown a low Se diet has a detrimental effect on testis size and associated synthesis of the sex hormones testosterone and oestradiol, sperm viability and motility (Li et al., 2020a). Breaks occurred in the midpiece region of the tail, where GPx4 is most prevalent (Rayman, 2000, Wu et al., 1973). Supplementation with 100µg/day of SeMet of men with sub-fertility showed an increase in sperm motility (Scott et al., 1998). Se is also thought to be involved in maintaining the balance between ROS

production and redox status during the promotion of ovulation (Pieczyńska and Grajeta, 2015, Takami et al., 2000, Takami et al., 1999). Low Se status has been linked to miscarriage in animals and humans (Stuart and Oehme, 1982, Barrington et al., 1996, Hidiroglou, 1979) as well as deficiency and excess consumption of Se being linked to other conditions such as pre-eclampsia, gestational diabetes, foetal growth resistance and preterm birth (Dahlen et al., 2022). Supranutritional (above adequate but below toxic) consumptions of Se at different stages of pregnancy and throughout gestation in ewes has also been shown to impact positively on foetal masses (and masses of specific organs of both ewe and offspring), positive impacts on vascularization of mammary gland and yield of colostrum have also been seen which impact positively on postnatal development of offspring. Further research into the impacts of Se in female fertility is needed.

1.7.9 Brain

The brain has been found to be the last organ to be depleted of Se under low Se diets (Chen and Berry, 2003). Grey matter and glandular parts of the brain contain higher concentrations of Se compared to white matter (Hock and Demmel, 1975, Drasch et al., 2000), both in humans and animals (Chen and Berry, 2003). Reductions in Se plasma content have been noted in Alzheimer's patients (Tancheva et al., 2023). In low Se diets the turnover rate of dopamine and serotonin is increased and noradrenaline and 5-hydroxy-3-indoleacetic acid decreased compared to the control (Hawkes and Hornbostel, 1996). This suggests a lack of oxidative protection, and dysregulation in neurotransmitter turnover linked to cognitive decline (Berr et al., 2000), Alzheimer's (Varikasuvu et al., 2019) and dementia (Karlsson, 1993).

Selenoproteins such as Sepp1, GPx1, GPx4 and TrxRs have been shown to play vital voles in normal brain function, including Se homeostasis and protection from oxidative stress (reviewed in Chen and Berry (2003)). Disruption to these selenoproteins have been suggested to be involved in a range of neurodegenerative disorders (reviewed in Pillai et al. (2014)). Mood can also be impacted by selenium deficiency. A cross-sectional study on US adults found a negative correlation between dietary Se intake and depressive symptoms (Li et al., 2018). A higher intake (226.5µg/day) of Se was also found to significantly improve mood and reduced depression and anxiety scores (Finley and Penland, 1998).

1.8 Work conducted in this thesis

The potential impact on health of Se status and supplementation is becoming an area of research interest. Multiple researchers have been attempting to biofortify crops to increase dietary Se intake within the general population, in the hopes that this will have a positive impact on health. However, the narrow therapeutic window of Se also raises concern about potential toxicity of these biofortified crops. The wide range of study designs and focus areas concerning crop biofortification make it difficult to compare methods of biofortification and subsequent impacts on health. Indeed, the literature highlights many gaps in knowledge not only about the best methods of biofortification and crops most amenable to enrichment but also the impact of different cultivars on Se accumulation and impacts on plant growth. There are also further gaps in knowledge about the impacts these Se-enriched crops may have on health and the mechanism by which they exert their effect.

The work in this thesis aims to fill some of the gaps relating to methods of Se biofortification and impacts of cultivar on accumulation and tolerance to Se as well as trying to further develop understanding into the impact of Se enriched crop extracts on mammalian cells. The four experimental chapters focus on, i) the assessment of foliar application of sodium selenate to manipulate plant tissue Se levels, ii) the analysis of hydroponic application of Se and impacts of Se enriched tissues on mammalian cells, iii) the assessment and validation of a cellular model to explore the cytotoxicity and cytotoxic mechanisms of Se compounds in mammalian cells, and finally, iv) the development and analysis of a novel chemical tool for use in Se research.

1.9 COVID statement

The work carried out in this thesis was impacted by some major unforeseeable events. Throughout this research the COVID-19 pandemic has caused disruption, from the lockdown in March 2020, lab access was restricted for a period of 7 months until October 2020 due to government guidelines and university restrictions. This delayed the start of the first polytunnel trial as there were difficulties in obtaining access to different departments within the university as well as materials for the trial. Following this, the repeated lockdowns alongside university guidelines, although they did not cause closure of facilities in the same manner further slowed lab work due to reduced lab occupancy. The university restrictions, such as reduced personnel were not removed until August 2021. Further disruption to work occurred between March and July 2021, as we had to close the labs and move them to a new

building. This resulted in 10 weeks of disruption and the start of lab work was further delayed due to flooding of the new facilities (a problem that has reoccurred multiple times from July 2021-until November 2023). During this research we have also struggled in obtaining reagents especially for cell culture and ICP-MS work, as well as nationwide CO_2 shortages. One of the main impacts of this has been the delay to ICP-MS work, Se speciation and cell culture work. We also had difficulty obtaining more Garlic bulbs for repeat plant trials due to bad weather and poor harvests on the Isle of Wight. Therefore, for the duration of the work, we have had to adapt rapidly in order to preserve the research and deliver on our original hypothesis.

1.10 Thesis Aims

1. To assess foliar application as a method of Se application and impacts on growth and antioxidant capacity of garlic cultivars.
2. To assess hydroponics as a method of Se application and determine the impact of Se enriched tissue extracts on HepG2 cell model.
3. To evaluate the cytotoxicity of Se species found in food crops on HepG2 cell model and further the understanding of the mechanisms of cell death.
4. To investigate the effect of a slow release H_2Se donor on markers of cell death in a HepG2 cell model.

1.11 Thesis Hypothesis

We hypothesise that:

Se enrichment will increase the bioactive properties of plants and tissue extracts by virtue of the accumulation of known Se compounds in plant tissues.

2 Materials and Methods

2.1 Materials

2.1.1 Plant materials

10 sets of Mersley, Lyubasha, Solent and Bulbils were kindly supplied by The Garlic Farm, Isle of Wight (https://www.thegarlicfarm.co.uk/) and 6 sets of Marco from Taylor & Sons Bulbs Ltd, Lincolnshire in October 2020. Marco, Mersley and Solent are softneck cultivars (*Allium sativum var L.*) and Lyubasha is a hardneck cultivar (*Allium sativum var. O)*.

2.1.2 Chemicals

Magnesium Chloride Hexahydrate, Methanol and Isopropanol VWR chemicals (Pennsylvania, USA). Dulbecco's Modified Eagle Medium (DMEM), Phosphate Buffered Saline (PBS), Sodium Selenite, Sodium Selenate, Selenomethionine (SeMet), Se-(Methyl)selenocysteine hydrochloride (SeMeSeCys), Albumin from bovine serum (BSA), Tris-Hydrochloride, SDS, Crystal Violet, 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI), Potassium chloride, D-(+)-Glucose (99.5% GC), Sodium bicarbonate, Sodium phosphate dibasic, Paraformaldehyde, Ethylenediaminetetraacetic acid (EDTA), Egtazic aicd (EGTA), Sodium fluoride, Sodium pyrophosphate, Sodium orthovanadate, sodium phosphate monobasic monohydrate, glycerol, deoxycholate, Hydrogen peroxide were obtained from Sigma-Aldrich (St Louis, USA). Nitric Acid (Primer grade plus), Foetal Bovine Serum (FBS), L-glutamine, Triton X-100, Sodium chloride, Calcium chloride, Trypsin 0.25% EDTA and Dimethyl Sulfoxide (DMSO), were from Fisher Scientific UK ltd (Loughborough, UK). Protein inhibitor cocktail (04 693 124 001) Roche (Basel,

Switzerland). Penicillin-streptomycin (Pen/Strep) was from Merck Life Science UK Ltd (Gillingham, UK). L-selenocystine ($SeCys_2$) was from Acros Organics (Geel, Belgium). Potassium phosphate monobasic and Magnesium sulfate heptahydrate were from Fisons (Glasgow, UK). Image iT Lipid Peroxidation Kit (C10455), Cumene Hydroperoxide, CyQuant LDH cytotoxicity assay kit (C20301) and Human PARP 214/215 ELISA kit (KH00741) and ECL were from Thermo Fisher Scientific (Waltham, USA). 2', 7'-Dichlorofluorescein diacetate (DCFDA) was from EMD Millipore Corp (Burlington, USA). Z-VAD-FMK was from APExBIO (Hsinchu City, Taiwan). Z-DEVD-FMK was from AdooQ Bioscience (Irvine, CA, USA). Milk -Marvel Original Dried Skimmed Milk (less than 1% fat) obtained from a local shop.

2.2 Sample preparation

2.2.1 Freeze drying and milling

Freeze drying the garlic tissues was used to facilitate nutritional assessments of plant tissues (eg ICPMS and Se Speciation) and to preserve the garlic, it has been shown to be one of the best methods of preserving the nutritional qualities of plant-based foods especially when operated under vacuum (Bhatta et al., 2020). Samples were pre-frozen at -80°C overnight in small plastic pots, before lids were removed and placed into the freeze dryer. Samples were dried in the Freeze dryer (Christ Gamma 1-16 LSCplus, with LyoCube) for 72hrs, -55°C and <0.4mmbars.

Freeze dried tissues were processed into a powder as previously described in (White et al., 2017, Mwesigye et al., 2019). Freeze dried samples were ground to a fine powder using a coffee grinder (Braun Aromatic) and stored in the dark in 50ml falcon tubes with added silica crystals to prevent rehydration until required.

2.2.2 Tissue extract

A methanol-water method was selected as previous studies have used this approach in working with Se enriched plant tissues (Newman et al., 2021, Viltres-Portales et al., 2024) and has been shown to be an effective method of extracting and maintaining phenolic content, antioxidant and bioactive properties from garlic plants in previous literature (Kallel et al., 2014). This method aids the extraction of polar metabolites (amino acid derivatives and other small molecular weight compounds), it also generates extracts that can be used in cell culture experiments as described in Rose et al. (2000) and Faulkner et al. (1998).

100mg of freeze-dried garlic sample was hydrated with 3ml of sterile water and vortexed 3 times for 15 seconds. This mixture was incubated for 1 hour at room temperature with occasional vortexing. 2ml of 70% methanol was added and the mixture was vortexed a further 2 times for 15 seconds and incubated at room temperature for a further 20 minutes. The mixture was then centrifuged at 3000rpm (MSE Centaur 2) for 5 minutes. 1ml of supernatant was aliquoted out into 1.5ml microcentrifuge tubes. The extraction solution was then vacuumed condensed in a MiVac DUO concentrator (GeneVac, Fisher Scientific) for approximately 3 hours until the volume of liquid had reduced from 1ml to 200µl (extracts 100 mg/mL).

2.3 Assessment of nutrient composition

2.3.1 ICP-MS

The elements B, Na, Mg, P, S, K, Ca, Ti , Li, Be, Al, V , Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Ag, Cd, Cs, Ba, Tl, Pb, U were measured via IC-PMS following the same method used by Thomas et al. (2016). Apart from Se, other key macro and

micronutrients were chosen for analysis because of their importance to plant growth and necessity in human health. These included Na, Mg, P, S, K, Ca, Mn, Fe, Cu, and Zn. Alongside the samples, a certified reference material (CRM) of tomato leaf (SRM 1573a, NIST) and the blank were digested and analysed in parallel with the samples.

Before ICP-MS could take place, samples must first undergo acid digestion. In brief, 100 mg of freeze-dried garlic sample was heated with 6mL of HNO_3 (PrimarPlusTM grade) in a microwave (Microwave Pro, Anton Paar GmbH, Graz, Austria) for 45 minutes. The sample was heated for 10 minutes to reach 140°C, held for 20 minutes at 140°C and then cooled for 15 minutes to 55°C. After cooling, the solution was diluted to 20mL using Mili-Q water followed by a 1:10 dilution prior to analysis using a triple quadrupole Inductively Coupled Plasma-Mass Spectrometer (ICP-MS)(Icap TQ, Thermo-Fisher Scientific, Bremen, Germany). Samples were run through the ICP-MS machine by Saul Vazquez Reina, ICPMS Technical Specialist in Gateway Building, Sutton Bonington Campus.

Samples were introduced via a single line from an autosampler (Cetac ASX-520) at a flow rate of 1.2ml/min through a perfluoroalkoxy (PFA) Microflow PFA-ST nebuliser (Thermo Fisher Scientific, Bremen, Germany). All samples, internal standards and calibration standards were diluted in 2% nitric acid with 4% methanol to enhance the ionization of some elements. Calibration standards included a i) multi-element solution with Ag, Al, As, Ba, Be, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, Tl, U, V and Zn, in the range 0 – 100 µg L-1 (0, 20, 40, 100 µg L-1) (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA), ii) multi-element calibration solution containing Ca, Mg, Na and K in the range 0-30 mg L-1

(PlasmaCAL, SCP Science, France) and iii) a phosphorus, boron and sulphur standard made in-house. Internal standards were used to correct for instrument drift and contained combinations of Sc (10 µg L-1), Ge (10 µg L-1), Rh (5 µg L-1), Re (5 µg L-1) and Ir (5 µg L-1). The ICPMS was operated in 'collison cell mode' with kinetic energy discrimination, switching between H_2 gas for Se determination and He gas for all other elements. Sample processing was undertaken using using Qtegra™ software (Thermo-Fisher Scientific).

2.4 General Culture of HepG2 cells

2.4.1 Culture Media

Human hepatoma (HepG2) cells (ATCC, Virginia, USA) were cultured in DMEM media (Sigma Aldrich, D6429), supplemented with 10% Foetal Bovine Serum (11550356), 1% Penicillin-Streptomycin (10,000U penicillin and 10 mg streptomycin/mL (P4333)), and 1% L-Glutamine (11534546). For all cell treatments FBS free DMEM (D6429) media was used containing 10% dH_2O, 1% Penicillin-Streptomycin (P4333), 1% L-Glutamine (11534546)). Cells were incubated at 37°C and 5% CO_2 until approximately 70% confluence.

2.4.2 Passage

Media was removed from adherent HepG2 cells and cells were washed with 10ml of pre-warmed PBS. Following this, PBS was removed from the flask and 2ml of trypsin EDTA was added and the flask was incubated for 3 minutes at 37°C and 5% CO_2 until HepG2 cells had detached from the flask. Media was then added and cells were centrifuged for 5 minutes at 1,000prm. Following centrifugation and formation of cell pellet, media was removed and discarded and the cell pellet was resuspended in

media. Cell suspension was then placed into fresh T75 flasks (3ml per T75, 9ml per T225) and 10ml of media was added to the flasks. Flasks were then placed back into the incubator and incubated at 37°C and 5% CO_2. Cells were generally passaged 3 times a week.

2.4.3 Cell seeding

For cell counting and seeding plates, the passage protocol was followed up to and including cell resuspension. Following this, 10µl of cell suspension was placed onto cell haemocytometer and cells were counted in 3 observation squares. For calculating the number of cells to be plated, the number of cells in 3 observation squares were averaged and multiplied by 10,000 to give the number of cells per ml. Total cell count was calculated using number of mls of stock x number of cells per ml. The number of cells needed per plate was calculated by number of wells being seeded x number of plates x desired cells per well. The calculation then followed:

$$\frac{number\ of\ cells\ needed}{total\ cell\ count}\ x\ volume\ of\ original\ stock = seeding\ volume\ (ml)$$

Seeding volume was then deducted from volume of liquid overall needed to seed all the plates. All experiments in 96 well plates were seeded with 100µl per well and all experiments carried out in 6 well plates were seeded with 2ml per well.

For fluorescent imaging in the case of DAPI and image iT Lipid Peroxidation, prior to cell seeding coverslips (24x50mm No.1 Scientific Laboratory Supplies LTD) were sterilised in 70% ethanol for 5 minutes, and placed individually into each well of a 6 well plate. Plates were dried at room temperature in a MSC hood. Cells were then seeded into the wells containing the coverslips at a density of 400,000 cells per well.

After seeding cells, plates were incubated for 24hrs to allow for cell adherence to the bottom of the plate. Serum containing media was removed prior to treatment and cells were washed with PBS.

2.4.4 Cryopreservation of cells-

Cells were frozen in 10% DMSO and 90% FBS freezing media. The normal cell passage protocol was followed up until resuspension where cells were resuspended in 3-5ml/T75 of fridge cold freezing media. 1ml of cell suspension was then aliquoted into cryovials and placed into a 'Mr Frosty' container and placed in -80°C overnight. Cryovials were then moved to liquid nitrogen dewars for long term storage.

2.5 Cell culture Assays

2.5.1 Cell viability - Crystal Violet Assay

Crystal Violet was used as a measure of cell viability, the membrane permeable crystal violet dye binds to DNA and proteins, however, cells that have died no longer adhere to the culture plate so are washed away during the wash step, therefore reducing the amount of dye present. Methanol is used to solubilise the stain and to obtain optical density readings. Crystal violet has been shown to be an effective method of assessing cell viability in adherent cells ((Asita and Salehhuddin, 2017, Cui et al., 2019, Carlisle et al., 2020).

The method was followed from (Feoktistova et al., 2016). In brief, media was removed from 96 well plates and 20µl of 0.5% crystal violet solution containing 20% methanol was added per well. Plates were incubated at room temperature for 10

minutes. After 10 minutes stain was washed off by rinsing the plates in water and using 200µl of methanol was added to each well. Absorbance was then measured at 550nm using a BIORAD plate reader (680XR Microplate reader). Results were expressed as % cell survival as previously described by Feoktistova et al. (2016).

2.5.2 Indication of DNA damage- 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) staining.

Cells were stained with DAPI using methods slightly adapted from the literature (Lee et al., 2014, Zhang et al., 2013). Adherent cells were fixed by applying 1ml of 4% paraformaldehyde to each well, and cells were incubated at 37°C and 5% CO_2 for 10 minutes. The 4% paraformaldehyde was removed from each well and they were washed with PBS three times. After fixing, 1ml of Triton X-100 was applied to each well to permeabilise the cell membrane, and plates were incubated at 37°C and 5% CO_2 for 30 seconds. Following washing PBS before, 1ml DAPI solution (300nM) was added to cells and incubated for 5 minutes at 37°C and 5% CO_2. After 5 minutes the DAPI solution was removed, and wells were again washed with PBS. Coverslips were removed from each well one by one and placed on a slide. Images were taken on EVOS fluorescent microscope.

2.5.3 Measure of cell membrane integrity- Lactate dehydrogenase (LDH) leakage

Leakage of LDH, a measure of membrane integrity was determined using a commercial validated kit, CyQUANT™ LDH Cytotoxicity Assay Kit (C20301 Invitrogen by Thermo Fisher Scientific) as per the manufacturer instructions. The kit works on the basis that NADH produced during the conversion of lactate to pyruvate via LDH converts the tetrazolium salt in the kit into a pink coloured formazon product that

can be measured using a plate reader. In brief, following the treatment period 10µl of 10X lysis buffer was added to maximum LDH activity wells and the plate was incubated at 37°C and 5% CO_2 for 45 minutes. 50µl of each sample was transferred to a 96 well flat bottom plate. For the positive control, 50µl of LDH positive control was added to wells that did not contain cells. Following this, 50µl of Reaction Mixture was added to each sample and the plate was incubated again for 30 minutes and protected from light. 50µl of Stop Solution was then added to each sample well and any bubbles formed were popped using a needle. The absorbance was measured on a BioRad plate reader at 415nm and 655nm. Data was exported to Microsoft Excel where the 655nm absorbance values were subtracted from the 415nm absorbance values. Percentage LDH activity was calculated by dividing absorbance value for each sample by the maximum LDH value and the resulting value was multiplied by 100 to give percentage.

2.5.4 Role of caspases within cell death mechanism - Caspase Inhibitors

Caspase inhibitors were obtained from APExBIO (Z-VAD-FMK) (Hsinchu City, Taiwan) and AdooQ Bioscience (Z-DEVD-FMK) (Irvine, CA, USA) and used as described in Rose et al. (2003). Cells were pre-treated for 1 hour with 75µM of pan-caspase inhibitor Z-VAD-FMK or caspase 3 inhibitor Z-DEVD-FMK. After 1 hour, cells were then treated with Se compounds and viability determined at 24 hours using the crystal violet method as described above.

2.5.5 Measures of Cleaved PARP via ELISA

For the determination of PARP, cleaved PARP was assessed in cell lysates using a commercial ELISA kit KH00741 (Invitrogen by Thermo Fisher Scientific) following the

manufacturer's instructions. In brief, cells were washed in PBS before being scraped into 200µl of PBS and centrifuged at 8,000xg for 10 minutes, with the resulting supernatant being discarded. Cells were then lysed in lysis buffer containing 10mM Tris pH 7.4, 100mM Sodium chloride, 1Mm EDTA, 1mM EGTA, 1mM Sodium fluoride, 20mM sodium pyrophosphate, 2mM Sodium orthovanadate, 1% Triton X-100, 10% glycerol, 0.1% SDS and 0.5% deoxycholate on ice for 30 minutes. Cells were then centrifuged at 13,000xg for 10 minutes at 4°C and supernatant retained and stored at -80°C until used. Standards of cleaved PARP were prepared in a serial dilution of 10ng/ml to 0ng/ml as per the manufacturer's instructions. 50µl of 1:5 dilution of cell lysate samples and standards were added per well into the coated ELISA plate before cleaved PARP detection antibody was added and the plate was incubated at room temperature for 3 hours on an orbital shaker, shaking at 500rpm. The plate was then aspirated multiple times before anti-rabbit IgG HRP was added and the plate was incubated for a further 30 minutes at room temperature. The plate was then aspirated multiple times again before stabilised chromogen was added to the wells and the plate was incubated for 30 minutes at room temperature in the dark. A stop solution was then added and the plate was read at 450nm on a BIORAD plate reader (680XR Microplate reader). Background values were subsequently deducted from the standards and samples and a 4-parameter algorithm was used to fit the standard curve. Cleaved PARP values were deduced for the samples from this standard curve and multiplied by the dilution factor of 5. Cleaved PARP was then normalised to protein levels via the Lowry assay (2.6.2).

2.5.6 Measures of intracellular ROS using Dichlorodihydrofluorescein diacetate (DCFDA)

DCFDA is a cell permeable ester of the dye fluorescein. Once taken up into cells, it is cleaved via esterases to form non-fluorescent H_2DCF, which is oxidised in the presence of ROS and generates fluorescein producing green fluorescence (Ng and Ooi, 2021, Sun et al., 2018a).

Measures of intracellular ROS production were determined using DCFDA (Vincent et al 2004) In brief, 50µl of 100µM of DCFDA working solution was added to each well of a 96 well plate and cells incubated for 1 hour at 37°C and 5% CO2. The DCFDA solution was then removed and cells were washed with Hank's Balanced Salt Solution (HBSS), (140mM Sodium chloride, 5mM Potassium chloride, 1mM Calcium chloride, 400µM Magnesium sulfate, 500µM Magnesium chloride hexahydrate, 300µM Monosodium phosphate, 400µM Monopotassium phosphate, 6mM D-Glucose (Dextrose) and 4mM Sodium bicarbonate).

Cells were treated with individual Se compounds or 1mM of H_2O_2 diluted in HBSS. H_2O_2 serving as the positive control (Sun et al., 2018a). Fluorescence was measured using the FluoStar Omega fluorescent plate reader (485/520nm) at 0, 30, 60 and 120 minute time points as conducted in previous literature (Park, 2016). Data was exported to excel, where background fluorescence (cell free + HBSS) was deducted from all measurements of fluorescence.

2.5.7 Measures of Lipid peroxidation

For the determination of lipid peroxidation the commercially available Image-It™ Lipid Peroxidation Kit (C10445 -Invitrogen by Thermo Fisher Scientific) was used.

Upon oxidation of lipids the peak emission shifts from red (~590nm) to green (~510nm). In brief after each treatment incubation time had passed, media was removed and cells were washed twice with PBS before 1ml of 10µM lipid sensor was added to each well and plates were again incubated for 30 minutes. After incubation, the lipid sensor was removed and cells were again washed twice with PBS, and 1ml of PBS was added per well to prevent dehydration. One at a time, each coverslip was removed from the well and placed on a slide for imaging. Images were taken on transmission, green fluorescence and red fluorescence of an EVOS microscope. Settings were kept the same for all images and all three independent repeats.

2.6 Western Blotting-

2.6.1 Protein Extraction

Following experimental treatments, the media was removed from cells and washed 3 times in 1x PBS. Following this, 200µl of freshly prepared lysis buffer containing 150mM NaCl, 50mM Tris HCl, 1% Triton X-100, 0.1% SDS and 1x/10ml protein inhibitor cocktail (Roche cOmplete Tablets, Mini EASYpack-04693124001) was added. Cells were then incubated on ice for 10 minutes to allow for lysis, and a 10µl sample checked under a haemocytometer to ensure cells had fully lysed. Cell lysates were then centrifuged at 15,000rpm for 15 minutes at 4°C, and the supernatant stored at -80°C until required as detailed in (Bio-Rad, 2012).

2.6.2 Protein Quantification Assay

Protein content of cell extracts was determined using the Lowry assay as described (Lowry et al., 1951). BSA was prepared in dH_2O and used to construct a standard curve and a concentration range of standards were prepared ranging from curve (1.2, 1, 0.8, 0.6, 0.4, 0.2 to 0mg/ml) respectively. Stocks of reagent 1 containing 5ml of 2% Na_2CO_3, 500µL of 1% $CuSO_4$ and 500µl of 2% potassium sodium tartrate and reagent 2 containing 500µl of Folin Ciocalteu phenol reagent and 5ml of NaOH were made. 50µl of 1:5 dilution of protein extract samples and standards were added to the wells in triplicate. 150µl of 0.1mM NaOH was added to all wells followed by 50µl of reagent, the plate was tapped to mix and incubated at room temperature for 5 minutes. After 5 minutes, 50µl of reagent 2 was added to each well and the plate was then incubated for a further 20 minutes before the plate was read at 655nm using the BIORAD plate reader (680XR Microplate reader).

2.6.3 Western blot analysis of cell lysates

Cell protein extracts were normalised to ensure equal loading of samples and western blotting was carried out as detailed per Bio-Rad (2012). Extracts (2mg/ml, 30µl total volume) were mixed with 10µl of 2x SDS-DTT mix (stock solution (10ml) contained 2ml glycerol, 1M Tris/HCl, 10% SDS solution, 0.154g DTT and a few grains of bromophenol blue) and were boiled at 95°C for 5 minutes to denature the proteins, and then centrifuged at 3000rpm for 3 minutes (Biofuge 13, Heraeus Instruments) to remove cell debris. Then 30µl/well of each sample was loaded into a 12% SDS page gel (Criterion™ TGX Stain-Free™ Precast Gel- BIORAD) submerged in a 1x running buffer contained 100ml of 10x Tris/Glycine/SDS running buffer (National Diagnostics) and 900ml of MilliQ water. 5µl of rainbow marker (Amersham™ ECL™

Rainbow™ Marker- High range (PRN756E)) was used for the protein ladder. All respective gels were run at 200 V for 45 minutes at room temperature. Gels were then transferred to pre-methanol wetted PVDF membrane (Amersham ™ Hybond™ 0.2µm Cat.No 10600021). The cassettes were placed into a tank (BIO-RAD TRANS-BLOT ® CELL) containing transfer buffer (30g Glycine, 3g Tris, 50ml Isopropanol and 950ml of MilliQ water), and ice blocks were used to prevent overheating of the transfer solution. Transfer was run at a constant current of 350mA for 2 hours. After transfer the PVDF membrane was removed and pre-stained using ponceau stain to confirm protein transfer onto the PVDF membranes.

Following removal of the ponceau stain, the membranes were blocked in 5% milk dissolved in 1xTBST (7.2g NaCl, 16ml 1M Tris pH 6.5, 1ml of tween-20 and 983ml of MilliQ water) for 1 hour, prior to the addition of the primary antibody. Membranes were probed overnight at 4°C (as per the dilutions noted in Table 1). Following blocking, membranes were then washed in 1xTBST (3X) for 5 minutes and then incubation with the secondary antibody at room temperature. Membranes were again washed in 1xTBST (3x) for 5 minutes. After washing, bands were visualised using ECL reagent (SuperSignal™ West Dura Extended Duration Substrate). The membrane was then incubated in the dark for 1 minute prior to being imaged on BIORAD ChemiDOC™ MP Imaging System. β-actin (A2066, Sigma) was used as the reference protein in all work. Relative band intensity was determined via densitometry calculated using ImageJ (version 64) software (NIH), as described in Stael et al. (2022). Blots were normalised to β-actin.

Table 2-1 Table of Antibodies, animals produced in, dilutions and company obtained from.

Antibody	Produced in…	Dilution	Product Code	Company
SelP (Anti-SePP1)	Rabbit	1:1000	SAB2103123	Sigma-Aldrich
GPx4 (Anti-Glutathione Peroxidase 4)	Mouse	1:500	MABF1969	EMD Millipore Corp.
SCLY (Anti-selenocysteine Lyase)	Rabbit	1:2000	CQA5827	Cohesion Biosciences
CBS (Anti-CBS)	Mouse	1:2000	MABS518	EMD Millipore Corp.
CSE (Anti-CTH)	Rabbit	1:1500	HPA023300	Sigma-Aldrich
Caspase 3	Rabbit	1:1000	D3R6Y	Cell Signalling
Caspase 8	Rabbit	1:1000	D35G2	Cell Signalling
β-actin (Anti-Actin)	Rabbit	1:1000	A2066	Sigma-Aldrich
Anti-Rabbit 2° (ECL™ Anti-rabbit IgG Horseradish Peroxidase Linked Whole antibody)	Donkey	1:5000	NA934	Amersham
Anti-Mouse 2° (Anti-mouse IgG Horseradish Peroxidase Linked Whole antibody)	Sheep	1:5000	NXA931	Amersham

2.7 Statistical Analysis

All data was inputted to Microsoft® Excel® for Microsoft 365 and subsequently organised to the correct formatting. All graphs were drawn using Graph Pad Prism 9. Statistical analysis was conducted in IBM SPSS Statistics 27.

3 Selenium accumulation in commercial garlic (*Allium sativum*) cultivars.

3.1 Abstract

Selenium (Se) is an essential micronutrient key to human health. Over the past 30 years Se intake in the UK population has been on the decline and is estimated to be at half the recommended daily allowance of 60µg for women and 75µg for men Se/day respectively. Alliums are a good candidate for biofortification as they are widely consumed, store well, and are added to a wide range of food products. To date, only a few studies have focused on members of the Allium family such as onion and garlic. Our aim was to assess the efficacy of Se foliar application on five commercial garlic cultivars grown under standardised polytunnel conditions, where growth parameters and climatic conditions were monitored throughout. Se was applied as sodium selenate at 0µM, 25µM or 50µM via controlled foliar application. Results showed Se enrichment via foliar application had no significant impacts on the majority of growth measures ($p>0.05$), although significantly increased pseudostem diameter and increased/decreased clove weights were seen with increasing Se application in certain cultivars ($p<0.05$) although these did not significantly affect overall growth measures such as bulb weight ($p>0.05$). Foliar Se application did not significantly impact on the accumulation of other macro and micronutrients in clove and shoot tissues ($p>0.05$). However, in our hands, foliar application led to minimal

and insignificant increases in Se accumulation ($p>0.05$) and significantly impacted on clove tissue antioxidant capacity Se across all cultivars ($p<0.001$).

3.2 Introduction

Humans require 22 mineral elements including the macronutrients, N, P, S, Ca, Mg, K, Na, Cl, and the micronutrients Zn, Fe, F, Mn, Cu, I, Se, Mo, Cr and Co (White and Broadley, 2009). Of these, deficiencies in Fe, Zn, Cu, Ca, Mg, Se and I_2 are common in the diets of humans (White and Broadley (2009). Selenium (Se) plays important roles in redox based metabolic systems including the immune systems, antioxidant and cytoprotective defences (Radomska et al., 2021). Globally, Se concentrations (both inorganic and organic sources) are low in most soils, and this results in a lack of Se in crop plants for human consumption. Consequently, since the 1970's, dietary Se intake has decreased from a mean of 63 µg/day to 48 µg/day in 2006 (FSA, 2009, Ysart et al., 1999). This has resulted in a reduction in the mean plasma Se concentration in humans (Rayman, 1997). Low levels of Se in the human body could also impact on antioxidant systems such as glutathione, a key selenoprotein and antioxidant, which could increase the risk of developing health conditions such as asthma or increasing the severity of pre-existing conditions (Fitzpatrick et al., 2012).

Low serum or plasma Se concentrations are common worldwide, and deficiencies are associated with increased risked of mortality (Akbaraly et al., 2005, Lauretani et al., 2008). Numerous epidemiological, animal, and human intervention studies have shown clear links between Se status and cancer incidence (Kok et al., 1987, Kornitzer et al., 2004, Narod et al., 2019), immune function (Hoffmann and Berry, 2008), obesity (Zhong et al., 2018), fertility (Qazi et al., 2019), and several other non-communicable diseases (reviewed in Rayman (2020)). These studies have led to efforts focused on increasing Se intakes in the general population via

supplementation of livestock feeds (Juniper et al., 2008) and inclusion of Se in fertilizers (Broadley et al., 2006).

The manipulation of micronutrient composition of crop is deemed a useful tool in targeting human malnutrition (Reviewed in Garg et al. (2018)). Biofortification of crops is now recognised as an efficient method to facilitate delivery of key nutrients including Fe, Zn, Mg and I_2 into the diets of humans (reviewed in White (2015))). Current biofortification strategies include soil fertilisation and foliar application (Reviewed in Puccinelli et al. (2017)). Interestingly, foliar application is emerging as preferred method of enriching crops with Se due to the lower amounts of Se fertilizer that need be applied compared to soil fertilisation (Puccinelli et al., 2020), thereby reducing the amount of Se that can leach and pollute groundwater (Yan et al., 2021). Foliar application remotes the negative interaction of Se with soil physicochemical properties like pH, presence of Fe and clay in the soil, and soil water characteristics that can influence Se root bioavailability (Kápolna et al., 2009). Foliar applied Se enters the plant by penetrating the leaf cuticle or via stomata (Saha, 2017) and was found in wheat to be transported to other areas within the plant by the phloem (Xia et al., 2020). Foliar application has been used in the biofortification of plants with Se in the following species, wheat (Delaqua et al., 2021, Wang et al., 2020a), rice (Boldrin et al., 2013, Hu et al., 2002), foxtail millet (Li et al., 2022b), broccoli (Šindelářová et al., 2015), carrot (Kápolna et al., 2009), tomato (Narváez-Ortiz et al., 2018, Zhu et al., 2018), potato (Zhang et al., 2019a), onions (Põldma et al., 2013) and basil (Kopsell et al., 2009).

Garlic (*Allium sativum*) is a globally important crop with an appealing flavour and history of therapeutic properties which is more commonly used today to add flavour to food (Nie et al., 2021). The current global garlic market is estimated to be worth $19 Bn and predicted to reach $32 Bn by 2031 (Anon, 2021). More than 28 million tons of garlic is produced each year with 20 million tonnes being produced solely in China (Polyakov et al., 2019). However, few studies have assessed Se enrichment of garlic (Ip et al., 1992, Slekovec and Goessler, 2005, Tsuneyoshi et al., 2006), and whether Se treatment impacts on growth, yield, and the quality of the bulbs. Even fewer have explored whether cultivar (cv.) differences influence Se accumulation in garlic tissues. This lack of research prompted the current study.

Aims and Objectives

Researchers at the University of Nottingham have a long-standing expertise of Se biofortification of crop plants across numerous genera. Therefore, the aims of the current research were to assess the impacts of foliar Se application on standard growth parameters and quality measures of five commercial cultivars namely, Marco (softneck), Mersley (softneck), Solent (softneck), Lyubasha (hardneck) and Bulbils (hardneck).

Hypothesis

We hypothesise that the foliar application of Se would positively impact on level of Se accumulated within garlic plants, plant growth and quality (antioxidant capacity) of garlic plants and bulbs.

3.3 Materials and Methods

3.3.1 Chemical and plant materials

All chemicals used were of the highest purity as detailed in 2.1.1. Chemicals specific to this chapter such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Trolox and potassium persulfate were obtained from Sigma (St. Louis, USA).

Regarding plant materials Mersley, Lyubasha, Solent and Bulbils were kindly supplied by The Garlic Farm, Isle of Wight (https://www.thegarlicfarm.co.uk/) and Marco was obtained from Taylor & Sons Bulbs Ltd, Lincolnshire in October 2020. Garlic cloves were randomised and weighed before planting.

3.3.2 Garlic cultivation and Se Treatments

Due to the impacts of COVID restrictions, this study elected to grow all plant tissues under standardised polytunnel conditions at the University of Nottingham, Sutton Bonington Campus, Sutton Bonington LE12 5RD. All garlic cultivars including Marco, Mersley, Solent Lyubasha and Bulbils were planted on 5/11/2020. Cloves were placed individually in 2L pots filled with Levington M3 Compost (pot and bedding-high nutrient). Using GenStat (17th Edition, VSNi), a randomised plot was designed, and the randomised blind block maintained for the duration of the study. Garlic cloves (n = 5 per treatment, per cultivar), totalling 75 garlic plants were grown in a 15 pot per block pattern, 3 pots per variety in each block (Figure 3.1). 1 spare pot of each respective garlic variety was also planted per cultivar in case of losses. Plants were watered once a week until February, and then twice per week until Se treatment in May 2021.

Prior to foliar application, garlic plants were removed from their randomised design by glasshouse staff blinded to the block design pattern, and these plants were placed into treatment groups ready for Se application. Cultivar information was not known by the glasshouse worker. This approach was used to avoid unblinding the trial and therefore maintaining the blinded nature of the experiment from the researchers. Each treatment group was (120 cm x 72 cm). Sodium selenate (Na_2SeO_4) was chosen for Se treatment as this inorganic source is widely used across numerous edible plant species (Tsuneyoshi et al. (2006), Põldma et al. (2011) and Hurst et al. (2010)). Na_2SeO_4 was diluted with water to attain 25µM and 50µM concentrations of Se, and applied at a rate of 86.8ml/m^2 using a Vermorel 2000 PRO Berthoud Jardin knapsack sprayer as previously described Hurst et al. (2010). Spraying rates were controlled using a MOREYES Mini digital Metronome. Control plants were sprayed with tap water at a rate of 86.8ml/m^2. A total of 150ml was sprayed per treatment area, covering over 25 plants respectively. Plants were treated on 10/05/2021 and again two weeks later on 24/05/2021. The treatment levels were as follows: control (0 µM), medium (25 µM sodium selenate) and high (50 µM sodium selenate).

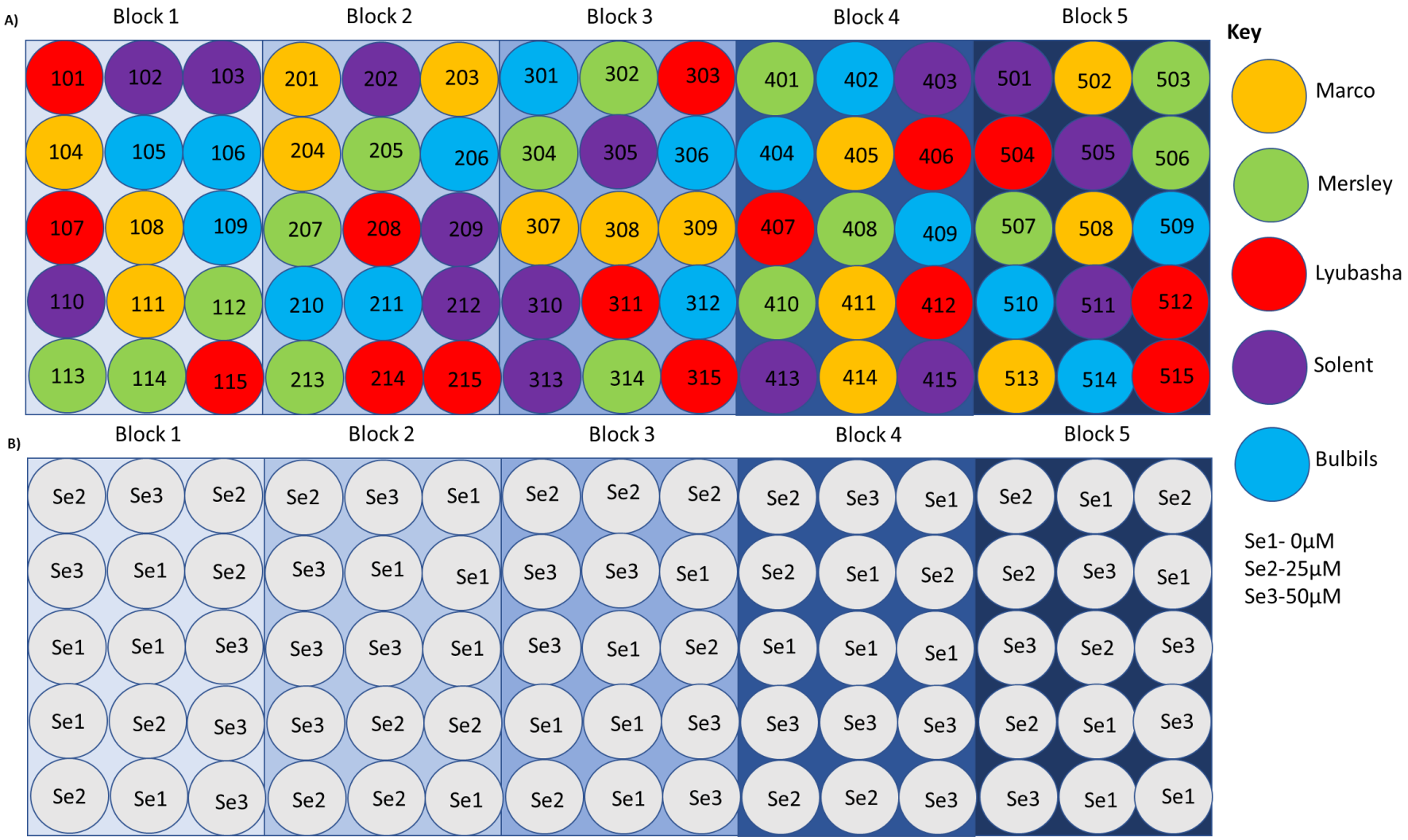

Figure 3.1- Randomised and Blinded Layout of polytunnel Se enrichment trial. A) Randomised chart showing location of cultivars within the randomised pattern. B) Shows Se treatment levels in the randomised plots.

3.3.3 Determination of growth data following harvest

Garlic plants were harvested on the 20/07/2021 (after leaf senescence had occurred). On harvest day multiple measurements were taken (similar to Põldma et al. (2011) and Brewster (2008)) including growth period, vegetative weight, bulb weight and bulb diameter, clove weight and number of cloves. Bulb diameter was measured using digital callipers (Workzone digital caliper). Bulb weight, clove weight, vegetative weight (weighed pot, soil and plant when harvested green tissue, then weighed pot and soil on harvest day once the senescent plant had been removed) using a SALTER portable balance. Harvest calculations were as follows:

$$Growth\ period\ (days) = harvest\ date - emergence\ date$$

$$Bulbing\ Index = \frac{bulb\ diameter\ (mm)}{pseudostem\ diameter\ (mm)}$$

(Guevara-Figueroa et al., 2015)

Yield was estimated by

a) $Area\ of\ pot = \pi r^2$

where r= diameter (in this case 8.5cm)

$Area\ of\ pot = \pi 8.5^2 = 226.98 cm^2\ (0.022698 m^2)$

b) Number of pots in a hectare (10,000m^2)

$$Number\ of\ pots\ in\ a\ hectare = \frac{ha\ (m^2)}{area\ of\ pot\ (m^2)}$$

$$Number\ of\ pots\ in\ a\ hectare = \frac{10{,}000 m^2}{0.022698 m^2} = 440{,}567.45$$

c) Yield of garlic (g) per hectare

$$Yield\ of\ garlic\ per\ hectare\ (g/ha)$$
$$= average\ weight\ of\ bulb\ (g) x\ number\ of\ pots\ per\ hectare$$

Average weight of bulb differed between cultivars (Marco 28.25g, Mersley 44.38g, Lyubasha 60.14g, Solent 47.06g)

d) converting from g/ha to tonnes/ha

$$Yield\ (tonnes/ha) = yield\ of\ garlic\ (g/ha)/1{,}000{,}000$$

3.3.4 Assessment of micronutrient composition using ICP-MS

Samples were freeze dried and ground into a powder as described in 2.2.1, prior to acid digestion and ICP-MS analysis 2.3.1. Apart from Se, other key macro and micronutrients were chosen for analysis because of their importance to plant growth and necessity in human health. These included Na, Mg, P, S, K, Ca, Mn, Fe, Cu and Zn.

3.3.5 Determination of the antioxidant status of leaf tissues.

Antioxidant status of leaf tissues were determined as described by Kasote et al. (2019) using the ABTS assay developed by Miller et al. (1993). In brief, 1ml of ABTS working solution was placed into each well of a 24 well plate containing an individual leaf disc and absorbance measured at 0, 5, 10, 15, 20 mins at 734nm. A time course was incorporated to confirm time dependant scavenging of the ABTS radical, although figure only displays 20 minute endpoint. The percentage of ABTS radical scavenging activity was determined using equation 1 below.

Equation 1.

$$\%\ radical\ scavenging$$

$$/FW\ of\ leaf\ disc: \left(\frac{Acontrol - Asample}{Acontrol}\right)\ x\ \left(\frac{100}{FW\ of\ leaf\ discs\ (mg)}\right)$$

Where, $A_{Control}$ was the absorbance of the reagent control and A_{Sample} was the absorbance of the leaf disc suspended reagent solution at the relevant time point.

For assessment of total antioxidant capacity in clove tissues, methanol/water extracts were first made using freeze dried and ground tissues as defined in 2.2.2.

Extracts of garlic were diluted to 50mg/mL before 10µl of extract was added to a 96 well plate and 190µl of ABTS working solution was added. Readings were taken on BioRad Model 680XR microplate reader at 0, 5, 10, 15 and 20 minute time points at 734nm. Absorbance of samples was divided by absorbance of control and multiplied by 100 to give radical scavenging (% ctrl). Figures only display 20 minute endpoint.

3.3.6 Statistical Analysis

Data was investigated using One-way ANOVAs (for comparing 3 or more groups) followed by post-hoc Tukey test. Two-way ANOVAs were used to assess if there was a significant interaction between two independent variables (eg. Se treatment x cultivar).

3.4 Results

The growth trial was conducted between 5/11/2020 and 20/07/2021. Local environmental conditions were monitored across the whole growth period of the garlic plants. These data are summarised in Figure 3.2A-C. The mean annual temperature across the growing season was 17.3°C ± 8.9. Where the annual temperature fluctuated from a mean minimum temperature of 0.9°C during January and elevated to 33°C in July. Day length paralleled seasonal temperature fluctuations and increased from a low of 7.56 hours in Dec to a high of 16.79 hours in June as the experiment progressed. Relative humidity decreased in the polytunnel from 98.5% in January to 68.7% in July.

During the growth period leaf tissues emerged in December following the cultivar order of Marco, Mersley/Solent and Lyubasha/Bulbils.

At harvest growth measures were assessed to, 1) assess variation between the growth characteristics of the garlic cultivars, and 2) to assess the impact of the general growing condition and application of Se on plants *viz. v*egetative weight, number of leaves, plant height, bulb diameter, bulb height, bulb weight, pseudostem diameter, clove number and clove weight. These assessments were conducted since the cultivars of Lyubasha and bulbils are not currently grown on a commercial scale but are of significant interest to producers.

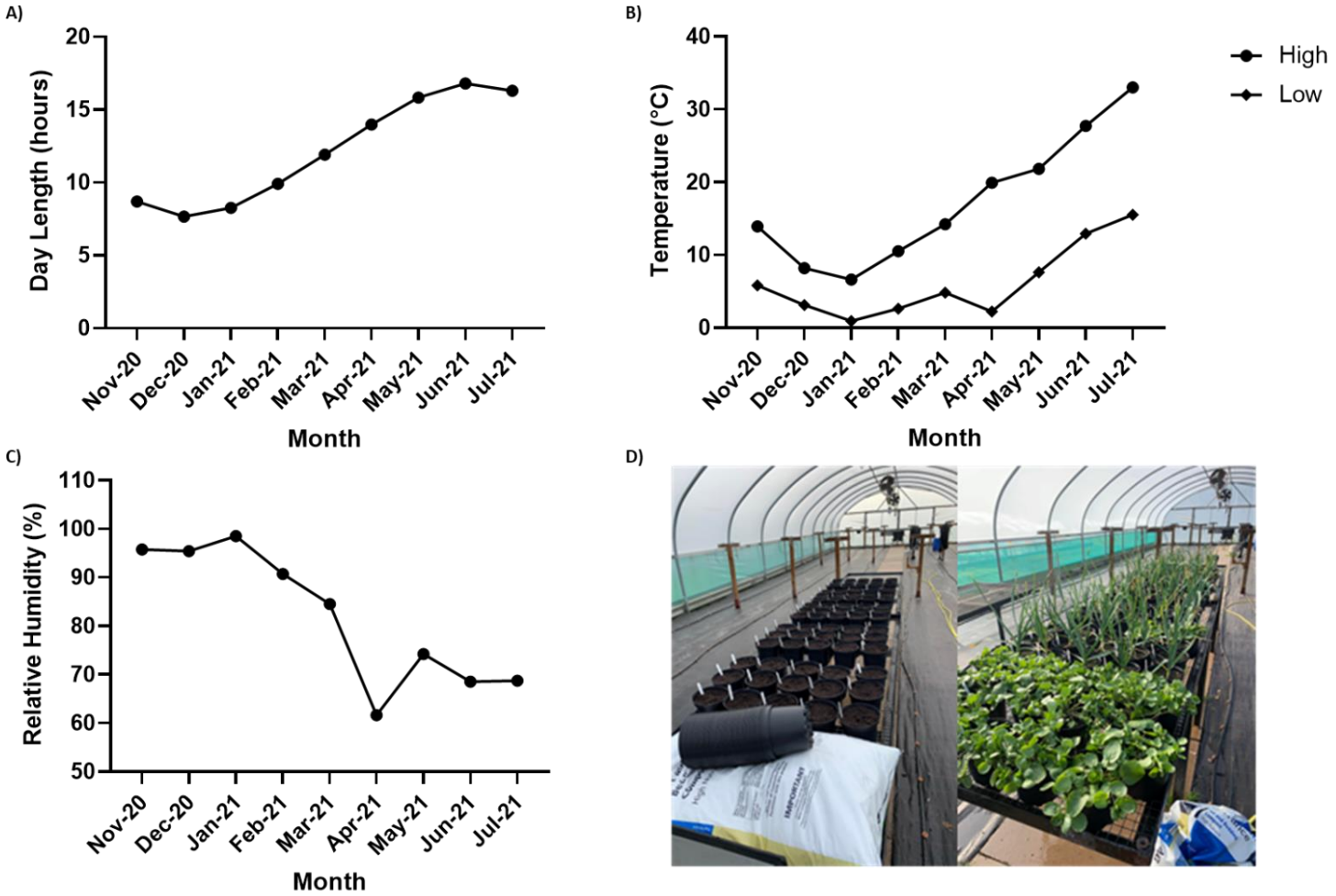

Figure 3.2-Day length and weather characteristics over the course of the trial 2020-2021, A) average day length in hours per month, B) average temperature high/low (°C) per month, C) average relative humidity (%) per month and, D) images showing growth of garlic plants from November 2020 (left) to May 2021 (right).

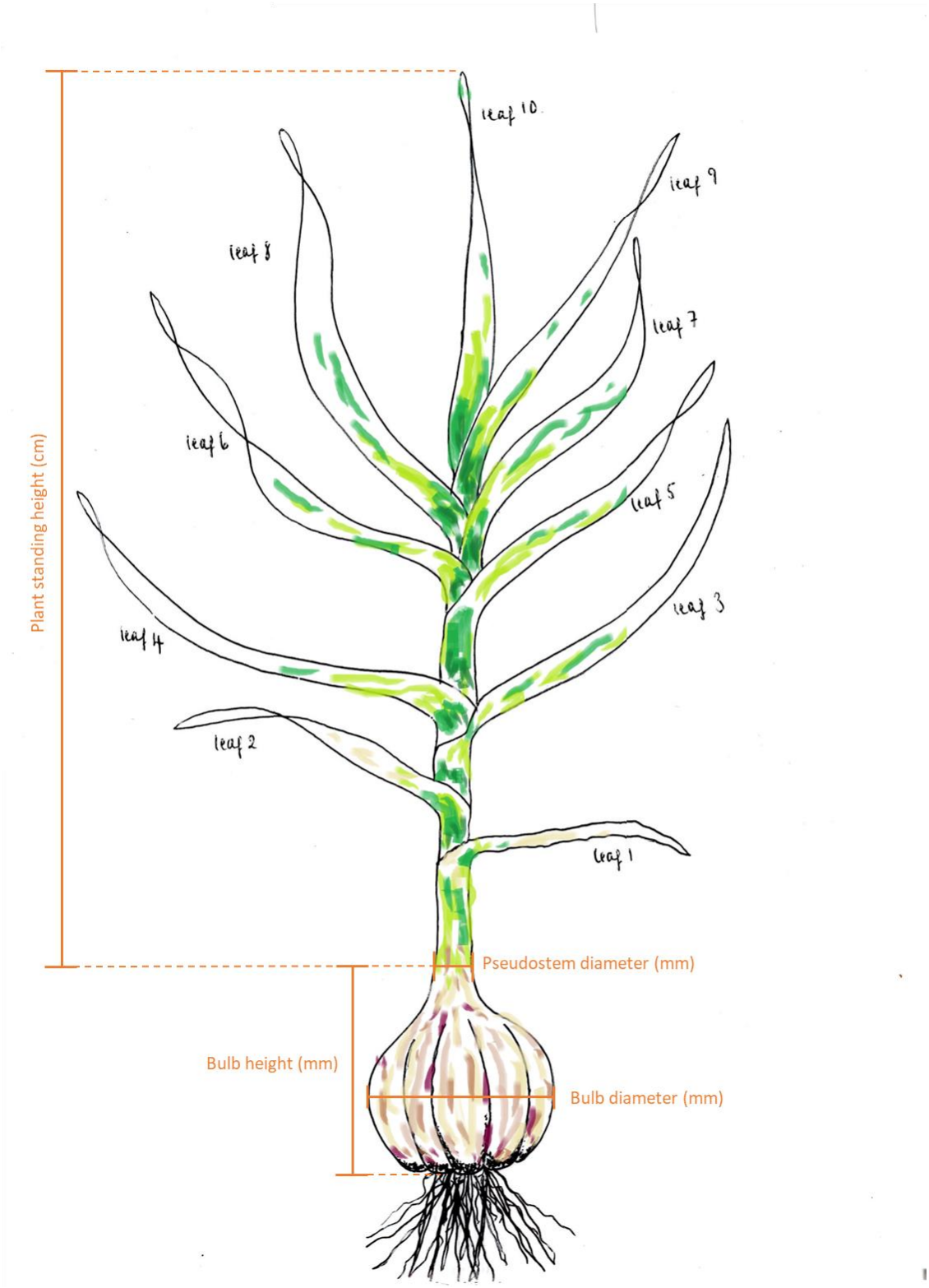

Figure 3.3- Drawing showing measures of harvest including, plant standing height (cm), Pseudostem diameter (mm), bulb height (mm) and bulb diameter (mm) (Section 3.3.3)

3.4.1 General growth across the trial

Plants began to grow rapidly from the months of March and April (Figure 3.2D). Measures including plant height and number of leaves were taken at monthly intervals to show the general growth habits of the cultivars on trial (Figure 3.4A and B). The cultivar Marco appeared to have the fastest start, displaying a trend of the greatest plant height and number of leaves up until March, after this point the hardneck cultivars Lyubasha and Bulbils had the greatest plant height until May where the other softneck cultivars, Solent and Mersley caught up and surpassed the hardneck cultivars. The cultivars Marco and Lyubasha had the greatest number of leaves month by month from February up until the month of June, where leaf number decreased for these cultivars as leaves began to senesce, perhaps suggesting that these cultivars reached maturity faster than Mersley, Solent and Bulbils.

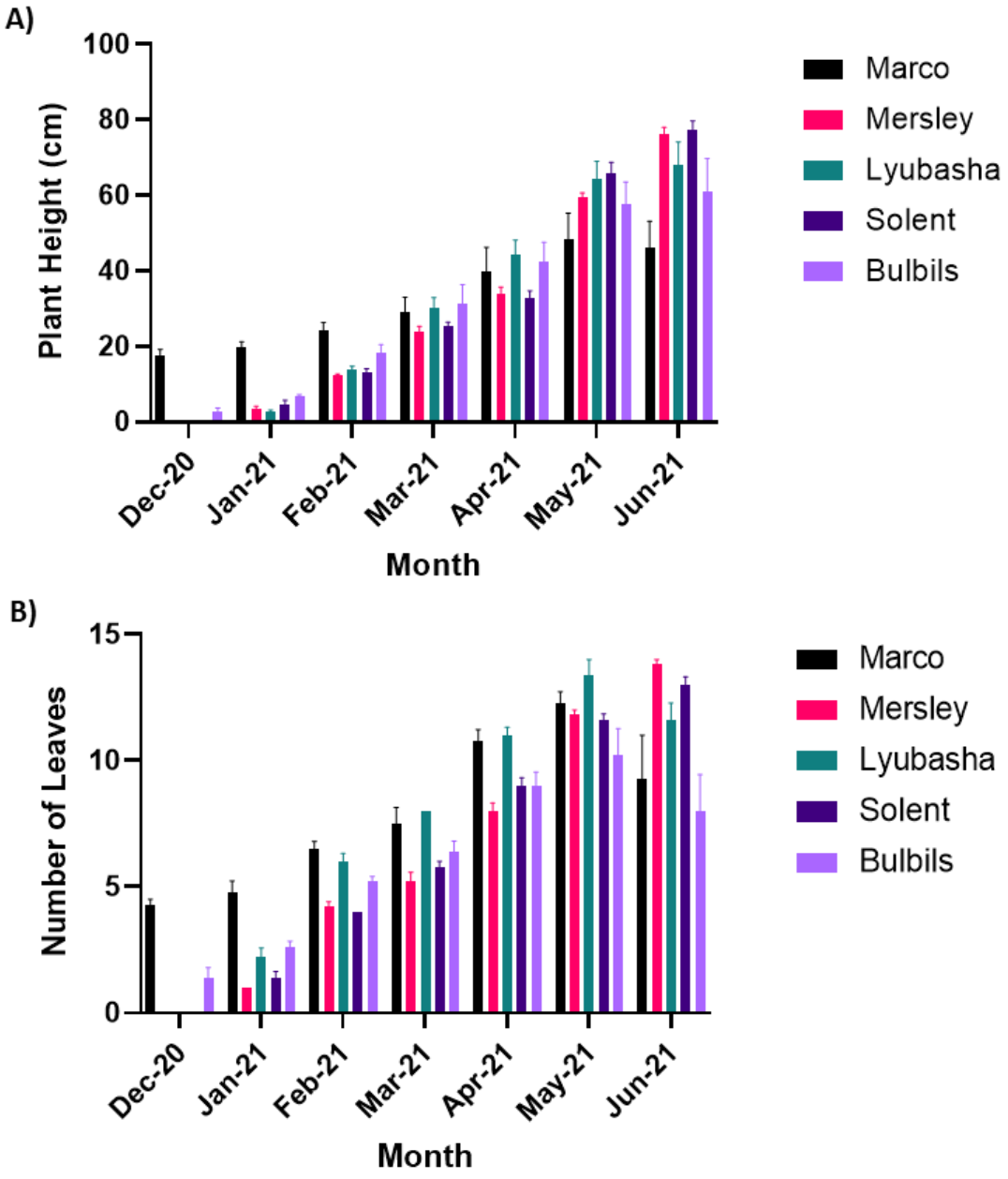

Figure 3.4-Monthly measures of A) plant height (cm) and, B) number of leaves for each cultivar on trial taken every month from emergence (December) until just before harvest. Data is representative of means ± standard error (n=5).

3.4.2 Variation in above ground growth characteristics between cultivars

The growth period of the garlic cultivars ranged from 208 days to 250 days, with the cultivar Marco having the longest growth period, due to an earlier emergence date,

as compared to the other cultivars on trial ($p<0.05$) (Figure 3.5A). The cultivar Bulbils also displayed a longer growth period than the cultivars Mersley, Lyubasha and Solent ($p<0.05$). At harvest vegetative weight ranged from 421g to 520g across the experiment with the cultivars, Mersley, Solent and Lyubasha tending to have a greater vegetative weight compared to Marco and Bulbils, although this was not significant (Figure 3.5B). Leaf number per plant ranged between 10-14 leaves per plant with Mersley, Lyubasha and Solent having a similar number of leaves, 13/14 at harvest. However, the cultivars Bulbils and Marco had significantly less leaves (10 and 11 respectively) as compared to the other cultivars ($p<0.05$)(Figure 3.5C). Plant standing height ranged from 49.75-76.21cm, however a general trend was observed of Mersley/Solent > Lyubasha/Bulbils > Marco, with Marco being significantly shorter in comparison to the other cultivars ($p<0.000$)(Figure 3.5D).

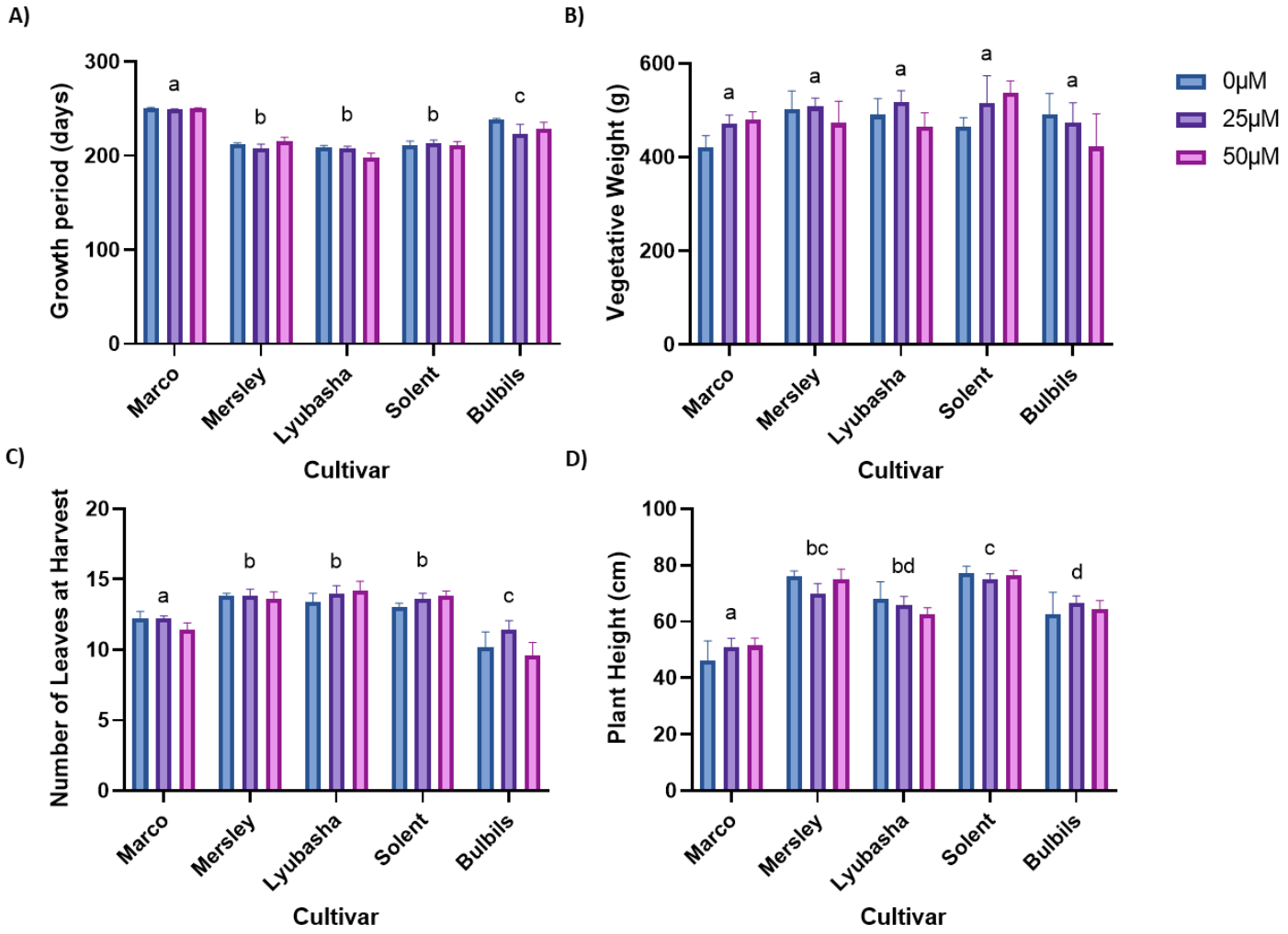

Figure 3.5-Above ground measures taken at harvest A) growth period, B) vegetative weight (g), C) number of leaves at harvest and, D) plant height (cm). Data is representative of means ± standard error (n=5). Mean values followed by the same letter are not significantly different at $p<0.05$ as determined between cultivars determined via one way ANOVA followed by post-hoc Tukey test.

3.4.3 Variation in bulb characteristics between cultivars

In general bulb diameter ranged from 46.17mm to 55.31mm. The cultivar Lyubasha was found to have significantly larger bulb diameter as compared to Marco and Bulbils ($p=0.001$ and $p=0.007$ respectively) (Figure 3.6A). Bulb height ranged from 32.40 to 43.24cm, Lyubasha was again found to have significantly greater bulb height as compared to Marco and Bulbils ($p=0.012$ and $p=0.021$ respectively) (Figure 3.6B). Bulb weight ranged from 29.64 to 63.00g, Lyubasha was found to have a significantly heavier bulb weight as compared to the cultivars Marco, Mersley and Bulbils ($p<0.05$)(Figure 3.6C). This suggests that the cultivar Lyubasha to have significantly larger bulb proportions compared to the cultivars Marco and Bulbils which may be of interest to commercial garlic growers. There was no significant difference pseudostem diameter or bulbing index between the cultivars. In general, the pseudostem diameter ranged from 5.27 to 12.17mm and bulbing index ranged from 4.39 to 10.85 (Figure 3.6D and E).

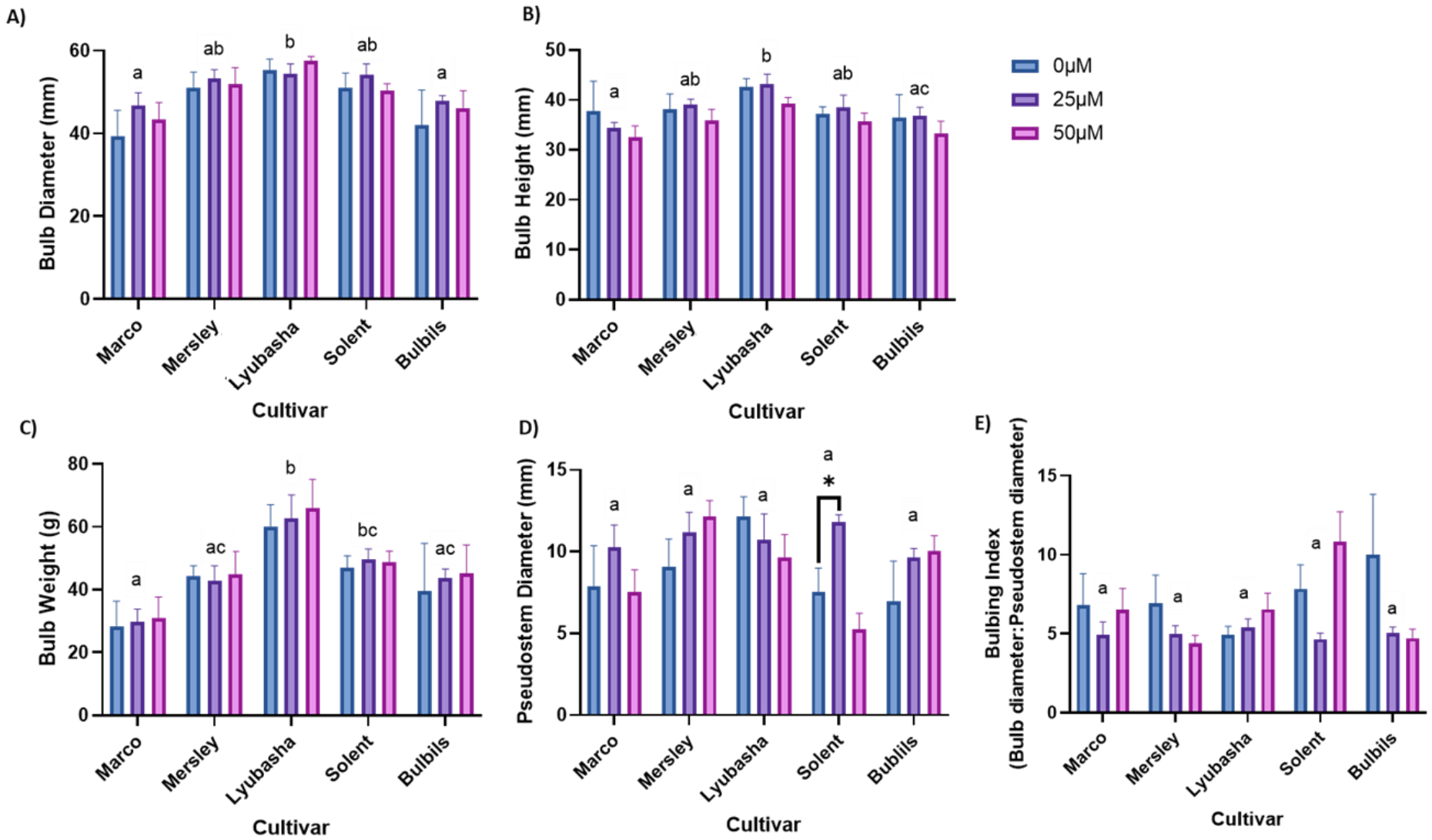

Figure 3.6- Bulb measures taken at harvest , A) bulb diameter (mm), B) bulb height (mm), C) bulb weight (g), D) pseudostem diameter (mm) and, E) bulbing index (bulb diameter:pseudostem diameter). Data is representative of means ± standard error, (n=5). Mean values followed by the same letter are not significantly different between cultivars, * signifies $p < 0.05$ between Se treatments within a cultivar, both were determined via one way ANOVA followed by post-hoc Tukey test.

3.4.4 Variation of clove characteristics between cultivars

The number of cloves per bulb ranged from 4.8 to 15 across the cultivars, in particular the cultivars Mersley and Solent had a significantly greater number of cloves compared to Marco, Lyubasha and Bulbils ($p<0.05$)(Figure 3.7A). However, the cultivars Mersley and Solent both displayed significantly smaller clove weights (average 1.98g/clove and 2.51g/clove respectively) compared to the Marco (4.79g/clove), Lyubasha (7.36g/clove) and Bulbils (4.96g/clove) ($p<0.000$) (Figure 3.7B). Lyubasha was also found to have the greatest clove weight compared to all the cultivars on trial ($p<0.05$), again suggesting this to be the highest yielding cultivar of garlic out of the current cultivars on trial.

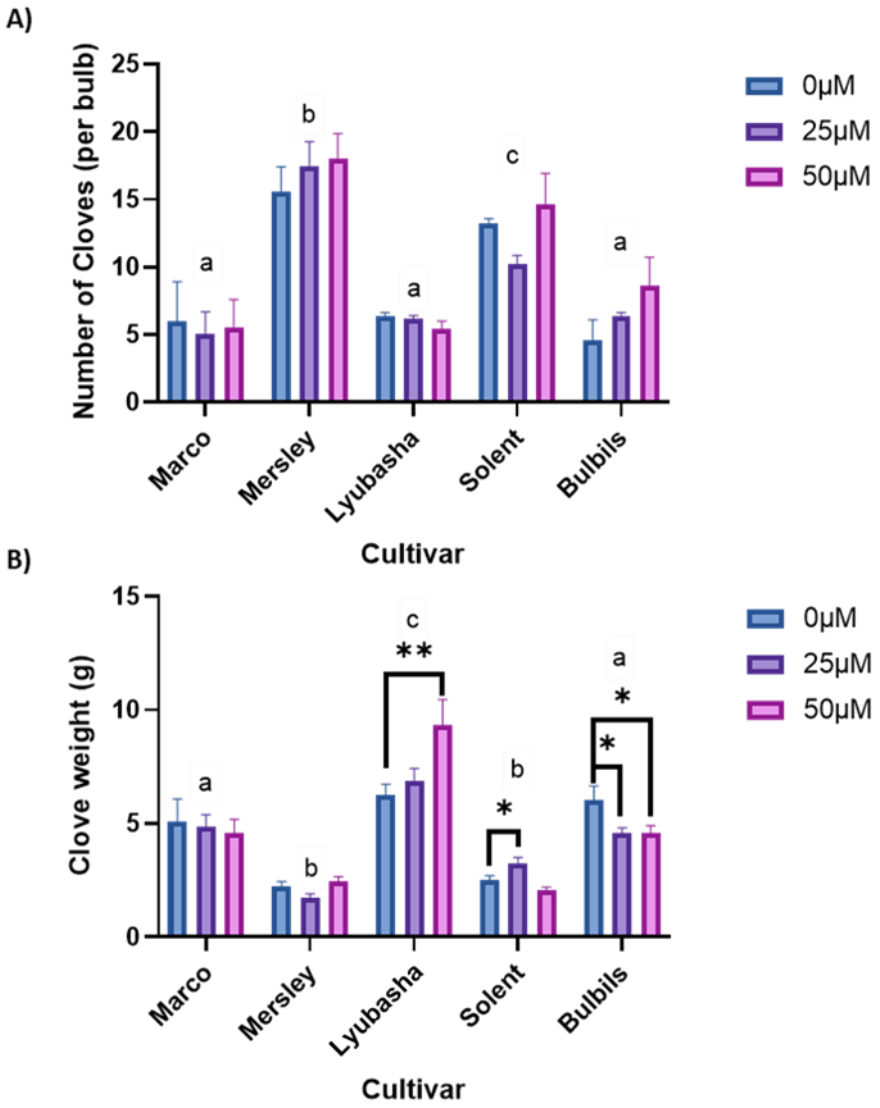

Figure 3.7-Clove measures taken at harvest , A) number of cloves per bulb and, B) clove weight (g). Data is representative of means ± standard error (n=5). Mean values followed by the same letter are not significantly different between cultivars, * signifies $p < 0.05$, ** $p<0.01$ between Se treatments within a cultivar, both were determined via one way ANOVA followed by post-hoc Tukey test.

3.4.5 Impacts of Se enrichment on growth parameters

Following assessment, there was no significant influence of Se application on the majority of above ground and bulb measures including growth period, number of leaves at harvest, plant standing height at harvest, vegetative weight, bulb diameter, bulb height, bulb weight and bulbing index of garlic plants ($p>0.05$) (Figure 3.5 and

3.6). Despite the lack of significant differences of Se application on growth measures some trends were observed with increasing Se application on bulb measures although this varied between cultivars. The application of 25µM Se marginally, but insignificantly, increased bulb diameter in all cultivars except Lyubasha. Bulb weight was also marginally, but again insignificantly, increased with increasing Se in Marco, Lyubasha, Solent and Bulbils. Bulb height however showed a decreasing trend with 50µM Se application for all cultivars. A mixed picture was seen in pseudostem diameter with some cultivars such as Mersley and Bulbils displaying a marginally but insignificantly larger pseudostem diameter with increasing Se application whereas other cultivars such as Lyubasha showed a downwards trend in pseudostem diameter. A significant increase in pseudostem diameter was found in the cultivar Solent at 25µM Se enrichment as compared to control ($p=0.034$), although 50µM enrichment did not result in similar significant increases as compared to control ($p=0.309$) (Figure 3.6D). This mixed picture of results likely contributed to the similar mixed picture seen in bulbing index (bulb diameter: pseudostem diameter).

Analysis of clove tissues were used to determine the impacts of Se on the edible parts of the garlic bulb. In this instance, size and abundance of clove tissues was determined. The number of cloves per bulb was not significantly impacted by the application of foliar Se ($p>0.05$) (Figure 6A). However, a significant two-way interaction was found between cultivar and Se treatment for clove weight ($p<0.001$). Significantly heavier cloves were found in Se treated tissues as compared to control for the cultivars Lyubasha and Solent at 50µM and 25µM Se enrichments respectively ($p=0.004$ and $p=0.033$ respectively) (Figures 3.7B). Interestingly in the cultivar Solent application of 50µM Se did not result in similarly enlarged clove

tissues as compared to control (p=0.170). In contrast to this Se enrichment of Bulbils resulted in significantly decreased clove weights at 25µM and 50µM as compared to control (p=0.030 and p=0.028 respectively). This suggests that the impact of Se treatment is concentration dependent and varies between cultivars. However, none of these significant differences whether it be increases or decreases in clove weight appear to influence the overall bulb weight (Figure 3.6C). Following the assessment of growth metrics, the mineral composition of clove and shoot tissues was analysed using ICP-MS.

3.4.6 Total Se and other macro and micronutrient content.

The average total Se content in the clove ranged from 0.064 to 0.128mg/kg DW and 0.034 to 0.114 mg/kg DW in the leaf (Table 3.1). There was a tendency for Se content to increase with higher Se applications in the cultivars Marco, Mersley, Lyubasha and Solent although significance was not achieved for clove nor leaf for any cultivars.

Table 3-1-Se content (mg/kg DW) of garlic tissues, clove and leaf for each cultivar under each treatment. Data is representative of means ± standard deviation (n=3).

		Se accumulation in garlic (mg/kg DW)				
Cultivar		Marco	Mersley	Lyubasha	Solent	Bulbils
0µM	Clove	**0.06** ± 0.01	**0.10** ± 0.09	**0.08** ± 0.03	**0.11** ± 0.03	**0.11** ± 0.01
	Leaf	**0.06** ± 0.01	**0.05** ± 0.01	**0.08** ± 0.02	**0.04** ± 0.01	**0.11** ± 0.05
25µM	Clove	**0.09** ± 0.04	**0.07** ± 0.01	**0.11** ± 0.02	**0.11** ± 0.02	**0.11** ±0.02
	Leaf	**0.08** ± 0.04	**0.04** ± 0.01	**0.07** ± 0.03	**0.03** ± 0.00	**0.05** ± 0.00
50µM	Clove	**0.11** ± 0.03	**0.10** ± 0.02	**0.12** ± 0.01	**0.09** ± 0.03	**0.12** ±0.05
	Leaf	**0.11** ± 0.04	**0.11** ± 0.09	**0.09** ± 0.06	**0.05** ± 0.01	**0.06** ± 0.02

In addition to Se, we also determined the levels of nutritionally important macronutrients, Na, Mg, P, S, K and Ca as well as micronutrients Mn, Fe, Cu, Zn in plant tissues since deficiencies in some of these macronutrients and micronutrients are common in human health, see table 3.2. Two-way ANOVAs were carried out to assess if there was a significant interaction between Se treatment and cultivar for the nutrients accumulated, however no significant interactions were found between Se treatment and cultivar for accumulation of any of the nutrients in clove or leaf tissues. Se enrichment resulted in no significant change in accumulation of the nutrients Na, Mg, P, S, K, Mn, Fe and Zn as compared to control for all cultivars (Table 3.2). However, enriching the cultivar Bulbils with 50µM Se, resulted in significantly increased levels of K and Cu ($p=0.038$ and $p=0.041$).

In parallel analyses of leaf tissues there was also no significant difference in Mg, P, S, K, Ca macronutrient and Mn, Fe, Cu, Zn macronutrient accumulation in the leaf tissues between Se treatments for the cultivars Marco, Mersley, Solent and Bulbils (Table 3.3). However, Se enrichment of the cultivar Lyubasha with 25µM Se, resulted in significantly increased Cu accumulation in leaf tissues as compared to control ($p=0.020$), although this was not reflected in 50µM Se enrichment ($p=0.884$).

The macro and micronutrient composition of clove tissues did however vary significantly between cultivars. In particular the cultivar Marco had significantly greater accumulation of Mg, P and Ca in clove tissues compared to the other cultivars ($p<0.05$), as well as significantly increased Mn, Fe and Zn accumulation compared to the cultivars Mersley and Solent ($p<0.05$). No significant differences were noted in the macro and micronutrient composition of leaf tissues between

cultivars. Interestingly, the accumulation of the majority of nutrients also differed between clove and leaf tissues, although this again varied between cultivars. In general, the nutrients Mg, P, K, Ca and Mn accumulated at a significantly higher level in leaf tissues as compared to clove tissues ($p<0.05$). Whereas clove tissues accumulated significantly greater levels of S and Zn ($p<0.05$).

Table 3-2- Macronutrient and micronutrient content in the cloves of Se enriched garlic cultivars (mg/kg DW) . Data is representative of means ± standard deviation (n=3). Significance calculated using one-way ANOVA and post hoc Tukey test and displayed as * $p < 0.05$ and indicates significant differences between Se treatments within a cultivar.

		Macronutrient (mg/kg DW)					Micronutrient (mg/kg DW)			
Cultivar	Treatment	Mg	P	S	K	Ca	Mn	Fe	Cu	Zn
Marco	0µM	**802.63** ±104.63	**5253.16** ±548.43	**5951.47** ±959.69	**20977.53** ±1809.70	**323.70** ±61.89	**10.66** ±0.80	**43.30** ±5.25	**0.65** ±0.06	**12.97** ±2.69
	25µM	**846.88** ±240.48	**6164.45** ±2142.00	**6584.89** ±2086.90	**20848.12** ±2980.00	**357.83** ±114.55	**9.63** ±1.96	**54.51** ±15.35	**0.70** ±0.23	**15.15** ±6.34
	50µM	**880.68** ±202.74	**5709.74** ±854.91	**6689.01** ±1056.08	**21748.46** ±2505.94	**376.28** ±99.80	**11.08** ±3.70	**47.47** ±4.23	**0.69** ±0.18	**14.90** ±2.72
Mersley	0µM	**520.71** ±37.15	**2850.26** ±120.42	**4561.78** ±319.08	**13092.53** ±689.53	**173.45** ±25.79	**6.85** ±0.43	**28.63** ±19.95	**0.57** ±0.02	**6.34** ±0.35
	25µM	**598.53** ±20.02	**3337.33** ±362.54	**5508.94** ±897.03	**14302.81** ±875.74	**197.68** ±5.722	**7.49** ±1.11	**31.53** ±23.90	**1.07** ±0.50	**6.94** ±1.85
	50µM	**632.68** ±202.10	**3408.19** ±765.16	**6425.77** ±1767.91	**13586.63** ±986.69	**311.77** ±204.71	**9.16** ±4.16	**29.18** ±5.00	**0.71** ±0.24	**8.51** ±2.04
Lyubasha	0µM	**570.54** ±17.38	**3275.63** ±211.06	**6093.76** ±555.44	**12553.52** ±890.38	**264.97** ±49.46	**8.61** ±1.03	**39.63** ±6.03	**0.90** ±0.43	**11.17** ±0.23
	25µM	**616.71** ±52.81	**3759.14** ±249.09	**7378.29** ±359.50	**13969.17** ±753.61	**216.34** ±6.31	**9.25** ±1.81	**39.34** ±6.72	**0.74** ±0.11	**12.75** ±1.95
	50µM	**558.27** ±41.61	**3357.80** ±496.52	**6474.34** ±1125.40	**13121.98** ±1870.03	**224.48** ±14.25	**7.66** ±0.73	**38.24** ±4.51	**0.67** ±0.17	**12.47** ±2.71
Solent	0µM	**595.31** ±10.62	**3331.75** ±422.46	**6040.69** ±1502.28	**13612.90** ±785.14	**220.65** ±9.76	**8.27** ±0.81	**31.42** ±16.29	**0.64** ±0.16	**10.53** ±2.61
	25µM	**543.90** ±32.18	**3351.45** ±46.83	**5210.31** ±532.86	**14011.36** ±277.64	**164.54** ±20.61	**6.72** ±0.44	**24.73** ±1.43	**0.58** ±0.52	**8.90** ±0.42
	50µM	**600.90** ±25.19	**3100.63** ±233.81	**5911.37** ±372.38	**14201.87** ±340.25	**236.14** ±40.41	**7.35** ±0.95	**23.07** ±0.51	**0.63** ±0.04	**10.08** ±1.05

Bulbils	0µM	**560.77** ±82.45	**3391.37** ±421.46	**5594.99** ±401.09	**12062.33** ±881.47	**225.25** ±14.99	**7.47** ±2.30	**37.58** ±3.09	**0.53** ±0.02	**10.83** ±0.45
	25µM	**571.45** ±72.24	**4431.23** ±308.41	**7049.35** ±1268.33	**14375.38** ±938.58	**222.56** ±28.14	**8.17** ±1.15	**41.30** ±3.88	**0.72** ±0.09	**12.95** ±1.90
	50µM	**640.17** ±87.12	**4287.18** ±965.53	**6597.72** ±1205.21	**15029.24*** ±758.14	**268.51** ±79.26	**9.65** ±2.69	**33.90** ±11.92	**0.74*** ±0.10	**11.56** ±4.23

Table 3-3 Macronutrient and micronutrient content in the leaf tissues of Se enriched garlic cultivars (mg/kg DW). Data is representative of means ± standard deviation (n=3). Significance calculated using one-way ANOVA and post hoc Tukey test and displayed as * $p < 0.05$ and indicates significant differences between Se treatments within a cultivar.

		Macronutrient (mg/kg DW)					Micronutrient (mg/kg DW)			
Cultivar	Treatment	Mg	P	S	K	Ca	Mn	Fe	Cu	Zn
Marco	0µM	**28726.50** ±16749.96	**7293.70** ±1642.71	**3331.86** ±1476.30	**21764.41** ±11429.20	**11478.08** ±4498.98	**224.28** ±64.07	**39.43** ±18.76	**1.18** ±0.41	**4.80** ±1.70
	25µM	**32292.92** ±10742.92	**13057.37** ±6415.65	**3676.28** ±1249.60	**23934.00** ±9983.43	**12327.54** ±2896.25	**216.79** ±6.83	**54.24** ±10.98	**1.05** ±0.47	**5.23** ±1.76
	50µM	**33116.40** ±5250.29	**14382.66** ±11781.82	**3725.40** ±1171.24	**26054.83** ±2411.57	**13230.80** ±3624.99	**194.55** ±33.21	**32.99** ±4.66	**1.06** ±0.38	**4.63** ±0.59
Mersley	0µM	**15886.03** ±11994.37	**4133.74** ±1409.79	**2413.64** ±746.63	**27193.22** ±3619.10	**7579.35** ±2844.13	**150.83** ±20.50	**23.07** ±8.79	**0.91** ±0.10	**3.04** ±0.92
	25µM	**12447.36** ±670.68	**5780.66** ±699.16	**2187.48** ±102.05	**27936.17** ±3335.52	**7051.79** ±1125.87	**156.77** ±62.25	**22.56** ±3.40	**1.08** ±0.25	**3.63** ±1.17
	50µM	**10380.71** ±446.56	**5919.45** ±1052.98	**2851.82** ±1585.74	**35428.95** ±11923.85	**7069.29** ±518.59	**163.42** ±36.80	**26.89** ±6.76	**1.10** ±0.50	**4.44** ±3.49
Lyubasha	0µM	**15154.73** ±2978.91	**5434.55** ±1221.20	**1992.30** ±761.75	**15123.82** ±1494.61	**10452.09** ±2121.62	**176.10** ±12.73	**32.50** ±7.04	**0.52** ±0.06	**3.62** ±2.81
	25µM	**15520.53** ±3557.60	**6507.60** ±2346.12	**2292.19** ±633.61	**17006.12** ±3125.75	**9736.86** ±2050.78	**226.90** ±70.38	**45.27** ±19.65	**1.10*** ±0.15	**4.22** ±1.32
	50µM	**14146.83** ±4217.72	**3741.18** ±2305.96	**2097.72** ±318.45	**14064.02** ±1413.55	**8880.71** ±2658.92	**192.86** ±58.10	**27.36** ±9.91	**0.60** ±0.28	**2.83** ±1.45
Solent	0µM	**11347.30** ±780.47	**5412.35** ±1384.14	**2172.21** ±219.56	**31343.34** ±2320.31	**7052.93** ±1111.12	**215.93** ±42.24	**24.81** ±3.61	**0.76** ±0.11	**2.73** ±0.41
	25µM	**11358.55** ±186.23	**5071.39** ±1370.90	**2161.69** ±379.97	**26993.88** ±1955.11	**7214.42** ±175.23	**236.09** ±7.74	**29.53** ±0.65	**0.72** ±0.08	**3.88** ±1.24
	50µM	**11059.33** ±2477.58	**5585.56** ±1143.10	**2729.86** ±432.65	**26747.42** ±2070.24	**8417.91** ±856.01	**192.81** ±43.37	**24.40** ±0.63	**0.78** ±0.34	**2.81** ±0.55

Bulbils	0µM	**13507.89** ±2595.08	**5137.64** ±2290.53	**4406.17** ±3799.87	**22867.72** ±14877.00	**10136.36** ±1105.56	**133.83** ±19.57	**30.63** ±13.04	**0.79** ±0.58	**6.55** ±5.77
	25µM	**12131.88** ±2443.42	**4815.75** ±1706.95	**2161.21** ±156.10	**20811.61** ±3586.66	**8669.01** ±1967.27	**126.67** ±19.16	**29.75** ±2.17	**0.52** ±0.06	**3.44** ±0.06
	50µM	**11470.71** ±1731.27	**3135.47** ±510.20	**2458.30** ±296.77	**20844.85** ±850.99	**7473.22** ±502.35	**115.58** ±15.86	**28.52** ±8.13	**0.93** ±0.09	**4.81** ±1.18

3.4.7 Impact of Se application on leaf antioxidant status

The final assessment of the garlic plant tissues was to measure antioxidant status since this parameter can be reflective of plant health, tissue quality and stress response. Antioxidant status was determined using a newly developed ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) leaf disc technique. For all cultivars of garlic and treatments, a time dependant scavenging of the ABTS radical was noted for individual leaf discs (Figure 3.8A). A general trend of a reduction in antioxidant status in the leaf was observed for all cultivars in the order of control > 25µM > 50µM. However, the reduction in antioxidant status was only significant for the cultivar Solent. Radical scavenging activity (%)/mg FW of leaf disc in Solent garlic plants enriched with 25µM or 50µM of sodium selenate was significantly lower as compared to the untreated control ($p=0.028$ and $p=0.010$ respectively).

To assess antioxidant capacity of clove tissues, extracts were made and ABTS antioxidant assay was again carried out. In almost all cultivars Se enrichment by foliar application of either 25µM or 50µM sodium selenate significantly decreased ($p<0.05$) the radical scavenging ability as compared to control (Figure 3.8B). The anomaly was 25µM foliar enriched Solent clove tissues that showed a significant increase ($p<0.001$) in radical scavenging ability compared to untreated control clove tissues. A two-way significant interaction between cultivar and Se treatment was observed ($p<0.001$). The general trend in the radical scavenging ability of cultivars differed between Se treatments. At control, Mersley, Lyubasha and Bulbils had the greatest radical scavenging ability, followed by Solent and finally Bulbils. Whereas at the 25µM treatment level, Solent had the greatest radical scavenging ability followed by Mersley and Bulbils, Lyubasha and finally Marco. Again, the general trend in

radical scavenging ability differed at the 50µM level, with Mersley, Lyubasha and Solent having the greatest radical scavenging ability followed by Bulbils and finally Marco.

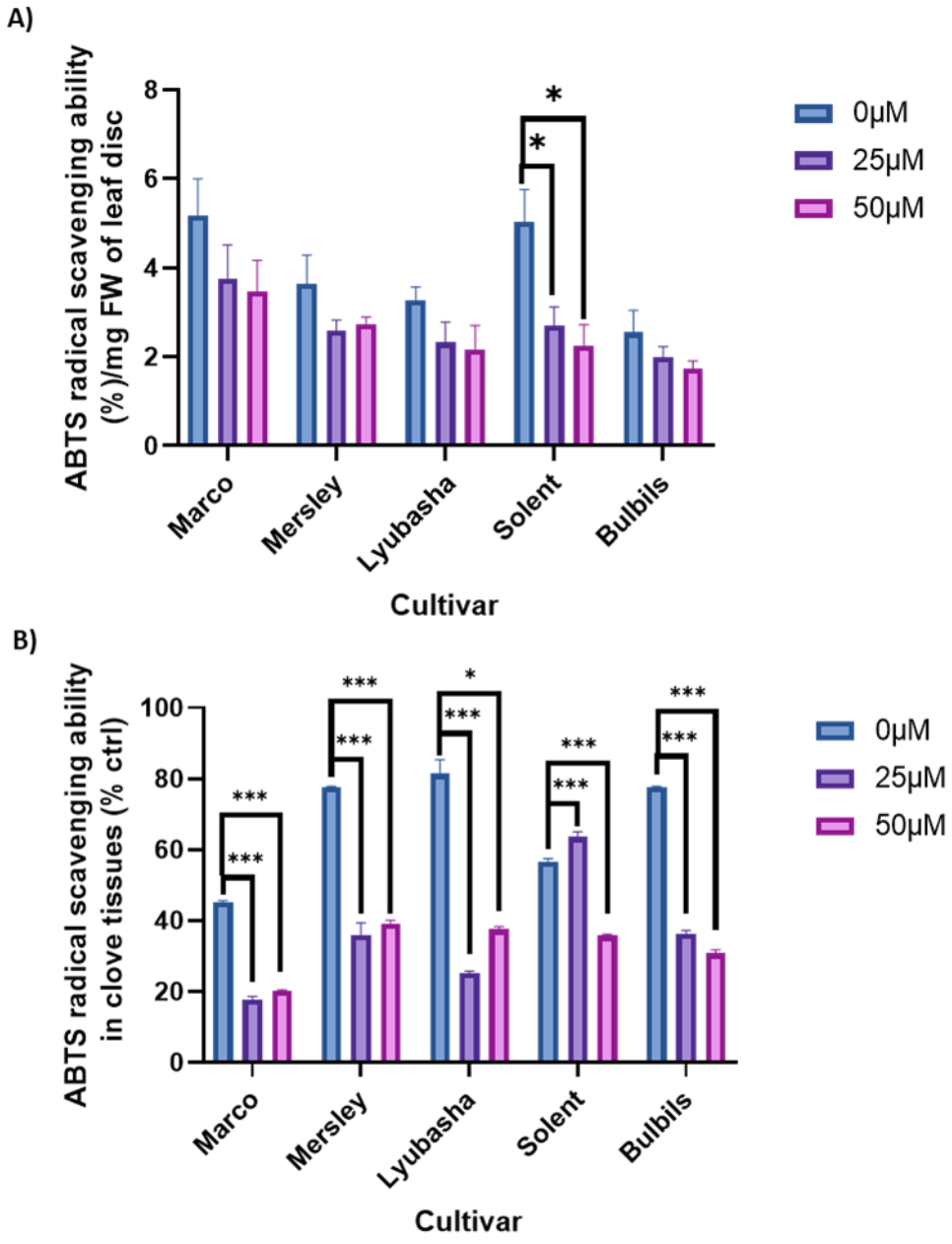

Figure 3.8- ABTS radical scavenging ability A) radical scavenging activity (%)/mg FW leaf disc at 20 minute endpoint for each cultivar at 0µM, 25µM and 50µM Se treatment. B) ABTS radical scavenging ability (% of control) taken at 20 minute end point using 50mg/ml clove extracts. Data is representative of means ± standard error (n=5). Significance calculated using one-way ANOVA and post hoc Tukey test and

displayed as * $p < 0.05$ and ***, $p<0.001$ and indicates significant differences between Se treatments within a cultivar.

3.5 Discussion

Alliums are an important crop family that are widely consumed across the globe. Although alliums are known to be accumulators of S (González-Morales et al., 2017), little is known about Se accumulation in this group nor other micronutrients. Indeed, few studies have assessed the cultivar impacts on micronutrient accumulation in response to biofortification in garlic hence the current research. This research is important given that there is now commercial interest in manipulating Se content of garlic for use in the nutraceutical industry (https://selenoforce.com/index.html) and different cultivars may provide unique properties for enrichment. In recent times, several approaches have been used to apply Se to crop plants namely soil Se fertilization, Se seed treatments, foliar/fruit spraying and hydroponic methods (Puccinelli et al., 2017). These studies have shown variable results. In alliums few have used this foliar application approach to enhance Se levels in plants, however these studies did not assess differences in cultivars nor fully understand the impact Se foliar enrichment has on growth parameters. As such, the current work was designed to explore the utility of foliar application in enhancing Se levels in the different garlic plant tissues.

In the current study, all plants grew successfully over the growing season, and only one plant out of 75 failed to sprout. Plants of all cultivars grew very well under polytunnels growth conditions and growth data obtained was comparable to field grown garlic in terms of bulb diameter, although bulb weight was marginally lower compared to a previous trial conducted in field by Põldma et al. (2011). Yield has

been estimated to have ranged between 12.4 - 26.5 tonnes/ha depending on cultivar.

Our research indicated that sodium selenate application at 25µM and 50µM does not have a negative impact on the growth of the garlic cultivars, Marco, Mersley, Lyubasha and Solent. Nor does it cause detrimental impacts on leaf tissues such as leaf burn as has previously documented in species such as apples (*Malus domestica Borth*), (Wójcik, 2023). Our results showed positive effects of Se enrichment on the cultivars Lyubasha and Solent, such as increases in clove weight and pseudostem diameter (Figure 3.7B and Figure 3.6D respectively). However, Se enrichment in the cultivar Bulbils resulted in significant reductions in clove weight. As far as we are aware this is the first time effects of Se enrichment on pseudostem diameter and clove traits have been examined as past literature has focussed on bulb traits. Similarly, other studies carried out in a range of allium species including *Allium sativum L.* and *Allium cepa L*. have also reported no significant change in bulb weight or bulb diameter with Se foliar enrichment (0-265µM) in the majority of treatment groups (Põldma et al., 2013, Põldma et al., 2011, Shafiq et al., 2019, Tsuneyoshi et al., 2006, Whanger et al., 2000). Climatic conditions between growth seasons have also been postulated to influence the effect of Se enrichment on growth measures. Indeed, Põldma et al. (2011 and 2013) found that significant increases in bulb weight compared to control seen in one growing season in 50µM enriched garlic and 265µM enriched onions could not be reproduced in subsequent years. In contrast, studies using lettuce (Hawrylak-Nowak, 2013, Xue et al., 2001), ryegrass (Hartikainen et al., 2000), potato (Turakainen et al., 2004), spinach (Saffaryazdi et al., 2012), green tea

(Hu et al., 2003) and peanuts (Irmak, 2017) have reported increases in plant growth when exposed to Se. These increases have only been noted when Se treatments are relatively low, and we and others speculate that inhibition of growth at higher Se enrichment concentrations results from the onset of Se toxicity (Hawrylak-Nowak, 2013, Dou et al., 2021, Fargasova, 2004). High concentration Se enrichment has been speculated to influence a multitude of factors involved in cell elongation and cell wall biosynthesis that result in growth inhibition. Downregulation of auxin, a key plant hormone in cell elongation, signal transduction genes and lignin biosynthesis genes have been found in Se treated Maize (Dou et al., 2021), as well as the down regulation of genes involved in the plant cell cycle and cell wall metabolism in Se enriched *Arabidopsis* (Van Hoewyk et al., 2008). It is however clear that the effects of Se enrichment are dependent on the form of Se supplied (Boldrin et al., 2013), timing of Se application (Zhang et al., 2019a), concentration of Se applied (Põldma et al., 2011, Dou et al., 2021), rate of application (Wang et al., 2020a), crop species applied to (Pannico et al., 2019) and potentially now cultivar it is applied to as well.

In this research, we found limited Se accumulation in Se treated garlic plants when applied using foliar application (Table 3.1). Põldma et al. (2011) also found that foliar enrichment of garlic of the cultivar 'Ziemiai' with 50µM resulted in a small but insignificant increase in Se content of garlic bulbs to 0.388mg/kg DW, although admittedly this is still higher than the Se accumulation in enriched clove tissues seen in the present study *viz* 0.09-0.12 mg/kg DW (depending on cultivar). Reasons for this could be again cultivar differences, climatic differences, timing of Se application or differences in leaf morphology such as the presence of epicuticular wax impacting on the accumulation of Se (Meucci et al., 2021). Significant accumulation was

however seen in the work of Põldma et al. (2011) at very high levels of foliar application *viz* 265µM and 531µM. In the current study we chose to not apply Se at such high concentrations due to the risk of pollution, furthermore it also raises the question of the efficiency of the application method if such high doses are needed to significantly increase Se accumulation within garlic plants. Indeed, other studies have noted that Se accumulation is much lower in foliar enriched plants compared to other enrichment methods (Wang et al., 2022b).

Assessing the Se accumulation within the clove and leaf tissues of Se enriched garlic plants is relatively novel. Based on the results of this study, Se accumulation in tissues was equally distributed between clove and leaf material which is in agreement with other Se enrichment studies conducted in onion (Kápolna and Fodor, 2006). In other monocotyledonous species like rice (*Oryza sativa L*.) (Boldrin et al., 2013), wheat (*Triticum aestivum L.)* (Wang et al., 2021) and maize (*Zea mays L.*) (Wang et al., 2020b), Se occurred in greater quantities in above ground tissues compared to underground tissues after foliar Se application with selenate. The organs in which Se is accumulated within plants has been said to be dependent on species, stage of development and physiological conditions (Wiesner-Reinhold et al., 2017) and usually follows seeds > flowers > leaves > roots > stems (Terry et al., 2000). Although further work is needed to determine if this is the case in bulbing species such as garlic which have long been postulated to remobilise nutrients to bulbs (Books, 2022).

Foliar application of Se in this study had no appreciable effect on other macronutrient and micronutrients nor their accumulation in Marco, Mersley and

Solent (Table 3.2 and Table 3.3). A significant increase in Copper (Cu) accumulation was found in Se treated clove and leaf tissues for the hardneck cultivars Bulbils and Lyubasha (Table 3.2). The impacts of Se enrichment on micronutrient composition in Alliums has been far less documented, however, increases in Cu accumulation has been previously seen in Oryza sativa L. with Se foliar application specifically (Boldrin et al., 2013) and some wildlife meadow plants (Drahoňovský et al., 2016). Cu is also a co-factor in the protein, copper-zinc superoxide dismutase (CuZnSOD) an important antioxidant in plants needed to help maintain reactive oxygen species (ROS) gradients that are not only important for plant stress tolerance but also key cues for plant development (Dreyer and Schippers, 2019). Potentially the application of Se may induce toxicity responses including the uptake/mobilisation of Cu in order to increase the amount of CuZnSOD and in turn increase the antioxidant capacity (Drahoňovský et al., 2016), however fluxes in Zn are not seen in this trial and mechanisms are only speculative. It is also understood that macro and micronutrient accumulation in particular Cu accumulation with Se application can differ between genotypes (cultivars)(Lidon et al., 2019, Marques et al., 2021) and therefore 'hard-neck' cultivars may be more predisposed hardwired to accumulate Cu compared to the other 'soft-neck' cultivars on trial. The absence of significant difference in accumulation of Mn, Zn, Fe during foliar Se enrichment is also supported by findings of no significant change in Mn accumulation in Se enriched Medicago sativa L. (alfalfa) (Petković et al., 2019), Zn accumulation in *Triticum aestivum L.* (wheat) (Nawaz et al., 2015) and Fe accumulation in Oryza sativa L. (rice)(Marques et al., 2021). A significant increase in K was also found in Se enriched clove tissues of the cultivar Bulbils (Table 3.2). K is another essential nutrient for human health, high

dietary K is associated with antihypertensive effects especially when in a high sodium diet (Weaver, 2013). A previous study conducted in wheat seedlings also found that accumulation of K is enhanced by the application of 5µM sodium selenate (Elkelish et al., 2019). However, previous enrichment studies conducted in garlic found that the application of high levels of sodium selenate reduced K accumulation (Põldma et al., 2011). The lack of significant difference in accumulation of macronutrients such as P, Mg and S found in this trial has also been seen in garlic foliar treated with 50µM of sodium selenate (Põldma et al., 2011) and broccoli treated with 25g/hectare and 50g/hectare via foliar application (Šindelářová et al., 2015). Ca accumulation was not significantly changed in foliar Se enriched wheat after 40 mg Se/L application of sodium selenate (Nawaz et al., 2015).

Pilot data from this trial suggests that Se treatment may negatively impact on the antioxidant capacity of garlic since Se application reduced ABTS scavenging capacity of isolated leaf discs (Figure 3.8A). Unfortunately, we were unable to measure leaf antioxidant species however, we do know that other studies reporting on the Se enriched lettuce and broccoli have observed similar trends in reduced antioxidant capacity and associated levels of tissue antioxidants including reduction in carotenoids and total polyphenols (Hawrylak-Nowak, 2013, Mahn, 2017, Pannico et al., 2019). Mechanistic data shows that selenate enrichment of *Arabidopsis* causes the downregulation of phytoene synthase a key enzyme in the biosynthesis of carotenoids resulting in decreased concentration of tissue carotenoids in plants (Sams et al., 2011). Pannico et al. (2020) recently showed that this enzyme is significantly reduced in selenate enriched basil and impacts on the levels of several carotenoids namely xanthophylls, neoxanthin + violaxanthin and lutein and

carotenes, β-carotene and α-carotene. A decrease in total carotenoid has also been seen in rice supplied with 135 or 405mg Se/L of selenate in Hoaglands' solution (D'Amato et al., 2018). Whether this is the case in the current work has yet to be determined, but this could perhaps explain why leaves of selenate enriched garlic in this trial had a reduced radical scavenging activity. A reduction in other antioxidants due to Se enrichment has been postulated to be due to a toxicity 'limit' of Se concentration being reached, where the plant can no longer detoxify the amount of Se accumulated and subsequent oxidative damage occurs (Mahn, 2017). Garlic plants supplemented hydroponically with increasing concentrations of sodium selenate found that L-phenylalanine ammonia lyase (PAL) activity, a key enzyme in the phenylpropanoid pathway, is significantly reduced as well as total phenolic activity at 4 and 16 mg/L of Se (Astaneh et al., 2018). Phenolic compounds such as caffeic acid hexose 1, kaempferol hexose pentose and p-coumaric acid (conjugate of rutin) have also been found to decrease in tomato leaves after 24hr exposure to hydroponic sodium selenate (Schiavon et al., 2013). In contrast, experiments carried out with selenite enrichment in Peanut (*Arachis hypogaea L.*) found that in plants treated with 3mg/ml and 6mg/L of sodium selenite, 3 key enzymes of the phenylpropanoid pathway were upregulated including phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate coenzyme A ligase leading to an increase in bound phenolic acids, *viz*. caffeic acid, p-coumaric acid and ferulic acid in a dose-dependent manner (Wang et al., 2016a). Therefore, antioxidant capacity and response to Se is based on the species of Se supplied as well as plant species. This supports the results seen in this trial. It is also thought that Se enrichment at low doses increases total polyphenol content as a defence mechanism to the salt-

stress that the plant is experiencing (Astaneh et al., 2018, Mahn, 2017, Wang et al., 2016a) as well as phenol leakage (Astaneh et al., 2018).

An increase in ascorbic acid (vitamin c) content has been found in shallot bulbs enriched with 63mg of sodium selenate/m^2 compared to non-treated control plants (Golubkina et al., 2019). Ascorbic acid is a key antioxidant in plants involved in the cell cycle and cell wall expansion (Noctor and Foyer, 1998) but also is involved in redox cycling and is the first line of defence a plant has against ROS (Ardebili et al., 2015), therefore maintaining or increasing levels of this antioxidant may be beneficial to crop quality. It has also been postulated that increased antioxidant capacity of selenite treated peanuts may be due to the presence of selenite mediating the ascorbate-glutathione cycle (a major H_2O_2 scavenging pathway), by increasing the activity of glutathione reductase which in turn increases glutathione levels and regeneration of ascorbate which are key cellular antioxidants (Wang et al., 2016b). This has been supported by the ca. 34-39% reduction in L-ascorbic acid concentration in 200µmol/L selenite or selenate enriched mustard sprouts compared to control (Woch and Hawrylak-Nowak, 2019). Furthermore, this literature illustrates the need for further antioxidant assays such as DPPH (Xu and Hu, 2004), as well as assays such as Oxygen Radical Absorbance Capacity (ORAC) which assess the ability of Se enriched garlic to scavenge naturally occurring radicals such as the peroxyl radical (Groth et al., 2020). Determining the total phenolic content via Folin-Ciocalteu may also be useful in assessing the impact of Se foliar enrichment as shown in Sabatino et al. (2019). HPLC could also be coupled to the ABTS assay in order to identify individual antioxidants (Gong et al., 2011).

In terms of antioxidant capacity of clove tissues, the literature is more limited, but generally finds that Se enrichment increases the total antioxidant capacity of the crops, as seen in garlic cultivars used in Põldma et al. (2011), Shafiq et al. (2019) and onion Põldma et al. (2013) which mirrors the significant finding of increased radical scavenging activity of 25µM Se enriched Solent clove tissues as seen in this trial. Põldma et al. (2011) in particular, but not solely noted that total antioxidant capacity is greater at lower Se enrichment levels, which perhaps suggests that 50µM Se foliar application on the cultivar Solent may tip the balance in being too high and therefore reducing the antioxidant capacity whereas 25µM is more preferable to the plant and avoids a stress response. The same could also be said for the other cultivars in this trial that a lower dose may have been more preferable. However, the significant decrease in radical scavenging activity as seen in the other cultivars on trial with Se enrichment has not been previously reported in other literature based on Se enrichment of bulbing species. The lack of literature surrounding the impacts of Se enrichment on bulbing species and in particular the direct effects to clove tissues illustrates the need for further experiments within this field. Differences in results could be due to a complex picture of genetic differences in cultivars, climatic effects, dosages of applied sodium selenate, the timing of foliar application and the number of foliar applications.

3.6 Conclusion

On the basis of this research foliar application may not be the most effective mechanism of enriching the particular garlic cultivars Marco, Mersley, Lyubasha, Solent and Bulbils with Se. However, application of 25µM or 50µM sodium selenate

did not significantly impact on growth in above ground and bulb measures, however, clove weight was significantly impacted by Se enrichment in the cultivars Lyubasha, Solent and Bulbils. Despite these significant differences in clove weights following Se enrichment, bulb weight was not significantly impacted. Furthermore, Se application at 25µM and 50µM did not significantly impact on the accumulation of the majority of macro and micronutrients assessed in this trial. Although the temptation may be to apply greater concentrations of Se, current antioxidant results show a concentration dependent decrease in radical scavenging ability in both clove and leaf tissues which could have detrimental effects, although further work is needed to assess the impact of Se enrichment on antioxidant species within the garlic cultivars. Our results suggest that foliar application of Se to the cultivars Marco, Mersley, Solent, Lyubasha and Bulbils would likely not be of benefit commercially, therefore alternative methods of Se enrichment need to be explored.

4 Assessment of hydroponics to improve selenium status in garlic.

4.1 Abstract

Se is an essential micronutrient for human health, our previous work in enriching garlic cultivars with Se via foliar application methods resulted in very low levels of accumulation (0.06-0.128 mg Se/kg DW in clove tissues)($p>0.05$). In this Chapter, we aimed to assess the accumulation of Se within four cultivars of garlic using hydroponic approaches and assess the impact of garlic tissue extracts on cell survival and the ability to alter Se status within HepG2 cells. Results showed that 50µM sodium selenate enrichment significantly increased Se accumulation within garlic tissues ($p<0.05$), with Se enriched clove tissues accumulating between 7 and 40mg Se/kg DW depending on cultivar ($p<0.05$). The highest levels of Se accumulated in the roots > shoot> clove with levels of Se accumulation differing between cultivars. Se speciation analysis via HPLC-ICP-MS revealed that Se enrichment significantly increased the accumulation of SeMet, $SeCys_2$, Selenate and SeMeSeCys in garlic tissues ($p<0.05$) with SeMet being the predominant form of Se in clove tissues. Se enrichment did not however alter accumulation of Na, Mg, P, S, K and Ca in clove tissues ($p>0.05$). Se accumulation was found to not significantly impact on the cytotoxicity of most of the cultivar extracts ($p>0.05$), however significant differences were seen in the IC_{50} values between control and 50µM Se enriched clove tissues of the cultivars Mersley and Solent ($p<0.001$). Se speciation of these tissues revealed differential accumulation of Se species between control and enriched tissues as well

as between these cultivars, however this did not fully explain the difference in cytotoxicity that was seen. Further analysis on markers of Se status, SCLY, SelP and GPx suggested that the incubation of HepG2 cells with 0.6mg/ml of garlic clove extracts for 24 hours did not significantly alter the Se status of cells ($p>0.05$).

Keywords: Hydroponics, Accumulation, Speciation

4.2 Introduction

Selenium (Se) is an essential micronutrient needed to maintain the healthy functioning of many cells and systems in mammals (Hartikainen, 2005) and is incorporated during translation into proteins (Schubert et al., 1987). Brazil nuts, fish, meat and eggs are a rich sources of Se, whereas cereal crops and vegetables are generally low in Se (Barclay et al., 1995, Holland et al., 1991, ODS, 2020). Several studies have shown that increased intake of Se in the diet is beneficial to health (Nordio and Basciani, 2018, Zhu et al., 2019), and in the last decade researchers have endeavoured to manipulate Se levels in the diets of humans using strategies including supplementation and biofortification. Indeed, current literature has explored manipulation of Se levels in several crop species (Puccinelli et al., 2017). These approaches have focused on increasing Se content via soil fertilisation in crops such as maize (Longchamp et al., 2013), wheat (bread and durum) (Galinha et al., 2013), lettuce (Hawrylak-Nowak, 2013), cucumber (Hawrylak-Nowak et al., 2015), lentil (Ekanayake et al., 2015), broccoli and carrot (Bañuelos et al., 2015), potato (Poggi et al., 2000), rice (Chen et al., 2002), buckwheat and pumpkin (Stibilj et al., 2004). However, a narrow range between deficiency (<40µg Se/day), and upper tolerable intake (255µg Se/day) is understood to apply to humans and therefore care is needed when manipulating dietary Se levels (Rayman, 2000).

Other strategies to manipulate Se in crop plants include hydroponic systems. These approaches have been developed to use an aqueous medium to deliver nutrients directly to the roots of crops and have been shown to be successful in Se enriching crops such as lettuce (Hawrylak-Nowak, 2013), basil (Puccinelli et al., 2017),

sunflower (Garousi et al., 2016), maize (Garousi et al., 2016), spinach (Saffaryazdi et al., 2012) and tomato (Pezzarossa et al., 2014). In the United States, commercial interest in hydroponics to manipulate Se levels in garlic has been reported however, limited research has been done in this field. Any use of this approach needs to determine impacts on Se tissue accumulation and toxicity.

Aims and Hypothesis

We hypothesize that Se supplied via hydroponic methods will increase the Se content of garlic tissues although the levels of Se accumulated will differ between cultivars. Moreover, increased Se accumulation in tissues will result in greater bioactivity of plant tissues.

1. Enrich four different garlic cultivars with Se via a hydroponic system and assess the accumulation of Se and other macronutrients in each cultivar and portion (clove, shoot and root) of the plant.

2. Assess the cytoprotective, anti-cancer properties and impact on key proteins and enzymes involved in Se metabolism of garlic extracts produced from the Se enriched garlic in a human hepatoma HepG2 model. This work to determine the capacity of these Se enriched garlic extracts to influence Se status of cells.

3. To determine whether differences seen in cytotoxicity of extracts is due to differential accumulation of Se species between control and Se enriched tissues as well as cultivars.

4.3 Materials and Methods

4.3.1 Plant and chemical materials

Mersley, Lyubasha, Solent were kindly supplied by The Garlic Farm, Isle of Wight (https://www.thegarlicfarm.co.uk/) in October 2020, unfortunately there was not enough Bulbils to conduct hydroponic trials with. Marco was obtained from Taylor & Sons Bulbs Ltd, Lincolnshire again in October 2020.

Ammonia citrate dibasic was obtained from Fisher Scientific UK (Loughborough, UK). Potassium nitrate, Ammonium nitrate, Monopotassium phosphate, Glutathione peroxidase activity kit (MAK437) were obtained from Sigma-Aldrich (St Louis, USA).

4.3.2 Hydroponic set up

The hydroponic system employed in the current work is described elsewhere (Tsuneyoshi 2006). Due to ongoing restrictions on access to facilities including the glasshouse hydroponics due to COVID, we adapted to run the hydroponic system in the lab. In brief, cloves were weighed prior to the trial starting. Following the polytunnel trial, we elected to concentrate on high (50µM) vs control (0µM) Se treatments for the hydroponic cultivation. For each cultivar cloves were separated into two treatment groups, control (0µM) and Se treated (50µM), (n=3, per treatment group). Each clove was placed in fine garden netting and suspended in a 600ml beaker such that the base of the clove touched the meniscus of the hydroponic fluid (100ml). The basic composition of the hydroponic solution was as follows; Potassium nitrate (400mg/L), Ammonium nitrate (350mg/L), Monopotassium phosphate (300mg/L), Calcium chloride dihydrate (150mg/L), Magnesium chloride hexahydrate (305mg/L) respectively. After two weeks of growth

at room temperature on an east facing windowsill, the hydroponic solution was refreshed and Sodium selenate (9.4mg/L) was added to the hydroponic solution for Se treatment group as previously described (Tsuneyoshi et al., 2006). Cloves were then cultured for an additional 2 weeks prior to harvest. At harvest, plants were rinsed in tap water, patted dry and separated into roots, shoots and cloves and the tissues were weighed. This process was repeated for three independent replicates.

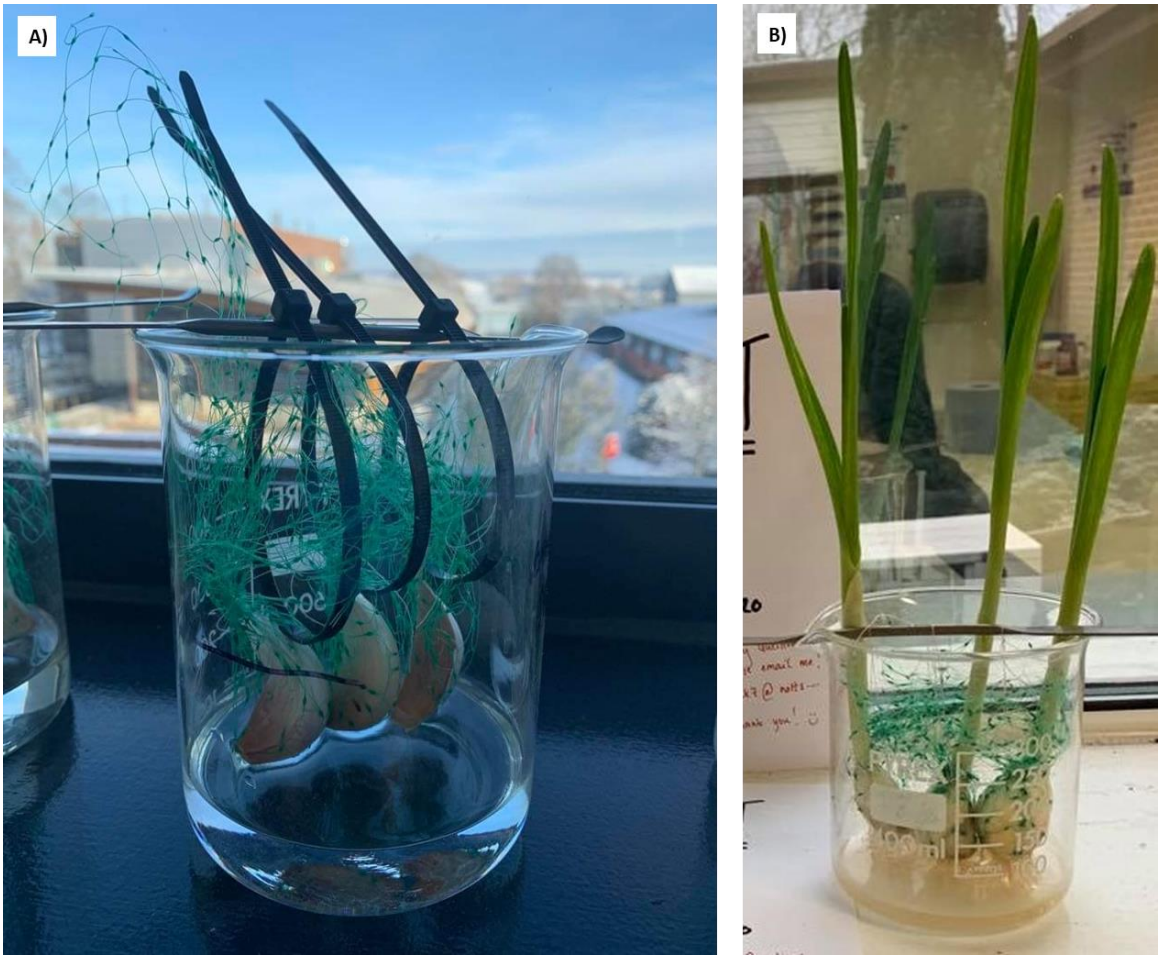

Figure 4.1-Photograph of cloves used in the hydroponic experiments A) beginning of the experiment and B) plants prior to harvest and analysis.

4.3.3 Freeze drying and milling

To facilitate nutritional assessments of plant tissues, all materials were freeze dried and ground to a powder as described in 2.2.1.

4.3.4 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Alongside the analysis of Se, the elements Na, Mg, P, S, K and Ca were also measured due to their importance in plant health and presence within the hydroponic solution. Acid digestion and ICP-MS were carried out as described in 2.3.1.

4.3.5 Se Speciation

Samples were digested and run on HPLC-ICP-MS as per Muleya et al. (2021) as detailed below.

200mg of ground garlic tissue was digested with 20mg of protease and 10mg of lipase in 5ml of MiliQ water, in an Enviro-Genie® (Scientific Industries, Inc) at 37°C and rotating at 60rpm for 24 hours. Samples were then centrifuged at 3600xg for 30 minutes (Fisherbrand GT 4R) before being filtered through a 0.2µM filter. Samples were then run by Saul Vazquez Reina an ICPMS Technical Specialist on the HPLC-ICP-MS. Standards containing 5ppb of sodium selenite, sodium selenate, selenocystine, selenomethionine and Se-methylselenocysteine were made and run after every 10 samples.

Speciation analysis was carried out on an LC ICS-5000 (Dionex, Thermo-Fisher Scientific) coupled to ICPMS. Samples were introduced into the column (Hamilton PRP-X100) via a mobile phase containing (20mM or 50mM ammonium citrate diabasic and 2% methanol adjusted to pH 4.3 by citric acid. Samples were introduced at a flow rate of 1ml/minute and 100µl injection volume. The same conditions for ICP-MS determination of Se as detailed in 2.3.1 was used. Peaks were identified by matching to retention times of standards and quantified via calibration standards.

To account for sample dilution, ppb was multiplied by 25, to convert from ppb to mg/kg results were divided by 1000.

4.3.6 Tissue extraction and confirmation of Se accumulation

Tissue extracts were prepared using a methanol/water extraction method as described in 2.2.2. To confirm the presence of Se within the extracts, extracts were diluted to 50mg/ml with MiliQ water and filtered using a 0.2µM sterile filter. The subsequent 200µl samples was then acidified using concentrated HNO_3 (2%) and diluted to a final volume of 10 ml (dilution factor 1:50). Se content was then determined using ICPMS as described in 2.3.1.

4.3.7 Cell Culture

Human hepatoma HepG2 cells were cultured as detailed in 2.4.1-2.4.4. For all cell viability analysis cells were plated at a seeding density of 10,000 cells/well in 96 well plates. Plated cells were left to incubate for 24hrs. The next day, plant extracts 0 – 10 mg/ml were prepared, diluted in FBS free media, and 100µl/well of each treatment was applied to cells. Exposed cells were then incubated for 24hrs before assessing a cell viability as described in 2.5.1.

4.3.8 Western Blotting

For western blotting, cells were seeded at a density of 500,000 cells/well into 6 well plates. Cells were treated with 0.6mg/ml of garlic extract (Marco control and treatment and Mersley control and treatment), a control well was used containing FBS free media and a positive control well containing 30nm of sodium selenite was also used as previous studies have found GPx1 protein to plateau in prostate cancer cells (LNCaP and PC3 cells) and SCLY gene expression increases noted in HepG2 cells

at this treatment level (Hendrickx et al., 2013, Seale et al., 2018). All treatments were diluted in FBS free media. Cells were exposed to treatments for 24 hours before cell scraping and protein extraction. Protein extraction, quantification and western blotting was carried out as detailed in 2.6.1-2.6.3.

4.3.9 GPX assay

Cells were seeded at 400,000/well in 6 well plates, 24 hours prior to being treated with 0.6mg/ml of control/Se enriched clove extracts of the cultivars Marco and Mersley, 30nm sodium selenite and control, solely FBS free DMEM media for 24 hours. Cells were scraped into 200µl of cold PBS and homogenised using a 26G ¾" needle. The samples were then centrifuged at 13,000xg and 4°C for 10 mins and the supernatant was used in the assay. The GPx activity was assayed using the Sigma kit MAK437. Manufacturers guidelines were followed and in brief, NADPH standards were prepared ranging in concentration from 0-6mM. 10µl of standards and samples and background (assay buffer) were added to a clear 96 well plate and 100µl of working reagent containing assay buffer, glutathione, NADPH and glutathione reductase was added to each sample well. Peroxide solution was then added and the absorbance at 340nm was measured immediately and again after 4 minutes. The optical density of the standards at 4 minutes was used to generate a standard curve. The optical density of samples and background at 4 minutes was deducted from 0 minute. The following calculation was used to calculate the glutathione peroxidase activity of the samples, activity normalised to protein levels via the Lowry assay detailed in 2.6.2).

$$\text{GPX Activity (U/L)} = \frac{\Delta\text{ODs} - \Delta\text{ODb}}{\text{Slope (mM}-1) \times 4\text{ (min)}} \times 1000 \times \text{DF Slope}$$

Where:

ΔODs = Change in OD readings at 340 nm of the Sample

ΔODb = Change in OD readings at 340 nm of the Background Control

1000 = Conversion factor from mmoles to μmoles.

DF = Sample Dilution factor (DF = 1 for undiluted Samples as in this body of work)

4.3.10 Statistical Analysis

Data is represented as mean ± standard deviation unless otherwise stated.

Independent t-tests were used when comparing between two groups whereas one-way ANOVA was used when comparing between three or more groups followed by post-hoc Tukey test. In certain cases, 2-way ANOVAs were used to determine if there was a significant interaction between two independent variables.

4.4 Results

4.4.1 Se accumulation in plant tissue

All four garlic cultivars grew well under the hydroponic conditions used with no losses. After the two-week initial growth period, the hydroponic medium was refreshed and 50µM sodium selenate was added to the Se treated group and plants were grown for an additional two weeks prior to Se analysis. In all cultivars Se accumulated readily in tissues with levels ranging between 7.14 mg Se/kg DW and 230.02mg Se/kg DW in Se enriched tissues (Table 4.1). Whereas, in control plants Se levels ranged 0.13mg Se/kg DW and 0.42mg Se/kg DW (Table 4.1). Se accumulation was also found to differ between portions of the plant, with the root tissues accumulating the highest levels of Se followed by the shoot tissues and lastly the clove tissues.

In clove tissues, a significant increase in Se accumulation was observed in the cultivars Mersley (19.47±4.86mg Se/kg DW, $p=0.020$) and Solent (32.51±5.24mg Se/kg, $p<0.001$) as compared to control clove tissues (Table 4.1). However, despite the increase of Se to 40.05mg Se/kg and 7.14mg Se/kg in the cultivars Marco and Lyubasha, no significant increase in Se accumulation was seen ($p=0.084$ and $p=0.148$ respectively). This is potentially due to the large variation between repeats, suggesting that different garlic plants albeit of the same cultivar can accumulate different amounts of Se potentially due to a slightly different genetic make-up. The levels of Se accumulated in clove tissues did not vary significantly between cultivars ($p=0.127$).

In shoot tissues, Se enrichment was found to significantly increase the Se accumulation of all 4 cultivars ($p<0.05$) (Table 4.1). A significant interaction between Se treatment and cultivars was also found ($p=0.003$), suggesting that the effect of Se enrichment differs between cultivars. In Se enriched shoot tissues, Solent was found to accumulate a significantly greater level of Se mg/kg as compared to the cultivars Marco ($p=0.042$) and Lyubasha ($p=0.034$), whereas in control tissues accumulation of Se did not significantly differ between cultivars ($p=0.337$). These results indicate that different cultivars accumulate differing amounts of Se when grown under standard conditions in the presence of a known Se source.

Finally, we also assessed the Se content of root tissues, unfortunately there was insufficient volume of root tissue from the cultivar Marco to allow for further analysis. Past studies have shown that some species hyperaccumulate Se in these tissues, however this work has yet to be assessed in alliums. Se enrichment was found to significantly increase the Se accumulated in the root tissues in the cultivars Mersley ($p=0.011$) and Lyubasha ($p=0.004$) as compared to control root tissues. Similarly to clove tissues, the levels of Se accumulated in root tissues did not significantly vary between cultivars ($p=0.110$).

Table 4-1- Se content of control and Se enriched (50µM) garlic tissues (clove, shoot and root) for each cultivar. ICP-MS was used to assess Se content in tissues. – denotes that there was insufficient volume of Marco roots for analysis. Data are representative of means ± SD, n = 3; significance between control and Se enriched tissues was determined via t-test * denotes $p<0.05$.

		Se Content (mg/kg DW)	
Cultivar	**Tissue**	**Control**	**+ Sodium Selenate (50 µM)**
Marco	Clove	**0.33** ±0.16	**40.05** ±30.12
	Shoot	**0.13** ±0.05	**47.96*** ±6.92
	Root	-	-
Mersley	Clove	**0.19** ±0.27	**19.47*** ±4.86
	Shoot	**0.23** ±0.19	**89.93*** ±29.65
	Root	**0.16** ±0.06	**230.02*** ±41.68
Lyubasha	Clove	**0.26** ±0.12	**7.14** ±5.07
	Shoot	**0.42** ±0.27	**45.65*** ±16.46
	Root	**0.32** ±0.12	**68.52*** ±7.39
Solent	Clove	**0.24** ±0.13	**32.51*** ±5.24
	Shoot	**0.21** ±0.16	**96.39*** ±8.49
	Root	**0.36** ±0.17	**143.85** ±142.37

4.4.2 Se biofortification impacts on mineral accumulation.

The accumulation of other minerals present in the hydroponic solution was also assessed in the current work. Previous research has shown that Se can interact with other nutrients such as sulphur. We therefore measured the levels of Na, Mg, P, S, K and Ca in plant tissues due to their importance in health and presence within the

hydroponic solution (Table 4.2). In clove materials Se enrichment resulted in no significant difference in the accumulation of Na, Mg, P, S, K and Ca in all four cultivars tested. In shoot tissues the accumulation of Na, Mg and P also did not significantly differ between control and Se enriched for all cultivars ($p>0.05$). However, Se enrichment of Marco led to a significant decrease in the accumulation of S in shoot tissues as compared to the control plants ($p=0.024$). A similar pattern of decreases for K and Ca was also noted for Solent ($p=0.034$ and $p=0.015$ respectively). In root tissues, there was no significant difference in the accumulation of macronutrients Mg, P, S, K and Ca between control and Se enriched tissues for all 4 cultivars. However, in the cultivar Mersley, Se enriched root tissues accumulated a significantly greater amount of Na compared to control ($p=0.041$).

In general, the macronutrient profile of the cultivars varied greatly between the garlic cultivars. Marco showed a significantly greater accumulation of Na, Mg, K and Ca compared to all other cultivars ($p<0.05$) in clove tissues. Hardneck Lyubasha had a significantly lower accumulation of S, K in cloves compared to softneck cultivars Mersley ($p=0.042$ and $p=0.008$ respectively) and Solent ($p=0.004$ and $p=0.003$ respectively) and again significantly lower accumulation of S and K in shoot tissues compared to Marco ($p=0.003$ and $p=0.016$ respectively). There was no significant difference in accumulation of any of the macronutrients between the cultivars Mersley, Solent and Lyubasha in the root tissues ($p>0.05$).

Table 4-2-Mineral content of garlic tissues of each cultivar treated with and without treatment of Se (50µM) in the form of sodium selenate on a dry weight basis . Data is presented as mean ±SD (n=3) for Clove (C), Shoot (S) and Root (R) tissues . Significance calculated by independent t-test between control and Se enriched tissues for each cultivar, mean followed by * signifies p<0.05.

		Mineral content (g/kg DW) displayed as mean ± SD											
		Na		**Mg**		**P**		**S**		**K**		**Ca**	
Cultivar	**Area**	**0µM**	**50µM**	**0µM**	**50µM**	**0µM**	**50µM**	**0µM**	**50µM**	**0µM**	**50µM**	**0µM**	**50µM**
Marco	**C**	**0.82** ±0.13	**0.88** ±0.06	**1.57** ±0.24	**1.54** ±0.08	**7.00** ±0.79	**5.86** ±1.07	**13.56** ±1.09	**12.75** ±0.72	**23.25** ±0.89	**25.14** ±3.51	**2.14** ±0.09	**1.76** ±1.12
	S	**0.23** ±0.14	**0.22** ±0.11	**1.91** ±0.32	**1.71** ±0.25	**9.57** ±2.62	**8.59** ±1.13	**19.62** ±0.47	**17.51*** ±0.91	**34.07** ±6.44	**32.87** ±4.49	**1.13** ±0.55	**0.90** ±0.34
	R												
Mersley	**C**	**0.03** ±0.02	**0.04** ±0.02	**0.91** ±0.19	**1.03** ±0.23	**7.75** ±0.937	**8.66** ±0.52	**14.07** ±2.89	**15.84** ±3.24	**18.78** ±2.63	**20.72** ±2.16	**0.96** ±0.18	**1.14** ±0.19
	S	**0.05** ±0.02	**0.05** ±0.02	**1.60** ±0.15	**1.59** ±0.19	**8.53** ±0.13	**8.66** ±0.27	**13.87** ±0.51	**14.13** ±0.98	**28.15** ±1.19	**27.67** ±0.33	**1.31** ±0.10	**1.21** ±0.02
	R	**0.08** ±0.02	**0.16*** ±0.04	**1.09** ±0.17	**1.21** ±0.27	**12.58** ±1.49	**14.09** ±2.88	**12.65** ±0.60	**13.36** ±1.64	**19.87** ±2.89	**20.53** ±4.19	**2.49** ±0.24	**2.32** ±0.11
Lyubasha	**C**	**0.07** ±0.07	**0.07** ±0.07	**0.70** ±0.14	**0.71** ±0.07	**4.94** ±0.22	**4.64** ±0.35	**9.07** ±1.86	**8.35** ±0.79	**11.73** ±1.80	**10.83** ±1.11	**1.03** ±0.08	**1.17** ±0.13
	S	**0.07** ±0.04	**0.06** ±0.03	**1.45** ±0.22	**1.29** ±0.25	**8.02** ±0.59	**7.23** ±0.19	**11.01** ±2.08	**9.61** ±0.61	**22.97** ±1.54	**20.33** ±1.22	**1.21** ±0.36	**1.23** ±0.47
	R	**0.12** ±0.08	**0.17** ±0.11	**1.62** ±0.51	**1.54** ±1.01	**11.15** ±2.26	**8.01** ±2.09	**10.13** ±2.08	**7.73** ±2.00	**21.66** ±1.50	**17.33** ±7.36	**2.80** ±0.63	**2.45** ±1.42
Solent	**C**	**0.03** ±0.02	**0.02** ±0.02	**0.95** ±0.22	**0.85** ±0.14	**7.90** ±1.25	**7.63** ±1.07	**15.97** ±0.79	**15.49** ±0.77	**19.06** ±184.21	**17.50** ±1.27	**1.11** ±0.36	**1.05** ±0.22
	S	**0.04** ±0.02	**0.04** ±0.02	**1.61** ±0.25	**1.37** ±0.19	**8.80** ±0.53	**8.56** ±0.27	**13.86** ±1.83	**13.29** ±1.28	**28.38** ±0.57	**25.89*** ±1.23	**1.54** ±0.02	**1.09*** ±0.19
	R	**0.09** ±0.01	**0.13** ±0.03	**1.39** ±0.40	**1.53** ±0.65	**11.62** ±1.66	**11.53** ±2.20	**11.42** ±6.21	**11.49** ±3.55	**21.59** ±3.47	**20.93** ±4.53	**2.88** ±0.46	**2.61** ±0.24

4.4.3 The effect of garlic tissue extracts on the cytotoxicity of HepG2 cells

Based on our initial ICP-MS analysis we found the Se levels varied between cultivars with Solent and Mersley accumulating the most Se in tissues. This posed the question as to whether plants that accumulate higher levels of Se were more cytotoxic to HepG2 cells or showed greater biological activity. Therefore, we next assessed the impact of individual cultivars on cell viability of cultured hepatoma HepG2 cells as the liver is the central organ of Se metabolism. All cultivars, regardless of Se enrichment caused a modest concentration dependent decrease in cell viability after 24 hours when treated across the concentration range of 0 - 10mg/ml of tissue extracts.

Despite the significantly increased Se accumulation in Se enriched tissues as compared to control tissues, the cytotoxicity profiles of Se enriched tissues appear to mirror that of the control tissues in the majority of clove, shoot and root extracts. Significant differences in cell survival between Se enriched and control extracts were only seen at specific concentrations in clove and root extracts. In clove tissues, Se enriched extracts of the cultivar Mersley were found to cause a greater loss in cell viability than control tissues at 0.3, 0.6 and 2.5 mg/ml concentrations ($p<0.05$), the cultivar Marco also displayed this trait at 2.5mg/ml concentration of Se enriched extract ($p=0.012$, Figure 4.2A and B). Whereas Se enriched extracts of the cultivars Lyubasha and Solent were found to cause a significantly lower loss in cell viability as compared to control extracts when applied to HepG2 cells at 1.25 and 2.5mg/ml respectively ($p=0.024$ and $p=0.006$, Figure 4.2C and D). Differences in cytotoxicity were also reflected in IC_{50} values of the cultivars Mersley and Solent in clove extracts. Se enriched Mersley clove extracts were found to have a significantly lower

IC_{50} value 1.51 ± 0.06mg/ml as compared to control 1.87 ± 0.06mg/ml ($p<0.001$), suggesting the increased Se accumulation in the cultivar Mersley results in increased cytotoxicity (Table 4.4). However, the opposite was seen in the cultivar Solent, where Se enriched tissues had a significantly higher IC_{50} value of 2.41 ±0.06 mg/ml as compared to control tissues 1.87 ± 0.06mg/ml ($p<0.001$) (Table 4.4). Therefore, despite the significantly increased Se accumulation in Solent clove tissues as compared to control and Mersley Se enriched tissues, suggesting the Se enriched extract is potentially less cytotoxic.

In addition to clove tissues, we also assessed shoot and root tissue extracts since our IC-PMS data indicated differential accumulation of Se in various organ tissues. Analysis of shoot tissue extracts (0-10mg/ml), resulted in no significant difference in the loss of cell viability between control and Se enriched garlic extracts at any of the concentrations tested for any cultivar (Figure 4.3). In root tissues, significant differences were only observed between Se enriched root extracts and control root extracts at specific concentrations, as per clove tissues. Se enriched root tissues of cultivars Mersley and Lyubasha resulted in higher cell survival as compared to control extracts at 1.25mg/ml ($p=0.001$) and 2.5mg/ml ($p=0.001$) respectively (Figure 4.4A and B). Whereas Se enriched Solent extracts had a significantly lower cell survival at 2.5mg/ml as compared to control ($p<0.001$) (Figure 4.4C). Despite these significant differences, no significant difference was found in IC_{50} values between Se enriched root tissues and control tissues for any of the cultivars examined (Table 4.4). Again, due to limited volume of tissue samples for the cultivar Marco the assessment of cell viability in this instance was not possible.

Interestingly, the cytotoxicity of garlic extracts also differed between clove, shoot and root tissues, this again appeared to be irrespective of levels of Se accumulation. Clove tissues were found to be the most toxic with ~30% cell survival at highest concentration of extract 10 mg/ml despite accumulating the lowest amount of Se, followed by root tissues (~50% cell survival at 10mg/ml) and least cytotoxic was shoot tissues (~60% cell survival at 10mg/ml).

From our research findings we were unable to see any correlation between tissue Se content and loss in cell viability. This finding made us question the possibility that our plant extracts may not contain any Se. Therefore, we determined the Se content of aqueous extracts used in our cell work from two cultivars having the highest Se content namely, that of Solent and Mersley. Using IC-PMS we confirmed that both cultivars and associated extracts contained appreciable levels of Se (Table 4.3). This result confirmed that our extracts contained high levels of Se but that the levels may not dictate impacts on cell viability and further research is needed to clarify this.

Table 4-3 Confirmation of Se present within extracts of garlic clove. Se (µmol/L), data is presented as mean ±SD (n=3).

	Control	**+ Sodium Selenate (50 µM)**
Mersley	**7.29** ± 8.49	**317.92** ± 104.67
Solent	**10.04** ± 3.61	**684.21** ± 80.90

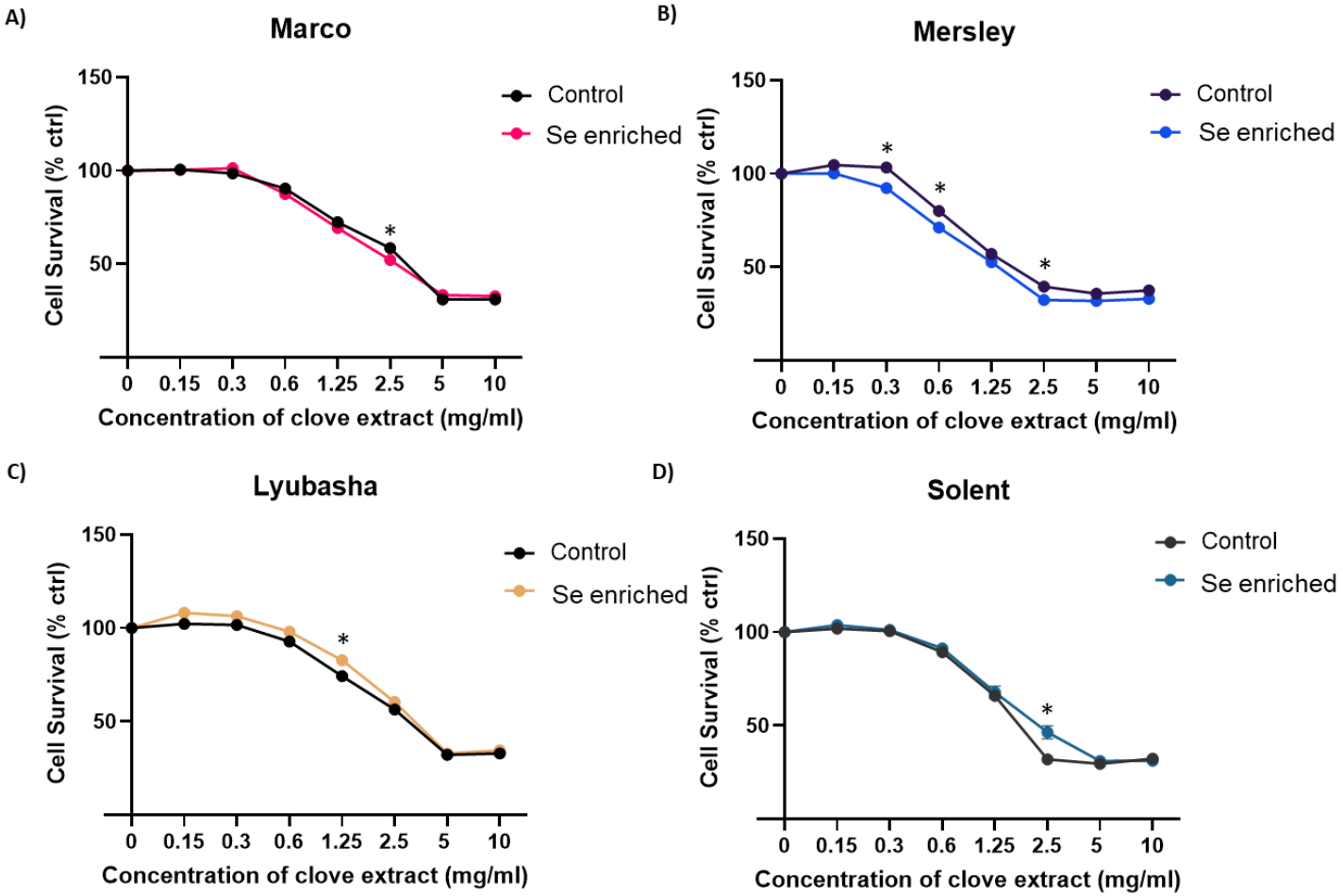

Figure 4.2- Cell survival/viability (%) of HepG2s after exposure for 24hrs to various concentrations (10mg/ml-0mg/ml) of either garlic control clove extract or Se enriched clove extract. Data represents mean ± standard error of the mean (n=27). Significance between control and Se enriched tissues is represented by *, $p<0.05$ as determined via independent t-test.

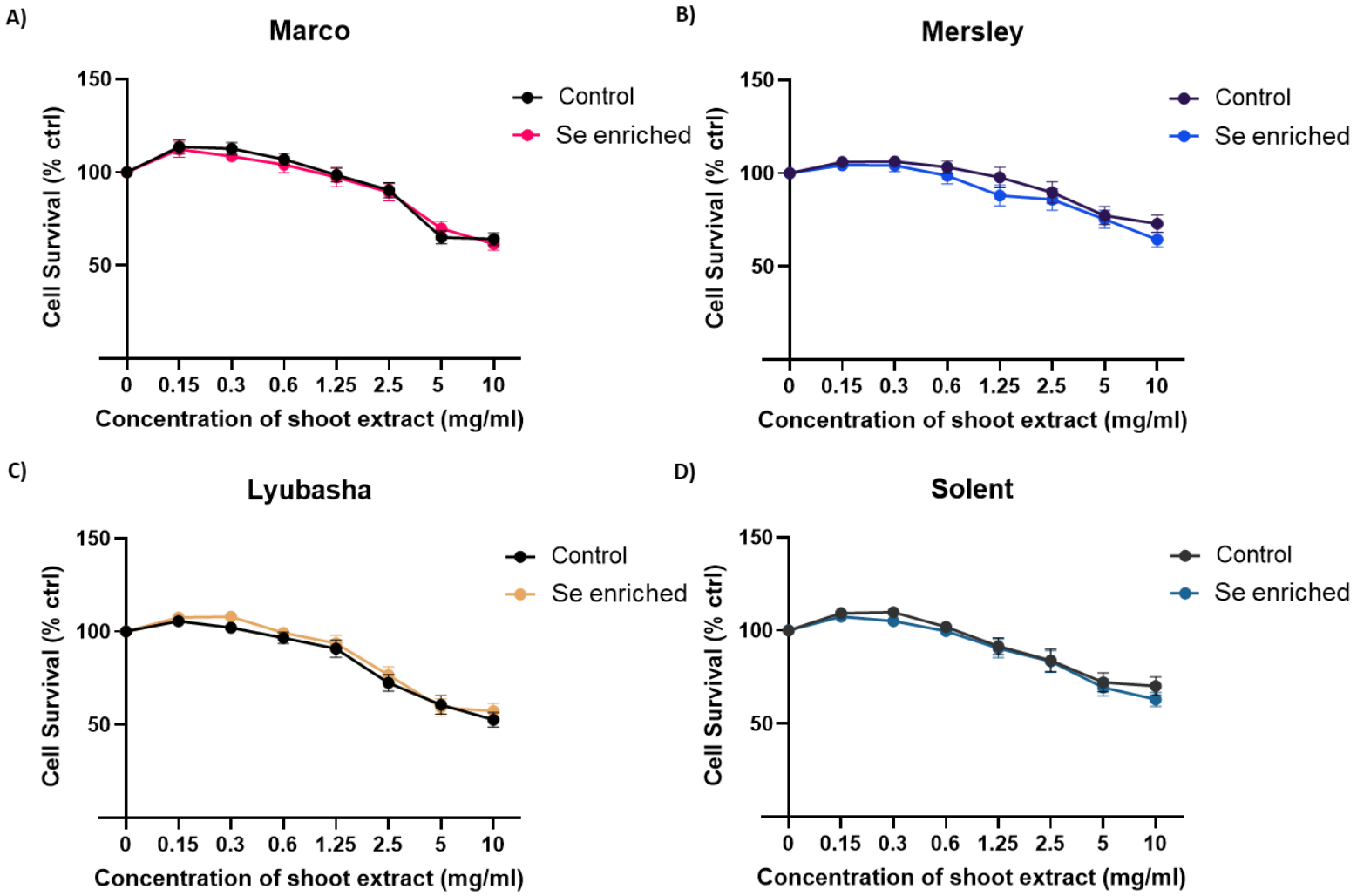

Figure 4.3- Cell survival/viability (%) of HepG2s after exposure for 24hrs to various concentrations (10mg/ml-0mg/ml) of either garlic control shoot extract or Se enriched shoot extract. Data represents mean ± standard error of the mean (n=27).

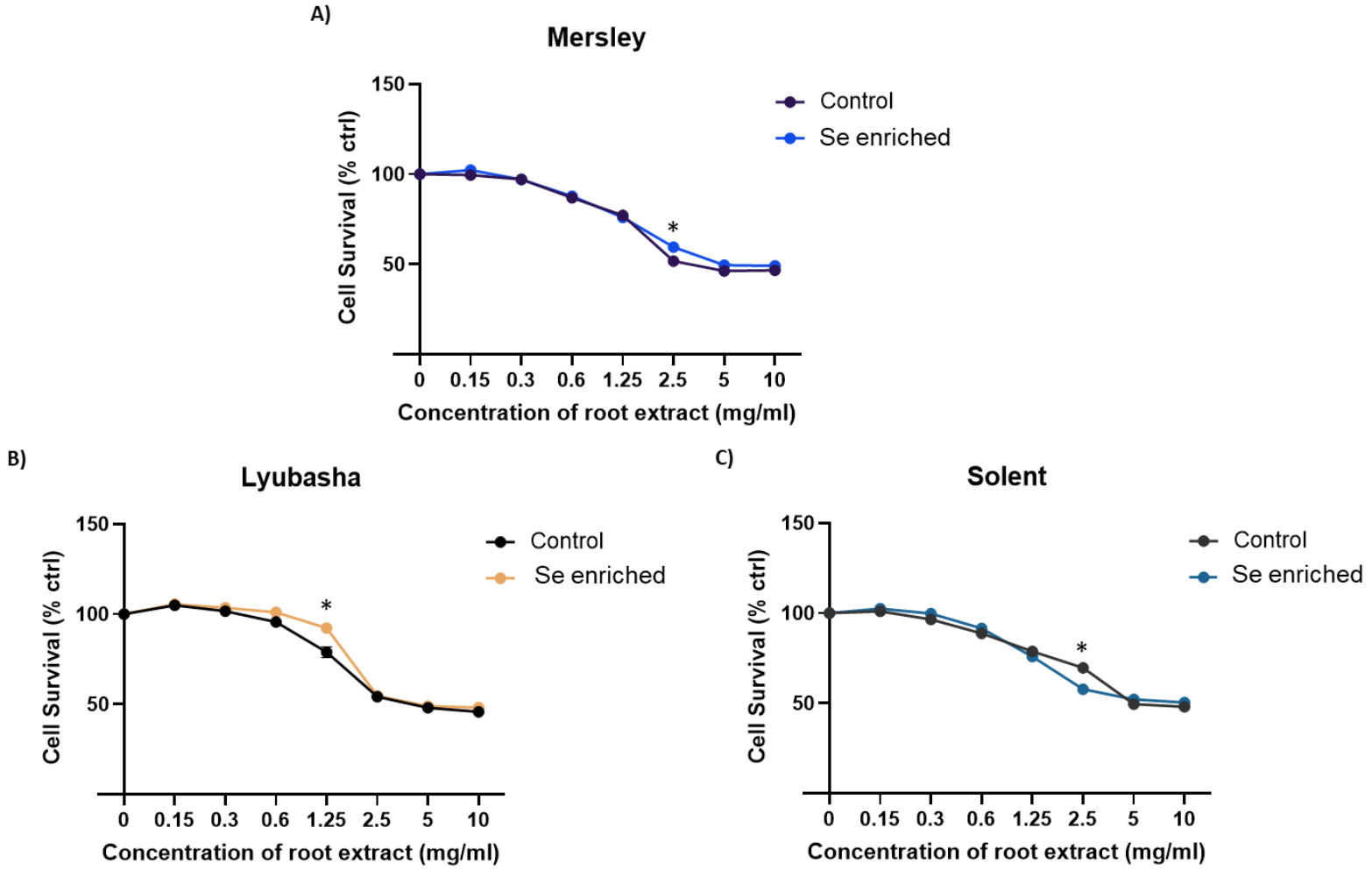

Figure 4.4- Cell survival/viability (%) of HepG2s after exposure for 24hrs to various concentrations (10mg/ml-0mg/ml) of either garlic control root extract or Se enriched root extract. Data represents mean ± standard error of the mean (n=27). Significance between control and Se enriched tissues is represented by *, $p<0.05$ as determined via independent t-test.

Table 4-4- IC_{50} values calculated from independent repeats of trial (n=27), data is representative of mean IC_{50} value ± SE. Significance was determined via independent t-test between control and Se enriched tissues, * signifies P<0.05. – denotes there was insufficient volume of Marco roots for analysis.

Cultivar	Tissue	IC_{50} values (mg/ml) (mean ± SE)	
		Control	Se enriched
Marco	Clove	**3.21** ±0.13	**3.07** ±0.13
	Shoot	**12.25** ±0.88	**12.44** ±1.17
	Root	-	-
Mersley	Clove	**1.87** ±0.06	**1.51*** ±0.06
	Shoot	**19.06** ±2.47	**18.37** ±3.02
	Root	**2.71** ±0.09	**3.76** ±0.33
Lyubasha	Clove	**3.17** ±0.21	**3.52** ±0.11
	Shoot	**11.10** ±1.14	**12.35** ±1.38
	Root	**2.85** ±0.14	**4.70** ±0.41
Solent	Clove	**1.87** ±0.06	**2.41*** ±0.06
	Shoot	**24.32** ±4.55	**13.66** ±1.13
	Root	**5.18** ±0.55	**2.83** ±0.09

4.4.4 Selenium Speciation of garlic extracts

One of the key questions that arose from this research was the form in which Se is found either as inorganic or organic Se compounds. Evidence shows that organic forms appear to be less toxic than their inorganic counterparts and this could go some way to explain the lack of correlation between Se content and loss in cell viability. Therefore, using HPLC-ICP-MS we identified and quantified the Se species accumulated in plant tissues (Figure 4.5 and 4.6).

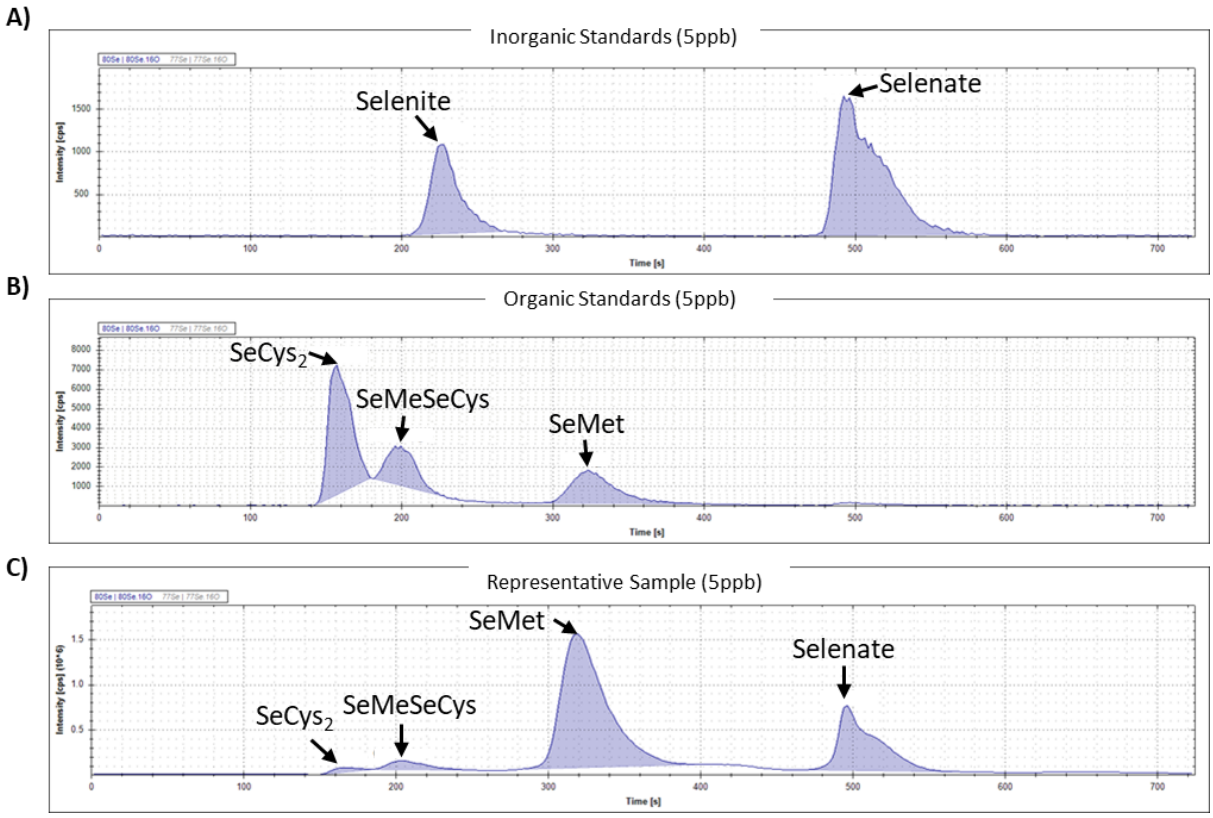

Figure 4.5-Chromatograms of Se speciation A) Inorganic, Selenate and Selenite standards, B) Organic standards, SeMeSeCys, SeMet and $SeCys_2$ at 5ppb. C) Representative sample showing peaks.

Using the cultivars Mersley and Solent as a tissue source we found that garlic preponderantly accumulates, SeMet > Selenate > $SeCys_2$ > SeMeSeCys in clove

tissues (Table 4.5). Other cultivars were not analysed due to limited tissue availability.

Hydroponically enriching the garlic cultivars Mersley and Solent with 50µM of sodium selenate was found to significantly increase the accumulation of the Se species, SeMet and Selenate in the clove tissues ($p<0.05$) compared to control tissues as well as $SeCys_2$ in the case of Solent ($p=0.004$) (Table 4.5). This suggests that the applied Se was rapidly incorporated into organic forms. The SeMet and Selenate accumulation in Se enriched tissues in proportion to other Se species was 93% and 4% respectively in the cultivar Mersley and 74% and 23% respectively in the cultivar Solent (Figure 4.6). This suggests that Mersley accumulated a greater proportional level of SeMet but a lower proportional amount of selenate as compared to Solent. However, Se enriched Solent clove tissues were found to accumulate significantly more SeMet (mg/kg) and inorganic Selenate (mg/kg) as compared to Se enriched Mersley tissues ($p=0.003$ and $p<0.001$). This suggests that Se speciation alone may not be fully responsible for the difference in cytotoxicity observed in Se enriched extracts as compared to control.

Table 4-5- Se speciation of control (C) and Se enriched clove tissues (E) from the cultivars Mersley (M) and Solent (S). Data is representative of mean ± standard deviation (n=3). Significance is determined by independent t-test and is displayed in light grey as a value below 0.05, the p value relating to C vs E tissues is to the right of the values, and the p value relating to M vs S is below the grey values.

	Organic Selenium Species (mg/kg)									**Inorganic Selenium Species (mg/kg)**		
	SeMeSeCys			**SeMet**			**$SeCys_2$**			**Selenate**		
Cultivars Clove Tissues	**Control**	**Enriched**	**Sig. C vs E p =**	**Control**	**Enriched**	**Sig. C vs E p =**	**Control**	**Enriched**	**Sig. C vs E p =**	**Control**	**Enriched**	**Sig. C vs E p =**
Mersley	**0**	**0.05** ±0.09	**.423**	**0.83** ±1.42	**7.07** ±0.28	**.014**	**0.08** ±0.08	**0.16** ±0.07	**.256**	**0.04** ±0.03	**0.33** ±0.13	**.020**
Solent	**0**	**0.08** ±0.14	**.423**	**0.01** ±0.01	**9.44** ±0.55	**.001**	**0.03** ±0.03	**0.22** ±0.05	**.004**	**0.01** ±0.01	**2.99** ±0.23	**.000**
Sig. M/S p =	**-**	**.774**		**.422**	**.003**		**.419**	**.257**		**.194**	**.000**	

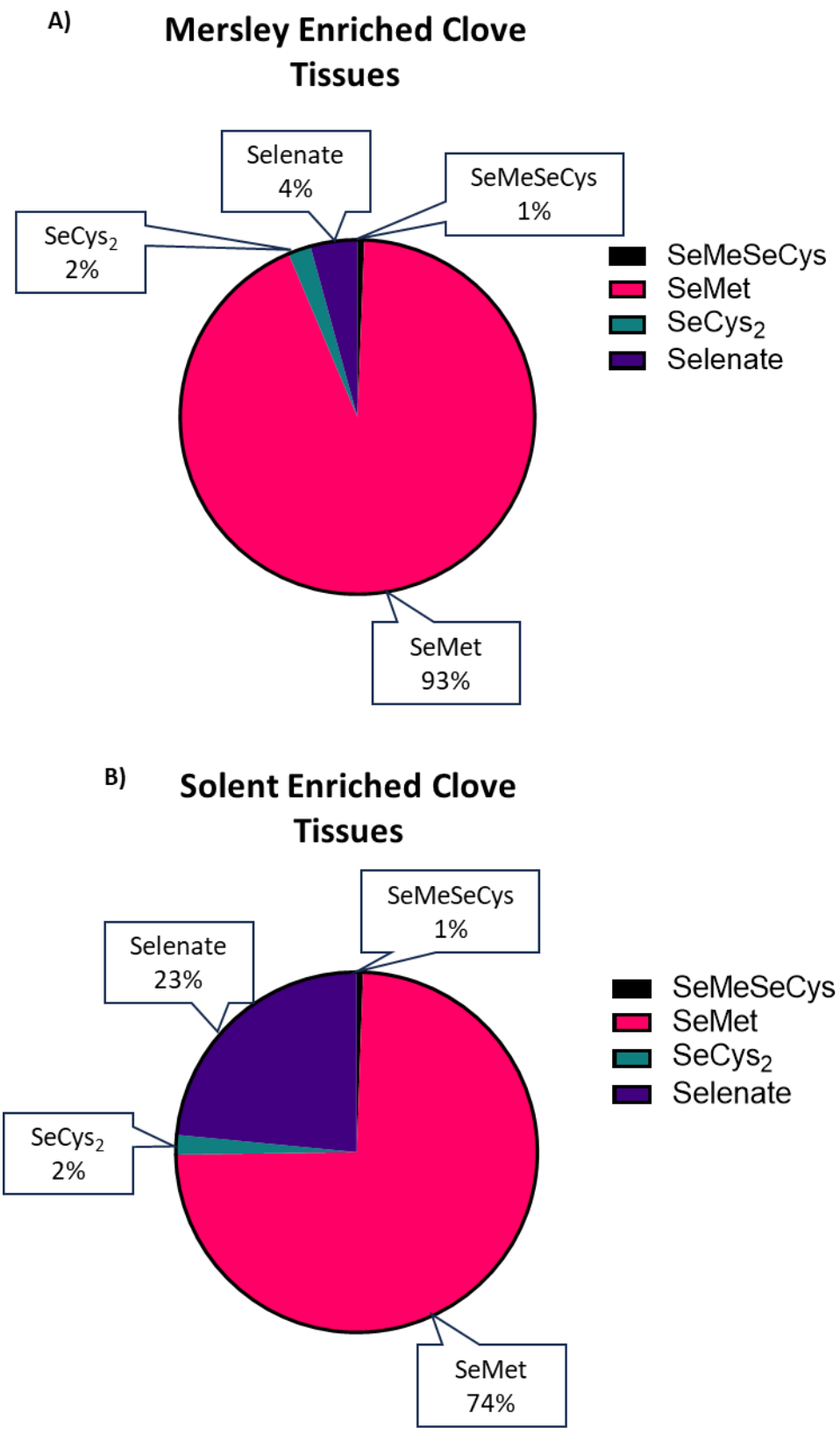

Figure 4.6- Proportion of Se Species in Se enriched garlic clove tissues (%) for A) Mersley and B) Solent

Hydroponically enriching Mersley garlic plants with 50µM of Se significantly increased the accumulation of the Se species, SeMet, $SeCys_2$ and Selenate in the shoot tissues and SeMeSeCys, SeMet, $SeCys_2$ and selenate in the root tissues compared to respective control tissues (Table 4.6).

The profile of Se species accumulated differed significantly between the tissues (clove, shoot and root) of the Se enriched garlic plants (Table 4.6). The roots accumulated the highest levels mg/kg of SeMeSeCys, SeMet and Selenate, whereas the shoots accumulated the highest levels of $SeCys_2$. In general, the clove tissues accumulated the lowest levels of Se species. However, proportionally, shoot tissues accumulated the greatest proportion of SeMet (96%) followed by clove (93%) and root (65%) (Figure 4.7). Root tissues accumulate the largest proportion of selenate (28%) as compared to 4% in clove tissues and 1% in shoot tissues (Figure 4.7). Our previous results show the greatest cytotoxic effects were observed in the clove and root tissues as compared to the shoot tissues. However, Se speciation reveals that despite differences in levels of Se species accumulated especially between clove and roots tissues, they exhibit a similar level of cytotoxicity. This suggests that Se species accumulated within these extracts might not be fully responsible for the differences in cytotoxicity seen (Table 4.4).

Table 4-6- Se speciation of control (C) and Se enriched clove (E), shoot and root tissues from the cultivar Mersley. Data is representative of mean ± standard deviation (n=3). Significance is determined by independent t-test between control and Se enriched tissues and is displayed in light grey box as a value below 0.05. Dark grey boxes containing a value less than 0.05 signify, a significant interaction between Se treatment and tissues (clove (Cl), shoot (S) and root (R)) in the accumulation of the specific Se species as determined by two-way ANOVA.

	Organic Selenium Species (mg/kg)									**Inorganic Selenium Species (mg/kg)**		
	SeMeSeCys			**SeMet**			**$SeCys_2$**			**Selenate**		
Mersley tissues	**Control**	**Enriched**	**Sig. C vs E p =**	**Control**	**Enriched**	**Sig. C vs E p =**	**Control**	**Enriched**	**Sig. C vs E p =**	**Control**	**Enriched**	**Sig. C vs E p =**
Clove	**0**	**0.05** ±0.09	**.423**	**0.83** ±1.42	**7.07** ±0.28	**.014**	**0.08** ±0.08	**0.12** ±0.07	**.256**	**0.04** ±0.03	**0.33** ±0.13	**.020**
Shoot	**0**	**0**	**-**	**0.87** ±1.44	**58.20** ±2.60	**<.001**	**0.13** ±0.03	**2.02** ±0.22	**.004**	**0**	**0.35** ±0.05	**<.001**
Root	**0**	**9.97** ±1.85	**.011**	**2.29** ±3.31	**106.17** ±9.66	**<.001**	**0.08** ±0.09	**1.00** ±0.11	**<.001**	**0.03** ±0.02	**45.05** ±9.13	**.013**
Sig. Cl/S/R p=	**-**	**<.001**	**<.001**	**.680**	**<.001**	**<.001**	**.633**	**.000**	**<.001**	**.160**	**.000**	**<.001**

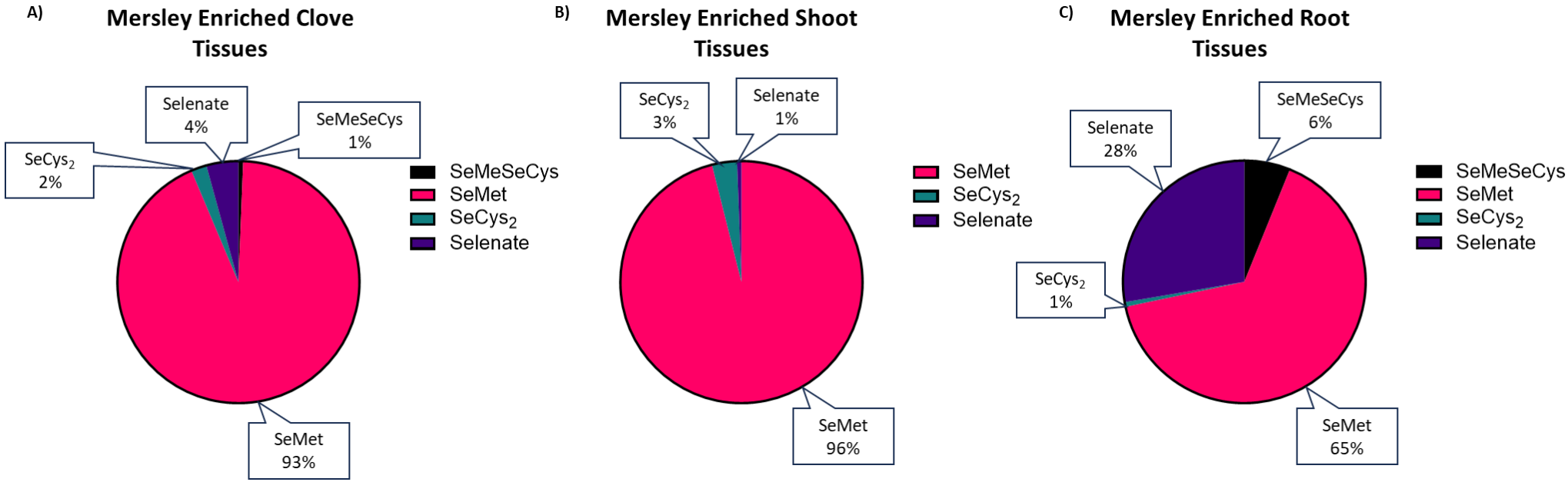

Figure 4.7- Proportion on Se species in Mersley garlic tissues (%) of A) Clove, B) Shoot and C) Root

4.4.5 Expression of SCLY, SelP and GPx activity after incubation with Se enriched extracts

To assess the impact of garlic extract on Se status of HepG2 cells, we measured the protein levels of Selenocysteine lyase (SCLY), Selenoprotein P (SelP) and Glutathione peroxidase (GPx) as they are markers of Se uptake and incorporation. The cultivar Mersley was chosen due to the significantly increased cytotoxicity between control and Se enriched tissues at the IC_{50} on HepG2 cells. Marco was used as a control as the cytotoxicity between control and treatment was not significantly different at the IC_{50} concentration. In order to maintain cell numbers and achieve enough protein for the assay, 0.6mg/ml dose of garlic extracts were chosen as they maintained >80% survival for both cultivars and treatments. A positive control of 30nm sodium selenite was used to compare the effects of garlic tissues to the application of solely Se. HepG2 cells were treated for 24hrs, before being scraped and a Lowry protein assay was carried out to normalise protein levels for gel loading.

Western blot analysis revealed that application of either control or Se enriched tissues of either Marco or Mersley had no significant impact on the expression of either SelP or SCLY compared to untreated control cells ($p>0.05$) (Figure 4.8A and B). Similarly, no significant differences in protein expression of SelP or SCLY were observed between Se enriched clove extract and control clove tissues for either cultivar ($p>0.05$). Although HepG2 cells treated with Se enriched Marco clove extract showed a marginally decreased expression of SelP, although not significant, as compared to HepG2 cells treated with control garlic clove extract. Our results also showed that there was an insignificant difference in SelP and SCLY protein expression between the cultivars Marco and Mersley regardless of Se treatment

(p>0.05). Treatment of cells with 30nm of sodium selenite also caused no significant increase in expression of either SelP or SCLY compared to untreated control cells as well as other garlic extract treatments (p>0.05).

Western blot analysis on the expression of the target protein GPx4, a key antioxidant, were attempted using GPx4 antibody. Unfortunately, the primary antibody for this target protein showed limited cross reactivity with the target enzyme. Manipulation of incubation times and temperatures failed to resolve this issue. A glutathione peroxidase (GPx) activity kit was used to assess the impact of Se enriched garlic extracts on HepG2 cells. No significant difference was found in GPx activity between control and any of the garlic extracts or 30nm selenite treatments (p>0.05)(Figure 4.8C). Although not significant a general trend in reduced GPx activity of Mersley treated HepG2 cells was seen as compared to Marco treated HepG2 cells. Se enrichment of either cultivar also appeared to further decrease GPx activity as compared to untreated control extracts although significance was not obtained. The high standard deviation between repeats may be causing this result, a larger number of repeats would have been desirable although this could not be done due to a limited amounts of garlic extract and delivery delays of the kits.

Overall, our results suggest that despite the application of Se enriched garlic extracts to HepG2 cells, there was a lack of Se incorporation into cells after 24 hours. This also may suggest that Se needs liberating from amino acid/protein sources by in vitro simulation of digestion processes in order to positively impact on Se incorporation.

To further understand the impact of Se enriched garlic extract on Se status of HepG2 cells, time and concentration dependent experiments are needed to assess the fluctuations of expression of SCLY, SelP and GPx activity. Sadly, time, availability of commercial kits and limited supply of garlic extract prevented these experiments from being possible in the current work. Further work assessing the repeated exposure of HepG2 cells to garlic extracts may also further clarify us about the toxicity and impact of regular consumption of Se enriched products.

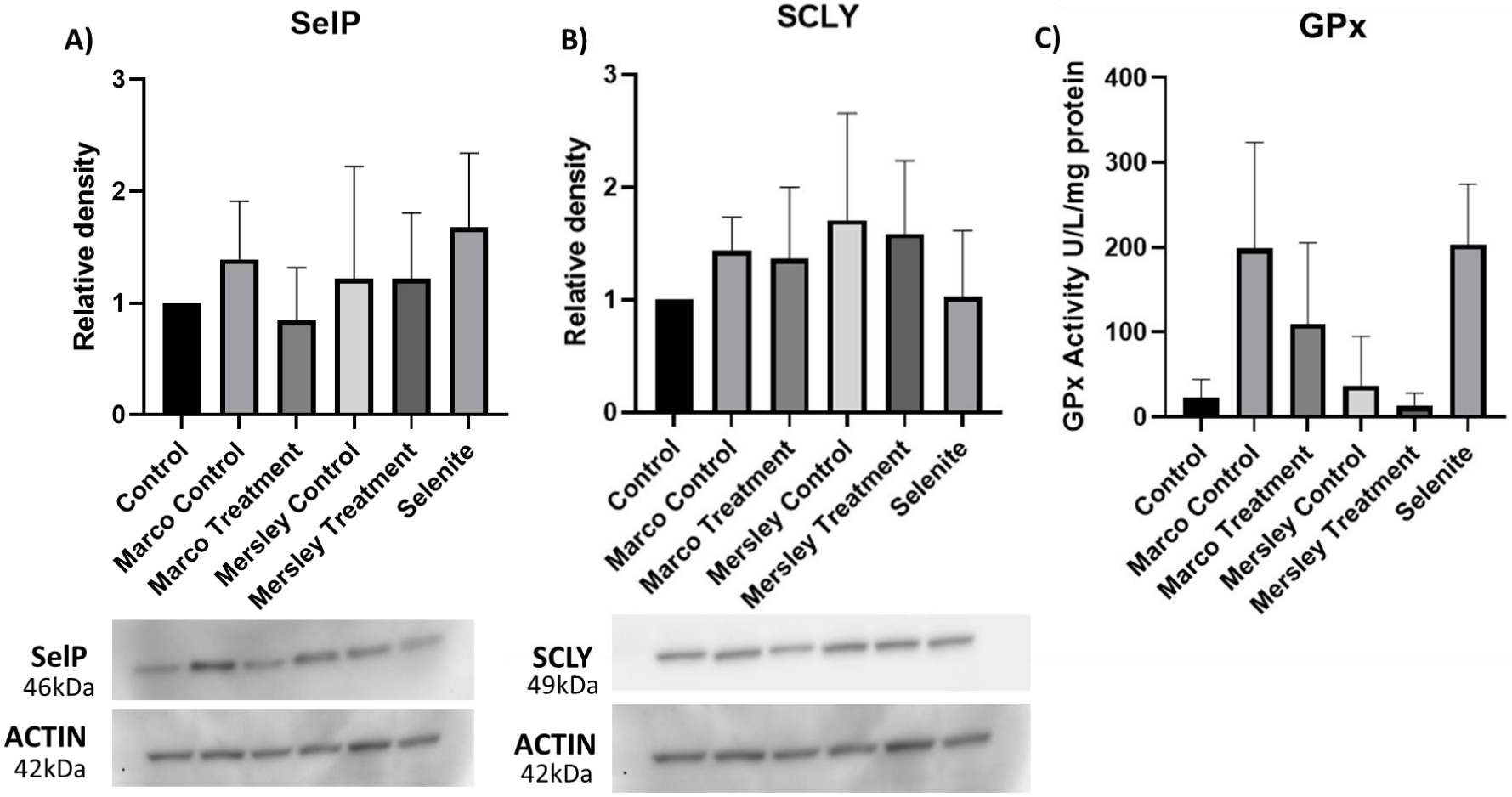

Figure 4.8- Western Blot analysis of SelP, SCLY and GPx activity in HepG2 cells after incubation with 0.6mg/ml garlic extracts for 24 hours A) Relative density of SelP expression/relative density of actin (loading control). B) Relative density of SCLY expression/relative density of actin (loading control) c) GPx activity U/L/mg protein of garlic extracts and 30nm sodium selenite applied to HepG2 cells. Unit stands for µmol/min normalised to protein (mg) via the Lowry protein assay. Data is representative of mean ±standard deviation (n=3).

4.5 Discussion

In Chapter 3 we found that foliar application of Se failed to cause significant accumulation of Se into plant tissues or organs. Therefore, in the current chapter we sought to determine if we could enrich garlic via a hydroponic method. The results from our study suggest garlic to be a secondary accumulator as shown by the total Se content being in the range of 100-1000ug/g DW as determined by Gupta and Gupta (2017). The total Se uptake in garlic plants was substantially higher in the current study compared to that of foliar enriched garlic from the previous chapter (0.09-0.12 mg Se/kg DW (depending on cultivar)) and Põldma et al. (2011) (0.626mg Se/kg DW) (Table 4.1). Several studies have reported conflicting information as to the provision of foliar application to enhance Se levels in alliums. To circumvent this problem some researchers and commercial producers such as SelenoForce® have suggested provision of hydroponics to biofortify crops like garlic. As compared to our foliar study this research indicates hydroponics to be a successful method of enriching garlic with Se. Se enriched cloves accumulated between 7.14 mg Se/kg DW and 40.05 mg Se/kg DW depending on cultivar. These higher Se levels compared to those in our foliar study support the efficiency of Se uptake in alliums under hydroponic conditions. This could be due to more efficient root uptake as compared to foliar uptake in garlic plants, as lower levels of Se accumulation has also been seen in Maize plants foliar enriched as compared to enriched via the roots (Wang et al., 2020b). Comparable levels of Se uptake and partitioning in garlic seedlings to the current study was reported by Li et al. (2020b), with roots accumulating the greatest amount of Se (100mg Se/kg DW), followed by shoots (75mg Se/kg Se) and cloves (60mg Se/kg Se) after treatment with 50µM of sodium selenate hydroponically. In

contrast to the vast majority of literature that analyses the uptake of Se within a single cultivar, we elected to compare and analyse several garlic cultivars in our work. This provided us with a better picture of the complexity of nutrient uptake between garlic cultivars and highlighted genetic variability in Se uptake between cultivars. The reasoning for this, although not studied in the current work could be due to expression of SULTR transporters, assimilation efficiency of Se (Zhang 2006) and antioxidant properties (Tamaoki 2008). Therefore, we assessed accumulation and speciation in hydroponically grown plants. Interestingly, we report that Se accumulation varied not only between tissues but also between cultivars. To date, a similar pattern of cultivar specific accumulation has only been described for maize (Płaczek and Patorczyk-Pytlik, 2021) and tomatoes (Zhao et al., 2017). We also found that Se was readily incorporated into clove tissues in the form of various organic amino acids namely, SeMet, $SeCys_2$ and inorganic selenate (Table 4.5). Importantly, the biological properties of these chemical species vary widely. Organic forms of Se are known to have anticancer (reviewed in Kuršvietienė et al. (2020), anti-inflammatory (reviewed in Duntas (2009)) and cardioprotective effects (reviewed in Sauheitl et al. (2009)). However, few studies have determined whether biofortification of crops with Se alters the bioactive nature of plant tissues and extracts. This could be important since Se biofortification approaches could be used to enhance the nutritional composition of crop plants or potentially raise the risk of toxicity. Interestingly, we report that the cultivar Mersley had significantly increased SeMet and selenate levels in clove tissues as compared to control tissues and significantly increased toxicity in our study (Table 4.4). This is in contrast to Se enriched Solent that accumulated significantly more SeMet, $SeCys_2$ and Selenate as

compared to control tissues and the cultivar Mersley, but had a limited toxicity in our study (Table 4.4). The finding of significantly different Se speciation not only between treatment groups but also between cultivars has previously not been seen as Perez et al. (2021) found no difference in Se species accumulation between four other cultivars of garlic (Nieve INTA, Union FCA, Gostoso INTA and Rubí INTA). Our findings suggest Se speciation is not solely responsible for the increased cytotoxicity observed in Se enriched Mersley extracts. Szychowski et al. (2018) found a difference in the production of ROS and LDH leakage of two cultivars of garlic, Harnaś and Morado when applied to human squamous cell line SCC-15 cells. Although we did not measure these indices within this trial perhaps it could explain the difference in cytotoxicity between Se enriched Mersley and Solent extracts.

Aside from the assessment of Se tissue distribution other researchers have also assessed the impact of Se on other key micronutrients such as S, P and Ca. Historically, Se has been shown to compete with S for accumulation in plants. Indeed, studies in *Arabidopsis thaliana* has shown that high sulfate in the rhizosphere results in the inhibition of the uptake of selenate, whereas high rhizosphere selenate promotes sulfate uptake (White et al., 2004). In contrast, other studies have shown only an antagonistic relationship between these two elements (Dhillon and Dhillon, 2000, Tsuneyoshi et al., 2006). This antagonistic response is thought to be due to the shared use of SULTR transporters located in the roots (Gupta and Gupta, 2017) although the level of antagonism varies across species (Terry et al., 2000). Therefore, in the current research we used a low-sulfate hydroponic solution as per the work of Tsuneyoshi et al. (2006) to facilitate Se uptake. In the present study, there was no significant change in the accumulation of

S between control and Se enriched plants in the majority of cultivars and tissues, only a significantly decreased S accumulation was reported in the clove tissues for the cultivar Marco (Table 4.2). Our results also showed no effect of Se enrichment on the accumulation of Na, Mg, P, K and Ca in clove tissues (Table 4.2) as supported by other studies conducted in basil, scallions and curly endive (Newman et al., 2021, Sabatino et al., 2019).

Treatment of HepG2 cells with ≥0.6-1.25mg/ml garlic extract (depending on cultivar) resulted in a decrease in cell survival similarly (Figure 4.2-4.4), supplementation of up to 1mg/ml was also found not to cause a significant decrease in HepG2 cell survival by Siegers et al. (1999). The cytotoxic effect of garlic has also been shown on other cancer cell lines such as breast cancer (MCF-7), gastric cancer (AGS) (Ghazanfari et al., 2011), prostate cancer (PC-3) (Bagul et al., 2015) and squamous carcinoma (SCC-15)(Szychowski et al., 2018). Our results found that Se enrichment did not impact on cytotoxicity of extracts for the majority of cultivars and tissues although significant differences in IC_{50} dose between Se enriched clove extracts and control clove extracts in the cultivars Mersley and Solent were seen (Table 4.4). We also observed that clove and root extracts were the most cytotoxic as compared to shoot extracts, despite clove tissues accumulating much lower levels of Se as compared to root and shoot tissues. Therefore, we postulated that these differences were due to the differential accumulation of Se species within control and Se enriched tissues as well as different tissues of the garlic plant and cultivars.

The majority of literature surrounding the Se speciation of garlic is conducted in field grown trials and focusses on the Se species accumulated within the clove tissues.

Although the drive to understand what species are accumulated in the edible portion of the plant is important, very few studies in garlic have assessed the accumulation of Se species in other tissues such as the shoots and roots, in order to provide a broader understanding of Se species accumulation and localisation within the plant. The present data reveals for the first time to our knowledge the Se speciation of hydroponically enriched garlic clove, shoot and root tissues of the cultivar Mersley (Table 4.6).

Previous literature has found SeMeSeCys to be the main form of Se species within Se enriched garlic (Dumont et al., 2006, Gupta and Gupta, 2017, Tsuneyoshi et al., 2006). However, in the current study, SeMet was found to be the major form of Se species in cloves, shoot and roots for the cultivar Mersley (Table 4.6). Our results mirror that of Se enriched grain crops such as wheat, barley and rye which have been found to accumulate SeMet as the predominant form of Se species making up 60-80% (Stadlober et al., 2001). SeMet has also been found to be the major Se species in other vegetable crops such as carrots (Bañuelos et al., 2016) and potatoes (Zhang et al., 2019a). Our results are also supported by that of Muleya et al. (2021) in which 90% of extracted Se from Se enriched maize, groundnut and cowpea was in the organic form. The lack of accumulation of SeMeSeCys in tissues, in particular clove tissues seen in the current work could be due to the juvenile nature of the garlic plants when harvested at 4 weeks of age, compared to fully bulbed, as in previous literature. Perez et al. (2021) assessed the distribution, accumulation and speciation of Se at different growth stages (6-8months) of garlic in a field trial setting enriching garlic cultivars with 0-15kg Se ha^{-1} sodium selenate and selenite liquid fertiliser. They found that organic Se species were mostly found in bulbs at the latter

stage of growth (8 months), compared to early stages and that organic species decreased in roots over time which was postulated to be due to the action of bulbing. Again, Se speciation does not clarify why roots and clove extracts are more cytotoxic compared to shoot extracts.

One method to assess the incorporation of Se into Se containing proteins of the garlic extracts applied to HepG2 cells was to assess the impacts on markers such as SCLY, which are involved with catabolism of selenoproteins and selenoproteins such as SelP and GPx (Figure 4.8). As far as we understand, no other study has assessed the impact of treating cells with Se enriched garlic extract on these markers of Se status. In this study, we observed no difference in SCLY and SelP expression or GPx activity between control and Se enriched extract treated HepG2 cells. However, additional replicates and further studies of exposure over time and concentration, as well as the use of invitro digestive methods to simulate the digestive processes and liberate Se from proteins, may give a better overview of the impacts of Se enriched garlic tissues and Se incorporation into mammalian cells. Similar results showing no significant difference in GPx activity have been seen in rat glial cells exposed to low Se broccoli (containing 0.58–2.31 nM Se) for 48 hours as compared to control (Yeh et al., 2006). The expression of SelP mRNA was also unaltered in Rats fed either Se enriched mushrooms or Se enriched yeast as compared to control (Maseko et al., 2014). This could potentially suggest that consumption of Se enriched foods needs to be over a longer-term in order to generate a positive impact on Se status in vitro and in vivo.

4.6 Conclusion

In conclusion, our study identifies hydroponics to be a successful method for the biofortification of garlic with Se. This comparing well to the studies of Tsuneyoshi et al. (2006) and Li et al. (2020b). Moreover, we found no significant impacts on the accumulation of other important minerals such as Na, Mg, P, S, K and Ca in clove tissues. We also report significant variation in the accumulation of Se within shoot tissues, that varies between commercial cultivars. While our initial hypothesis proposed that differences in cytotoxicity of plant extracts would correlate with Se levels, our results suggest additional research is needed. Se enrichment of garlic was found to increase the levels of SeMet, $SeCys_2$, selenate and SeMeSeCys within plant tissues, however, speciation analysis does not fully explain the differences in cytotoxicity seen in the cultivars Mersley and Solent. Overall, this illustrates that future studies need to consider the impact of cultivar Se accumulation, speciation and impact on cytotoxicity, in order to select the best cultivar of garlic for Se enrichment. Our results suggest that hydroponic cultivation offers an effective method of Se enrichment, although further studies are needed on whether hydroponic Se enrichment impacts on growth and yield of garlic.

5 Development of a human hepatoma HepG2 cell culture model to assess Se cytotoxicity.

5.1 Abstract

Selenium (Se) is an essential micronutrient for human health. Current research is centring on increasing the amount of Se in food via biofortification. We have validated a human hepatoma liver cell model to assess the biological activities of Se species, SeMet, $SeCys_2$, SeMeSeCys, Selenite and Selenate found in edible plants. Our results show that the toxicity of Se in HepG2 cells is directly related to the Se species supplied. Treatment of cells with up to 125µM of Se species for 24 hours resulted in a concentration dependent decrease in cell survival with $SeCys_2$ (IC_{50} 73µM) and selenite (IC_{50} 75µM), whilst SeMet, SeMeSeCys and Selenate had no appreciable effects on cell viability. However, in the presence of reduced glutathione (GSH), a key intracellular antioxidant, the toxicity of selenite is significantly increased (IC_{50} 15µm) ($p<0.05$). Time dependent cytotoxicity curves and fluorescent microscopy indicate that toxicity of selenite + GSH mix is driven by a short-lived (< 10 minute) toxic reaction intermediate. Additional studies to understand the molecular mechanisms of action were conducted to assess the reaction between selenite and GSH. Our research shows that although selenite mediated cell death occurs via caspases ($p<0.05$), pre-treatment of cells prior to the addition of selenite + GSH with caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK does not rescue cell survival ($p=0.273$ and $p=0.974$ respectively), potentially suggesting caspase independent

apoptosis. Significantly increased PARP cleavage was found in cells exposed to selenite +GSH ($p=0.002$). We demonstrated that despite a time dependent increase in ROS ($p<0.05$) and lipid peroxidation, incubation of cells with a range of radical scavenging compounds including hydroxyl, peroxyl and H_2O_2 scavengers alongside selenite + GSH mix did not significantly increase cell survival ($p>0.05$). This suggests ROS production may not solely be responsible for cell death. H_2Se is another short-lived reaction intermediate produced in the reaction between selenite and GSH and is postulated to be at least partly involved in mediating cell death. This research highlights the need for further understanding into the toxicity mechanisms of Se species that may be potentially accumulated in Se biofortified crops in order to provide a safe and effective solution for increasing Se intake within the UK population.

Keywords: Selenium, Apoptosis, Glutathione.

5.2 Introduction

In chapter 4 we showed that Se application to garlic cloves using hydroponic systems resulted in increased amounts of inorganic and organic forms of Se in plant tissues. Indeed, biofortification to aid Se enrichment promoted the accumulation of Selenocystine ($SeCys_2$), Selenomethionine (SeMet) and Selenomethylselenocysteine (SeMeSeCys) and inorganic forms such as selenate in garlic tissues. This correlates with past research conducted in garlic (Dumont et al., 2006, Perez et al., 2021, Tsuneyoshi et al., 2006), although Se enrichment studies have also found the presence of selenite (Luo et al., 2021). To develop this work further and better the understanding of the mechanism of Se bioactivity we developed a human hepatoma HepG2 model as a screening system to evaluate Se containing compounds found in biofortified food.

Previous studies highlight oxidative stress mechanisms in the biological role of Se, since Se is important in maintaining several antioxidant enzymes in mammalian cells. To date, most work has explored DNA damage (Wallenberg et al., 2014), cell cycle inhibition (Cheng et al., 2021), ROS production (Gonçalves et al., 2013) and subsequent interaction with known signalling pathways (An et al., 2013, Guan et al., 2009). However, studies by Tarze et al. (2007) indicated an alternative mechanism of toxicity caused by Se compounds in yeast. This seminal piece of research proposed that, aside from ROS mediated mechanisms of toxicity, other chemical processes that involve the generation of hydrogen selenide and other Se containing chemical species may also be important. Indeed, Tarze et al. (2007) and others have shown that the interaction of Se compounds with cellular thiols generates the superoxide

anion ($O_2^{\cdot -}$), and in turn promotes DNA damage (Kim et al., 2003, Peyroche et al., 2012, Tarze et al., 2007). In addition, the possibility of generating H_2Se is intriguing since this molecule serves as the metabolic intermediary of inorganic and organic Se metabolism in mammals. To date, no study has yet repeated the work of Tarze and colleagues in mammalian systems. This is important since recently, some researchers have proposed that H_2Se could be the fourth gaseous signalling molecule in mammalian systems produced following the catabolism of Se containing amino acids and via the reaction of Se compounds with cellular thiols (Kuganesan et al., 2019). Crucially, exogenous application of H_2Se mirrors many of the known biological properties of other gaseous signalling molecules such as nitric oxide, hydrogen sulfide and carbon monoxide. Therefore, the current chapter contains research focused on the development and validation of a human hepatoma HepG2 cell model to assess the biological activities of Se species identified in our hydroponic study (Chapter 4). This cell model has the advantage that HepG2 are already widely used for drug and toxicity testing with the liver being the main detoxifying organ and place of Se metabolism in the human body (Roman et al., 2014). In addition, I also wanted to explore the work of Tarze et al. (2007) and determine whether reactions with extracellular thiols could enhance Se toxicity.

Aims and Objectives

- to assess the toxicity of different Se species, $SeCys_2$, SeMet, SeMeSeCys, selenate and selenite on HepG2 cells
- to assess the toxicity of these Se species in the presence of thiols and the impact on cytotoxicity

- to investigate the potential mechanism of toxicity

Hypothesis

The main hypothesis was that the cytotoxicity of Se compounds in our cellular model would differ between inorganic and organic species. Moreover, this toxicity would be enhanced by the addition of biologically important thiols.

5.3 Materials and methods

5.3.1 General Chemicals

Reduced Glutathione (GSH), L-Buthionine-sulfoximine (BSO), N-acetyl-L-cysteine (NAC), Uric Acid, Sodium hydrosulfide (NaHS), Cytochrome C (bovine heart) and Catalase (bovine liver) were all from Sigma-Aldrich (St Louis, USA). Ascorbic acid was from Fisher Scientific UK ltd (Loughborough, UK). Trolox was from EMD Millipore Corp (Burlington, USA).

5.3.2 Cell culture

Human hepatoma cells (HepG2 cells) were cultured as detailed in 2.4.1-2.4.4. Cells were seeded at a seeding density of 10,000 cells/well in clear 96 well plates (Costar Flat Bottom with Lid) for the assays of Selenium compounds +/- GSH, pre-treatment and co-treatment with NAC and BSO, reactions with other thiols, caspase inhibition and Se + antioxidants. Cells were seeded at 20,000 cells/well in clear 96 well plates for the lactate dehydrogenase assay. Cells were seeded at 10,000 cells/well in black (with clear bottoms) (µCLEAR®, BLACK CellStar® F-Bottom) plates for DCFDA assays. Cells were seeded at 400,000 cells/well in clear 6 well plates for the measuring GSH and cleaved PARP ELISA.

5.3.3 Cell cytotoxicity

Cell viability was assessed using the crystal violet assay as detailed in 2.5.1 and results are expressed as % cell survival as previously described (Feoktistova et al., 2016).

5.3.4 Cell viability Assays

Cytotoxicity of Se species

Cells were treated with a range of concentrations of each Se compound, sodium selenite, sodium selenate, selenomethionine and Se-(Methyl)selenocysteine hydrochloride, L-selenocystine, 0, 1.5, 3, 6, 15, 30, 60 and 125µM dissolved in FBS free media. Cell viability was assessed after 24 hours as detailed in 2.5.1.

Cytotoxicity of Se species in presence or absence of glutathione

In studies using thiol manipulation cells were treated with a range of concentrations of each Se compound, 0, 1.5, 3, 6, 15, 30, 60 and 125µM +/- 500µM of GSH. Plates were again incubated for 24 hours at 37°C and 5% CO_2 after which cell viability was assessed as detailed in 2.5.1.

Manipulation of intracellular glutathione levels

The cellular biosynthesis of GSH can be manipulated to assess the effect of the presence of intracellular GSH using L-buthionine sulfoximine (BSO) an inhibitor of γ-glutamylcysteine synthetase and N-acetylcysteine (NAC) a GSH biosynthesis stimulant (Shen et al., 2000). Cells were pre-treated for 24hrs with 2.5mM BSO or 500µM NAC diluted in FBS free DMEM before being treated with a range of concentrations (0, 1.5, 3, 6, 15, 30, 60 and 125µM) of sodium selenite, sodium selenate and selenocystine diluted in FBS free DMEM for a further 24hrs. For co-treatment, cells were treated with Se species diluted in FBS free DMEM containing 2.5mM BSO or 500µM NAC for 24hrs and pH was checked prior to addition to cells. After 24 hours, cells were imaged using EVOS and cell viability was assessed as detailed in 2.5.1.

Reaction with other thiols

Cells were treated with either control (FBS free media) or 500µM of either glutathione (GSH), glutathione disulphide (oxidised glutathione) (GSSG), L-cystine, L-cysteine +/- 75µM of sodium selenite. Cells were then incubated for 24 hours at 37°C and 5% CO_2, before media was removed, cells were washed in PBS and cell viability was assessed as detailed in 2.5.1.

Age dependent cytotoxic effect of Selenite + GSH mix

A reaction mixture containing 15µM selenite + 500µM GSH (IC_{50}) was made up and a timer was started after both components had been added. Reaction mixture was added to cells at 0, 5, 10, 20, 40 and 60 minute time points. Plates were then incubated at 37°C and 5% CO_2 for 24 hours after which cell viability was assessed as detailed in 2.5.1.

Incubation of Selenite ± GSH with Antioxidants

Cells were treated with 75µM of sodium selenite +/- 500µM of GSH in the presence of 500µM of Ascorbic acid, N-acetyl cysteine, Trolox, Catalyse, Cytochrome C, Uric acid and Sodium hydrosulphide for 24 hours before cell viability was assessed as detailed in 2.5.1.

5.3.5 Measures of DNA damage - 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) staining

Cells were treated with 15µM sodium selenate and 500µM reduced glutathione mix prepared moments before treatment, referred to as 'fresh mix' and 15µM sodium selenate and 500µM reduced glutathione mix prepared and allowed to age for 1hr prior to treatment, known as 'aged mix'. Control was FBS media and positive control

consisted of 1mM H_2O_2 dissolved in sterile Earle's balanced salt solution (EBSS) containing 1M Calcium chloride, 810µM Magnesium sulfate heptahydrate, 5M Potassium chloride, 26mM Sodium bicarbonate, 117mM Sodium chloride, 1mM Sodium phosphate monobasic monohydrate and 6mM D-Glucose (Dextrose) and the pH was adjusted to pH7. Plates were then incubated again at 37°C and 5% CO_2 for 24hrs before DAPI staining as described in 2.5.2. Images were taken on EVOS Fluorescent microscope.

5.3.6 Measure of membrane leakage as a marker of apoptosis- Lactate dehydrogenase (LDH) leakage

To detect leakage of LDH the CyQUANT™ LDH Cytotoxicity Assay Kit (C20301 Invitrogen by Thermo Fisher Scientific) was used as described in 2.5.3.

Cells were treated with either FBS free media (control), 15µM sodium selenite and 500µM reduced glutathione mix diluted in FBS free media prepared moments before treatment referred to as 'fresh mix', 15µM sodium selenate and 500µM reduced glutathione mix diluted in FBS free media prepared and allowed to age for 1hr prior to treatment known as 'aged mix'. The kit also required maximum LDH activity wells, cells were treated with 100µl of FBS free media only. Plates were again incubated for 24 hours. LDH leakage was measured according to manufacturer's instructions.

5.3.7 Caspase and PARP determination of involvement

To assess a role of caspase in the cytotoxic response of cells to Se compounds inhibitory studies were conducted using caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK as detailed in 2.5.4. Cells were treated with control (FBS free media), 75µM selenite (IC_{50}) or 75µM selenite + 500µM GSH for 24 hours after pre-treatment with

caspase inhibitors before cell viability was assessed via crystal violet assay as described in 2.5.1.

For determination of cleaved PARP, cells were treated with control FBS free media, 75µM of sodium selenite +/- 500µM of GSH and 1 hour aged 75µM of sodium selenite + 500µM of GSH mix for 24 hours before cells were harvested. ELISA cell extraction and PARP measurement followed according to cleaved PARP 214/215 ELISA kit (Thermo Fisher Scientific) following manufacturers guidelines as detailed in 2.5.5.

5.3.8 ROS Detection methods

Measures of intracellular ROS production were determined using DCFDA and lipid peroxidation using a commercially available Image-It^{TM} Lipid Peroxidation Kit (C10445 -Invitrogen by Thermo Fisher Scientific) both methods are detailed in chapter 2. Cells were incubated with DCFDA (as detailed in 2.5.6) for 1 hour prior to cell treatment with 15µM sodium selenate and 500µM reduced glutathione mix diluted in HBSS prepared moments before treatment referred to as 'fresh mix', 15µM sodium selenate and 500µM reduced glutathione mix diluted in HBSS prepared and allowed to degrade for 1hr prior to treatment known as 'aged mix'. 1mM of H_2O_2 diluted in HBSS was used as positive control during this experiment. 100µl of HBSS was added to cell-free wells and this was referred to as 'background'. Each treatment was replicated 6 times per plate with 3 independent replicates performed on separate days with freshly prepared reagents. Fluorescence was measured using the FluoStar Omega fluorescent plate reader (485/520nm). Data was

exported to Microsoft excel, where background fluorescence was deducted from all measurements of fluorescence.

For assessment of lipid peroxidation, Image-It™ Lipid Peroxidation Kit (C10445 - Invitrogen by Thermo Fisher Scientific) was used to label oxidised and reduced cell membranes. Upon oxidation of lipids the peak emission shifts from red (~590nm) to green (~510nm).

Cells were treated with 15µM sodium selenate and 500µM reduced glutathione mix prepared moments before treatment referred to as 'fresh mix' and 15µM sodium selenate and 500µM reduced glutathione mix prepared and allowed to age for 1hr prior to treatment known as 'aged mix'. Control was FBS media and positive control consisted of 100µM of cumene hydroperoxide 2ml/well diluted in PBS (provided in kit). 'Fresh mix' and 'aged mix' were imaged at 30, 60 and 120 mins, meanwhile control and positive control were imaged at the 120 min endpoint. Lipid peroxidation was measured as per the manufacturers instructions as detailed in 2.5.7.

5.3.9 Determination of Intracellular Glutathione

HepG2 cells were treated with 500µM NAC, 2.5mM BSO or solely FBS free media for 24 hours prior to cells being scraped into Eppendorf tubes. Reduced glutathione (GSH) was measured using 'Quantificantion kit for oxidised and reduced glutathione-38185' from Sigma-Aldrich. The manufacturers guidelines were followed in full. In brief, scraped cells were centrifuged at 200xg for 10 minutes at 4°C, after which the supernatant was discarded. Cells were then washed with PBS and the above step repeated. 80µl of 10mmol/L HCl was then added to the cell pellet and cells were

lysed via freeze-thawing for 2 cycles. 20µl of 5% SSA was then added to the lysed cells and centrifuged for 10 minutes at 8,000xg. The resulting supernatant was transferred to a new Eppendorf and dH_20 was added to reduce the SSA concentration to 0.5%. A concentration range of GSH standard solutions provided in the kit were serially diluted in 0.5% SSA to give the concentrations of 50, 25, 12.5, 6.25, 3.13, 1.57 and 0µmol/L GSH. 40µl of standards and samples were added to a 96 well plate followed by 120µl of buffer solution. The plates were then incubated at 37°C for 1 hour. After incubation, 20µl of substrate working solution, 20µl of coenzyme working solution and 20µl of enzyme working solution were added to each well and the plate was incubated for 10 minutes at 37°C. Plates were then read on a BMG Fluostar plate reader at a wavelength of 415nm. The concentration of GSH was then determined following instructions from the manufacturer by using y=mx+c equation of the standard curve.

5.3.10 Data handling and stats

Data is represented as mean ± standard error of the mean (SE). Independent t-tests were used when comparing between two groups whereas one-way ANOVA was used when comparing between three or more groups followed by post-hoc Tukey test.

5.4 Results

5.4.1 $SeCys_2$ and Selenite promotes cell death in HepG2 cells.

Preliminary experiments were conducted to determine the cytotoxic profile of five selenospecies commonly reported in biological systems namely selenocystine ($SeCys_2$), selenate, selenite, selenomethionine (SeMet) and Se-methylselenocysteine (SeMeSeCys) which are commonly found in food. The addition of up to 125µM of $SeCys_2$ and sodium selenite for 24 hours caused a concentration dependent decrease in cell survival. Significantly increased cytotoxicity was observed in cells treated with >7µM of $SeCys_2$ and >15µM sodium selenite respectively as compared to control ($p<0.05$). The IC50 for both $SeCys_2$ and sodium selenite compounds were 73±12.14µM and 75±8.30µM respectively. In our hands, neither SeMet, SeMeSeCys or sodium selenate caused significant induction of cytotoxicity even at the highest concentration (125µM). However, cells treated with low concentrations ≤3µM of $SeCys_2$, Selenite and selenate showed an increase in cell proliferation (Figure 5.1).

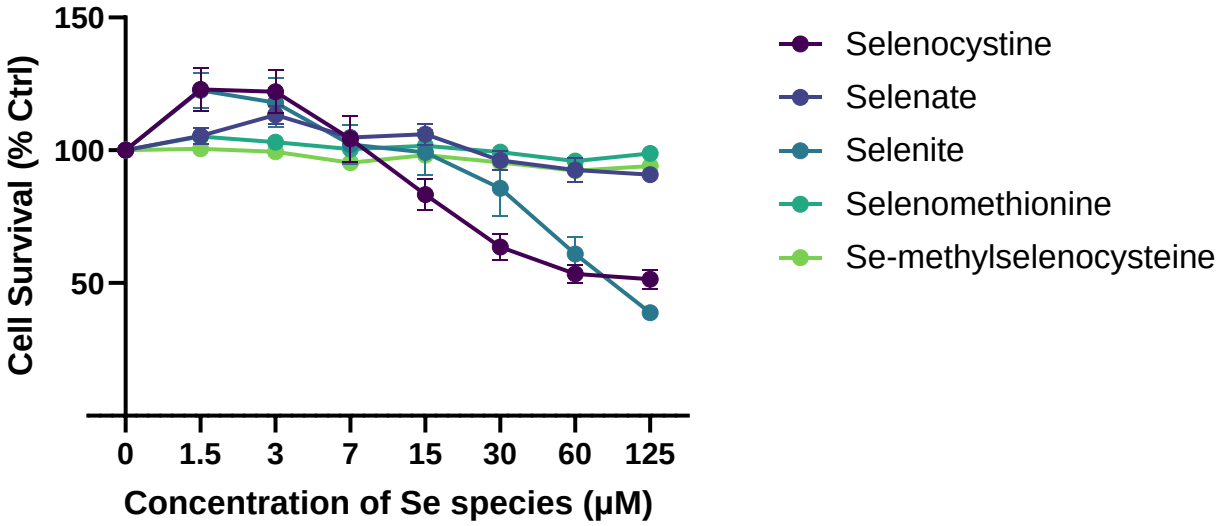

Figure 5.1- A concentration dependent loss of cell viability induced by Se species , selenocystine, selenate, selenite, selenomethionine and Se-methylselenocysteine determined at 24 hours using the crystal violet viability assay. Data represents mean ± SE (n=9).

5.4.2 Interaction of Se compounds with cellular thiols.

Evidence suggests that during uptake and metabolism Se compounds react with cellular thiols, therefore we next assessed the interaction of the selected Se species with the main intracellular thiol, Glutathione (GSH). The co-treatment of HepG2 cells with selenite and 500µM reduced GSH significantly increases the cytotoxic effect of selenite at all concentrations ($p<0.05$) apart from 0µM and 125µM selenite. The addition of GSH to selenite reduced the IC_{50} from 75±8.30µM to 15±3.32µM (Figure 5.2B). The presence of 500µM GSH in combination with low concentrations of $SeCys_2$ did not significantly impact on the cytotoxicity of $SeCys_2$ at the majority of concentrations of $SeCys_2$, and therefore the IC_{50} was not impacted by the addition of GSH. A significant decrease was however seen in the initial proliferation of cells at

1.5µM $SeCys_2$ + GSH as compared to $SeCys_2$ alone (p=0.018) (Figure 5.2A). The application of exogenous GSH did not negatively impact on cell survival when co-treated with selenate, SeMet or SeMeSeCys (Figure 5.2C-E).

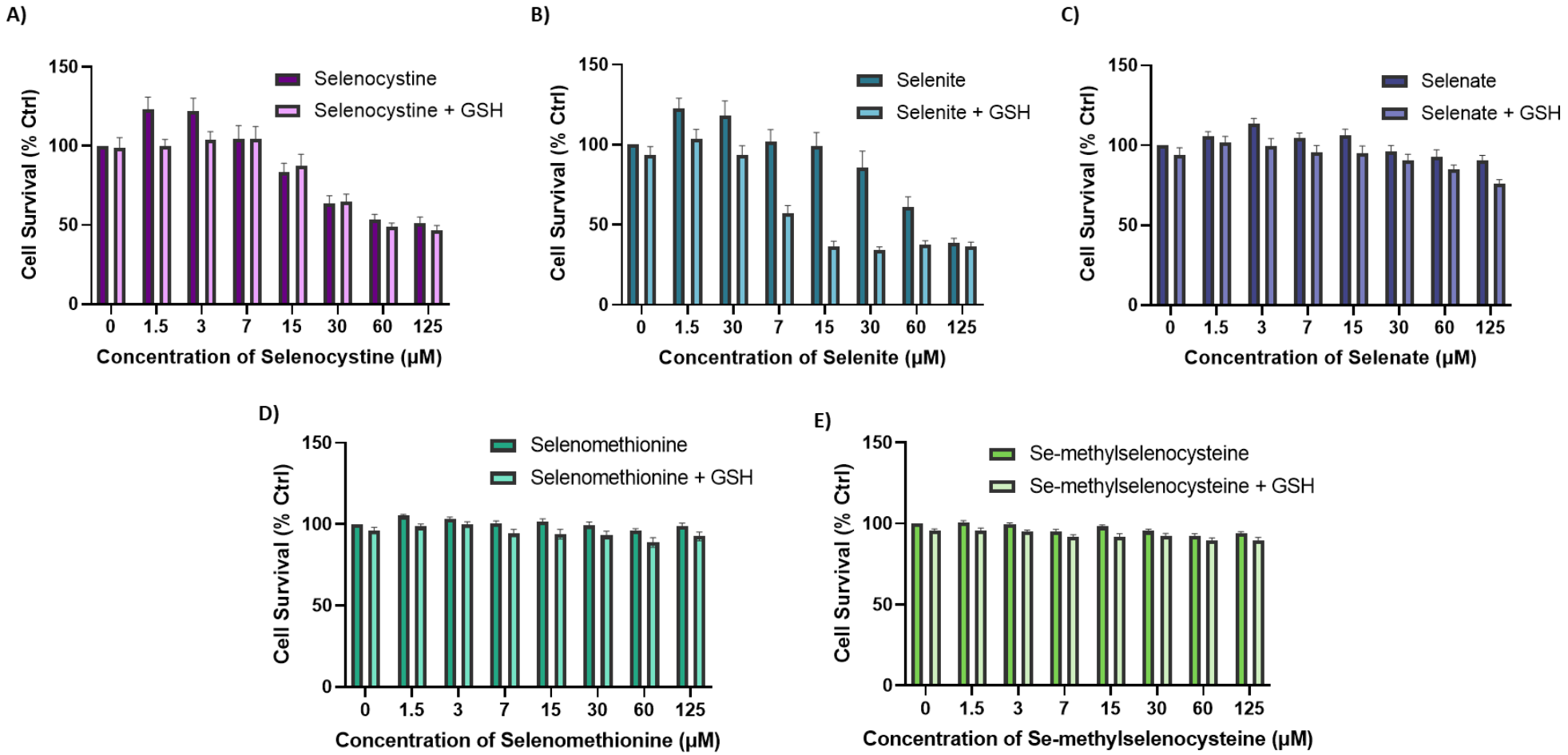

Figure 5.2- Concentration dependent loss of cell viability induced by selenospecies selenocystine, selenite, selenate, selenomethionine and Se-methylselenocysteine in the presence or absence of 500µM reduced glutathione (GSH) as determined at 24 hours using the crystal violet assay. Data is representative of three or more separate experiments and expressed as percentage survival compared with control (mean±SE).

Levels of intracellular GSH can be manipulated through the addition of Buthionine sulfoximine (BSO), a GSH biosynthesis inhibitor and N-acetylcysteine (NAC), a GSH precursor. The treatment of HepG2 cells for 24 hours in the presence of BSO virtually eliminated the presence of GSH 0.001 ±0.026µM/mg protein whereas cells treated with NAC had 2.87 ±0.13µM/mg protein GSH concentration as compared to 2.27 ±0.18µM/mg protein in untreated control cells.

The pre-treatment or co-treatment of NAC/BSO in the presence of $SeCys_2$ and selenate had no significant impact on cell survival at any concentration tested as compared to control ($p>0.05$) (Figure 5.3A - F).

Manipulation of intracellular levels of GSH via the co-treatment of selenite treated cells with NAC resulted in a concentration dependent decreased cell survival which was significant at all concentrations tested ($p<0.05$) (Figure 5.3D). Pre-treatment of cells with NAC prior to incubation with selenite did not result in any significant increase or decrease in cell survival as compared to control ($p>0.05$) (Figure 5.3C).

Co-treatment with BSO resulted in a significant decrease ($p<0.05$) in cell survival at selenite concentrations of 7µM and above (Figure 5.3D), whereas pre-treatment with BSO only caused a significant decrease in cell survival at a 15µM, 30µM and 125µM selenite concentrations (Figure 5.3C). This suggests that although GSH mediated cell death was reduced, cells could not tolerate redox stress in the absence of GSH.

Cell survival was lower in pre-treated cells across the board and irrespective of cell treatment, which could be explained by the 24 hour longer incubation time in FBS free media containing no growth factors due to the application of pre-treatments.

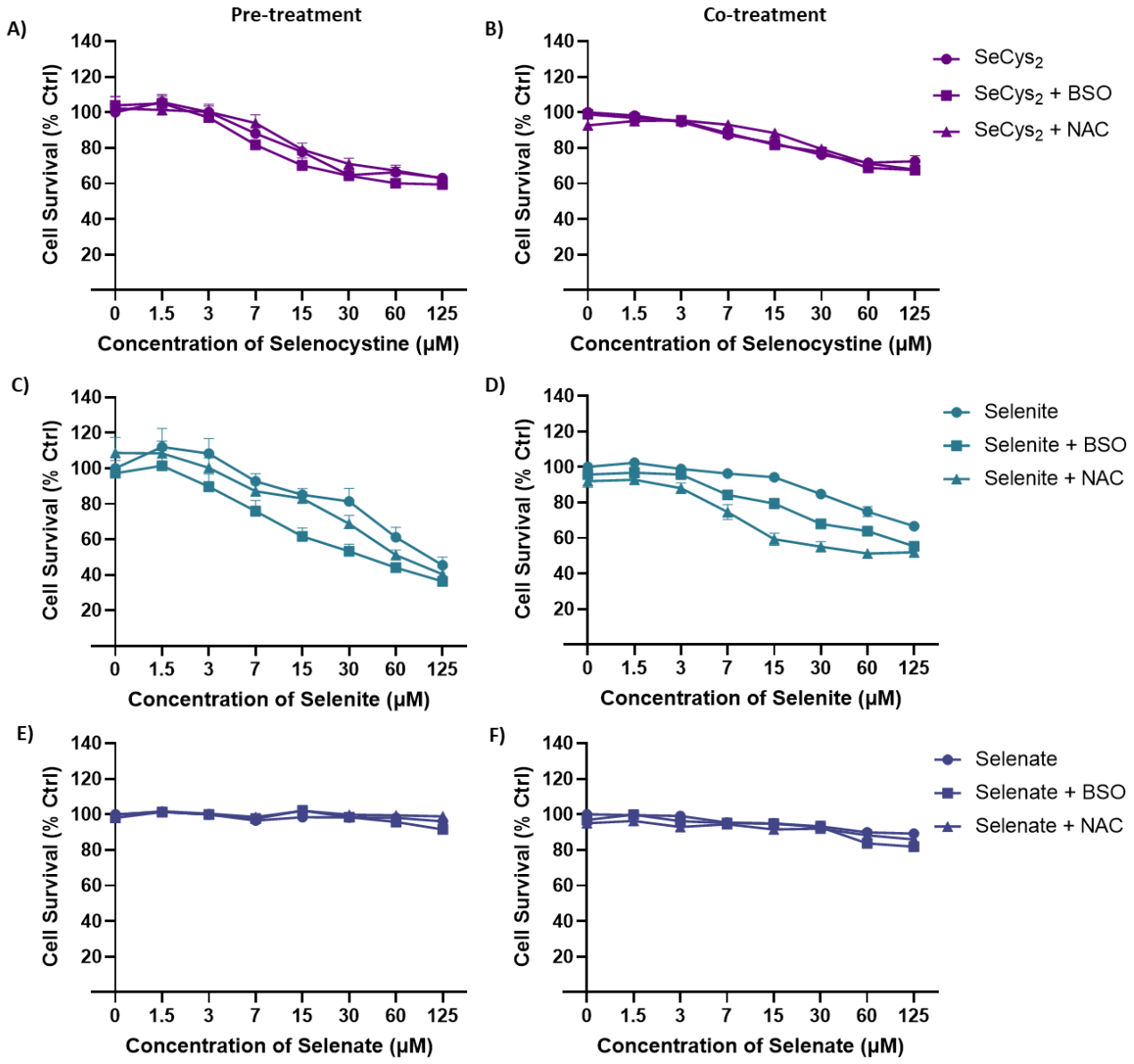

Figure 5.3- Concentration dependent loss of cell viability induced by Se Species, $SeCys_2$, selenite and selenate pre-treated or co-treated with 500μM NAC/ 2.5mM BSO for 24 hours prior to treatment with Se compounds as determined at 24 hours using the crystal violet viability assay. Data is representative of three or more separate experiments and expressed as percentage survival compared with control (mean±SE)

Selenite was found to be the most cytotoxic in the presence of GSH and therefore we focused on this molecule to explore its toxicity. Selenite is generally found in low quantities in crops, as selenite is rapidly converted to selenate in plant roots (possibly as a toxicity avoidance mechanism), however selenite has been detected in varying levels within plants that have been biofortified with Se. More recently researchers have begun to postulate that Se speciation may determine toxicity of Se more so have a bigger effect on Se toxicity than the level of Se accumulated. To ascertain if this increase in cytotoxicity of selenite in the presence of GSH is solely an effect of GSH, or more widely thiol containing compounds we tested selenite alongside thiol and disulphide containing compounds. A decrease in cell viability was observed in those compounds containing thiols such as GSH and L-cysteine, however the severity of the effect was the greatest with GSH ($p<0.001$)(Figure 5.4). The cytotoxicity of selenite + disulphide containing compounds such as GSSG, L-cystine did not significantly differ from selenite only control, suggesting that a redox mechanism may be involved in the reaction between selenite and thiol containing compounds.

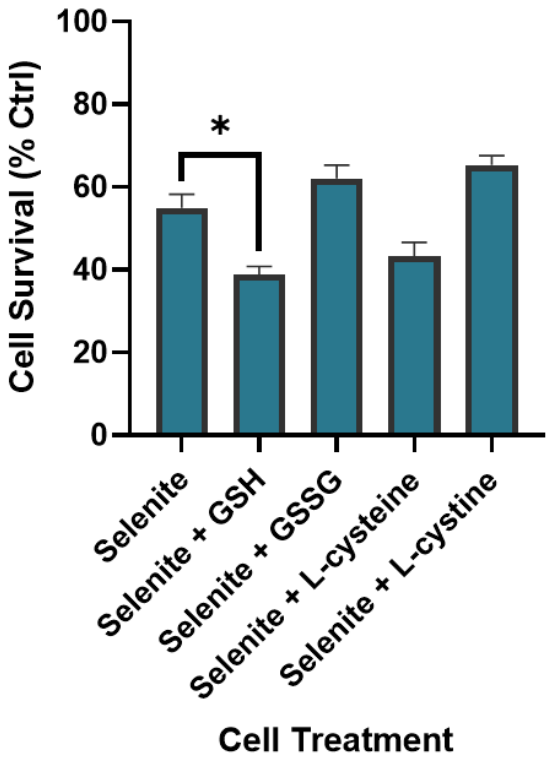

Figure 5.4- Effects of various compounds containing either disulfide such as glutathione disulfide (GSSG) and L-cystine or free sulfide group (thiol) such as GSH and L-cysteine on the toxicity of selenite on HepG2 as determined at 24 hours via the crystal violet cell viability assay. Data is expressed as mean ± SD (n = 3). * indicated significance, $p<0.05$ as determined via ANOVA and Tukey post hoc test.

5.4.3 Age of selenite: GSH mixture impacts on cell toxicity

During the original set of experiments assessing the viability of cells with Selenite in combination with GSH, we noted that there was variability in the cytotoxicity of selenite + GSH mix depending on how long the mix had been incubated on the bench. This made us consider whether the toxicity of the selenite + GSH mix is caused by a short lived reaction intermediate. We therefore set up an experiment to assess the cytotoxicity of selenite + GSH mixtures aged for varying lengths of time.

HepG2 cells were treated with a mixture of 15µM of selenite + 500µM of GSH that had been incubated prior to treatment for 0, 5, 10, 20, 40 and 60 minutes. Each treatment was then applied to cells for 24 hours. The 15µM concentration of selenite and GSH was chosen as it killed 50% of cells in the preliminary results. Results showed that the toxicity of the mixture decreases with increasing incubation time of mixture prior to treatment (Figure 5.5). An incubation time of 10 minutes was sufficient to significantly increase cell survival to 80% compared to 50% at 0 minute incubation, after 60 minutes cells survival reached 86%. This suggests that the promotion of toxicity occurs by a short lived reaction intermediate such as a free radical or short-lived gas, or potentially a mixture of both.

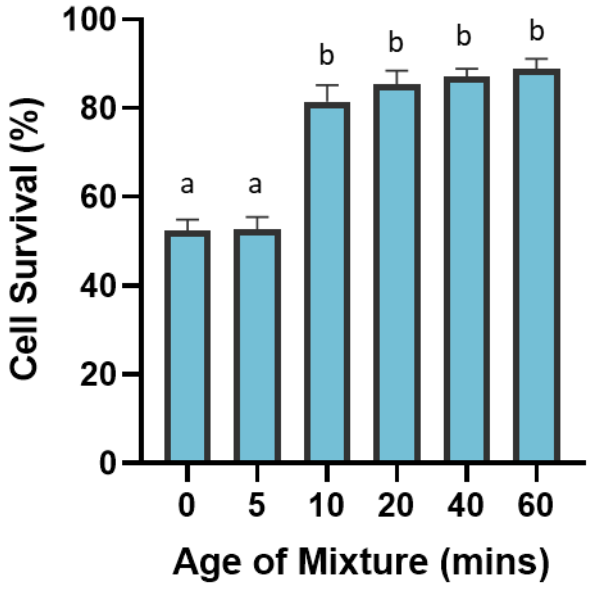

Figure 5.5- Toxicity of selenite:GSH (IC_{50} 15µM selenite +500µM) with increasing age of the mixture as determined at 24 hours using the crystal violet viability assay. Data is expressed as mean±SE (n=18), significance was determined via one-way ANOVA followed by Tukey post-hoc tests, mean followed by same letter indicates no significant difference, therefore mean followed by different letter indicates a significant difference ($P<0.001$).

Following on from the cytotoxicity results I next assessed indices of apoptosis. This was to aid in determining the molecular mechanisms by which Se was promoting cytotoxicity. To facilitate this part of the work I elected to look at three key hallmarks of apoptosis, DNA damage, LDH leakage and caspases. 4',6-diamidino-2-phenylindole (DAPI) staining was used to assess DNA damage, nuclear condensation and nuclear blebbing after 24hrs of treatment with respective Se compounds. In cells treated with fresh selenite + GSH mix it was found that this mix promoted increased fluorescence correlating with nuclear condensation and blebbing in cells which was comparable to 1mM H_2O_2 positive control, whereas aged selenite + GSH mixture shows nuclei blebbing at a similar incidence to untreated control (Figure 5.6). Thus confirming that a possible mechanism of cell death in fresh selenite + GSH mix was via DNA damage.

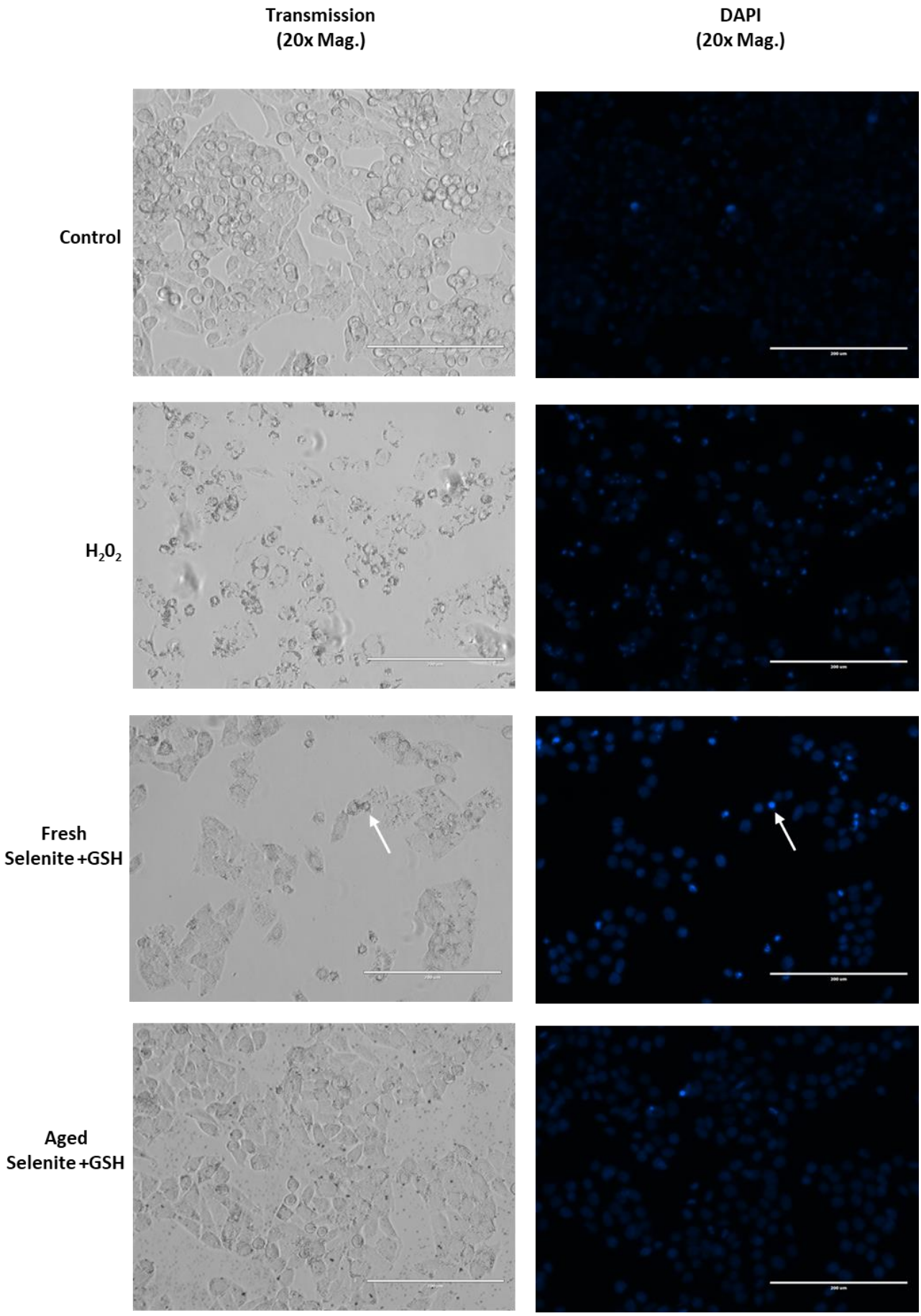
Transmission
(20x Mag.)
DAPI
(20x Mag.)
Control
H_2O_2
Fresh
Selenite +GSH
Aged
Selenite +GSH

Figure 5.6- Morphological observation of HepG2 cells using DAPI staining (20x) : The cells were treated for 24 hours prior to staining with DAPI. Images were taken on fluorescent EVOS microscope. Arrows represent nuclear condensation and blebbing. Scale bar represents 200µm.

Extracellular LDH leakage can also be used to indicate apoptosis, as a stable cytoplasmic enzyme LDH is released into the cell culture medium upon damage to the plasma membrane. Cells treated with fresh and aged selenite + GSH mix showed no significant difference ($p=0.234$) in LDH leakage suggesting there is no damage in plasma membrane at 24 hours (Figure 5.7). Significantly greater LDH leakage ($p=0.008$) was however seen in cells treated with degraded selenite + GSH mix compared to untreated control, suggesting presence of elemental Se or another compound that is redox cycling (Figure 5.7).

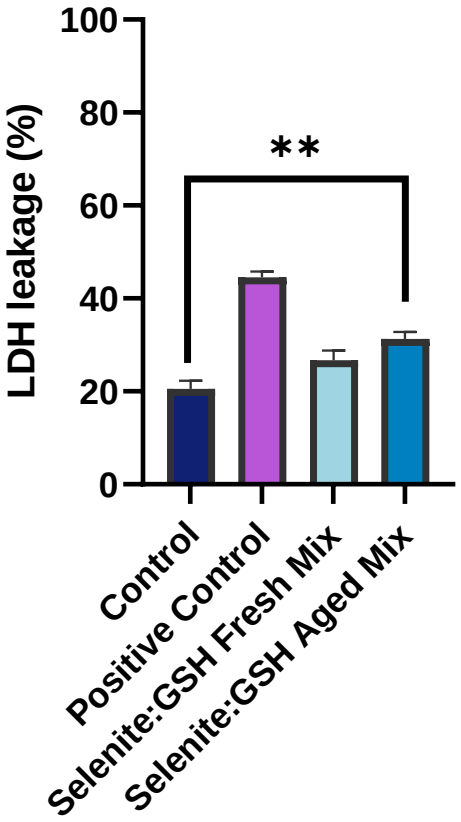

Figure 5.7- Effects of fresh vs aged selenite + GSH mix on LDH leakage determined at 24 hours via the *CyQUANT* ™ LDH Cytotoxicity Assay Kit. Data is expressed as percentage of maximum LDH activity as mean ±SE, and is representative of 3 or more separate experiments (n=18), ** signifies significance $p<0.001$ as determined via one-way ANOVA and Tuley post-hoc tests.

5.4.4 Involvement of Caspases in Selenite + GSH mediated apoptosis.

Caspase Inhibitors Z-VAD-FMK (pan-caspase inhibitor) and Z-DEVD-FMK (caspase 3 inhibitor) were used to assess the involvement of caspases in selenite + GSH mediated loss of cell viability. In our hands pre-incubation with individual caspase inhibitors for 1 hour prior to exposure to selenite prevented death by up to 45% and 30% respectively. Although a significant increase in cell survival was only seen in HepG2 cells treated with pan-caspase Z-VAD-FMK ($p=0.014$) as compared to un-pretreated control. This observation suggests the involvement of a caspase mediated

mechanism, but potentially not the involvement of caspase 3 in selenite mediated cell death (p=0.376), (Figure 5.8A). However, HepG2 cells treated with selenite alone resulted in a small but insignificant increase (p=0.113) in cleaved PARP as compared to control, suggesting cell death may happen independently of PARP (Figure 5.8B). Further experiments, over a larger range of concentrations and time course analysis will help ascertain if apoptosis of selenite treated cells happens independently of PARP and caspase 3.

Pre-treatment with either caspase inhibitors for 1 hour prior to treatment with selenite + GSH mix prevented death by up to 30% and 15% respectively (Figure 5.8A). Although this was found not to be significant for either caspase inhibitor (p=0.273 and 0.974 respectively) as compared to un-pretreated control cells exposed to Selenite + GSH. This suggests that apoptosis may occur in a caspase independent manner when cells are exposed to selenite + GSH mix (Figure 5.8A). A significant increase (p=0.002) was however seen in cleaved PARP after 24 hours in cells treated with selenite + GSH mix compared to untreated control (Figure 5.8B), suggesting that other suicidal proteases may play a role in the chain of events in selenite + GSH cell death.

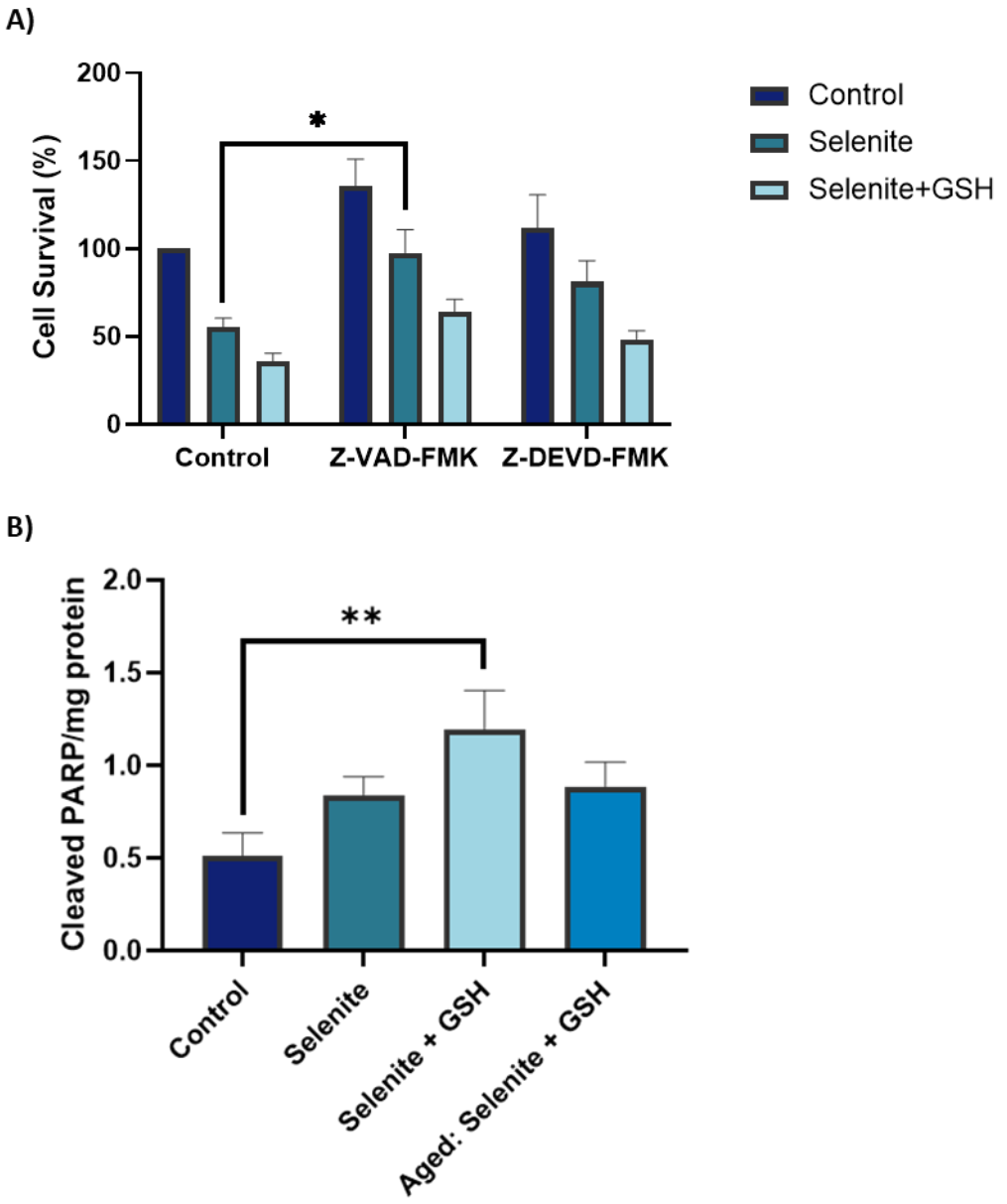

Figure 5.8- Assessment of involvement of caspase PARP cleavage in the presence of selenite ± GSH A) Assessing the role of caspases within selenite +/- GSH mediated cell death. HepG2 cells were pre-treated for 1 hour with 75 µM of caspase inhibitors Z-VAD-FMK/Z-DEVD-FMK prior to cell treatment with 75 µM selenite +/- 500µM GSH. Cell viability was determined after 24 hours using the crystal violet cell viability assay. Data is representative of three separate experiments and expressed as percentage survival compared with control (n=9) (mean±SE). B) The impact of 75µM selenite, 75µM selenite+ 500µM GSH and one hour aged 75µM selenite+ 500µM GSH treatment of HepG2 cells on cleaved PARP determined after 24 hours exposure to Se. Data is representative of three separate experiments and expressed cleaved PARP/mg protein (n=3) (mean±SD). * indicates significance $p<0.05$, ** indicates significance $p<0.01$ as determined via ANOVA and Tukey post hoc test.

5.4.5 Se compounds promote ROS

ROS has been widely reported to initiate the apoptotic cascade in mammalian cells. Using the positive control (H_2O_2) we noted significant induction of ROS in hepG2 cells treated with this compound. This confirmed ROS production in our model and our ability to detect this using DCFDA. Following validation, we next explored if selenite + GSH also induced ROS in our model. Treatment with either fresh or aged 15µM selenite + 500µm GSH promoted a time dependant increase in ROS (Figure 5.9). Incubation of HepG2 cells with fresh selenite + GSH mix resulted in significantly increased ROS production as compared to control at every time point measured ($p<0.05$). Whereas HepG2 cells treated with aged selenite + GSH mix did not significantly differ as compared to control. However, the ROS production of fresh selenite + GSH mix was only significantly different to aged selenite + GSH mix at 0 mins ($p=0.013$), whereas at all other time points there was no significant difference between fresh and aged mixes as indicated by DCFDA fluorescence intensity (Figure 5.9). Increased levels of lipid peroxidation were also seen in the cells treated with fresh selenite + GSH at the 30 minute time point, however this effect dissipated over time (Figure 5.10), again suggesting the cytotoxic effects seen are due to a short-lived reaction intermediate such as ROS.

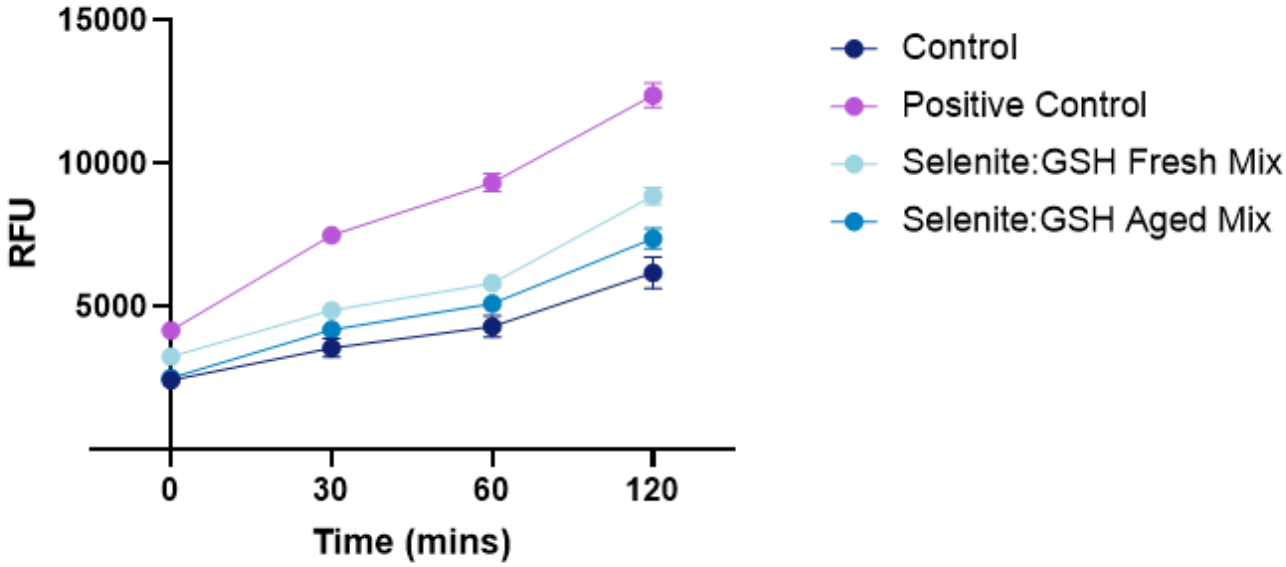

Figure 5.9- Time dependent increase in RFU after staining with the ROS probe, DCFDA (100µM) for 1 hour prior to treatment with control FBS free media, 1mM H_2O_2 and IC_{50} selenite + GSH mixture either prepared fresh and applied immediately to cells or aged for 1 hour prior to application to cells. Plates were read at 485/520nm at 0, 30, 60 and 120 minute time points and background readings was deducted from all values. Data is representative of three or more separate experiments (mean ±SE) (n=18).

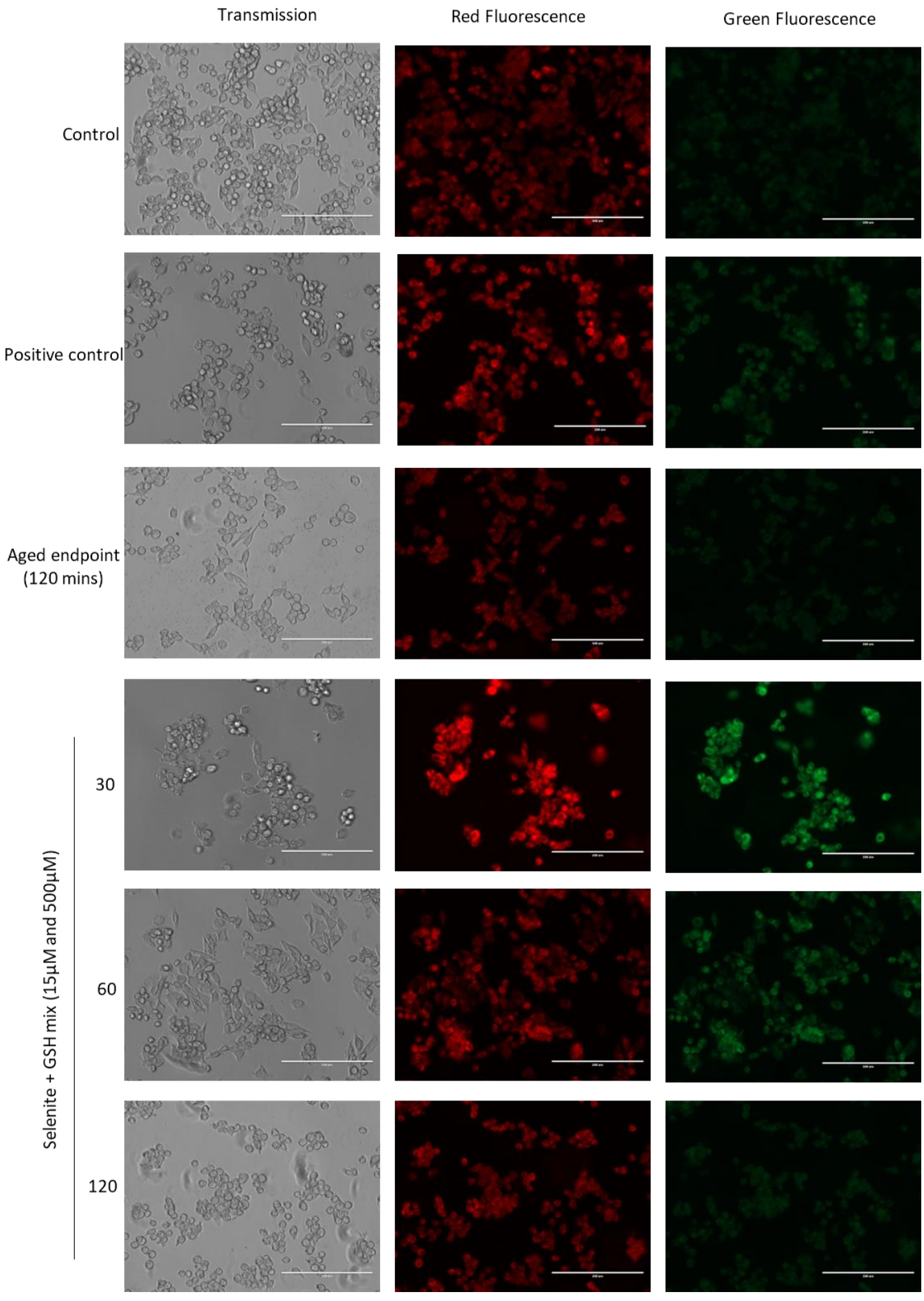
Transmission
Red Fluorescence
Green Fluorescence
Control
Positive control
Aged endpoint (120 mins)
Selenite + GSH mix (15µM and 500µM)
30
60
120

Figure 5.10- Transmission and fluorescence images (20x) of HepG2 cells stained with lipid/lipid peroxide detection reagent after 30, 60, 120 incubation with selenite + GSH mix. Images show initial bust of lipid peroxidation after 30 minutes treatment with selenite + GSH which then dissipates over time as well as similar levels of lipid peroxidation in 1 hour aged selenite + GSH as compared to control. Scale bar represents 200µm.

Previous research has found that ROS are responsible for damage caused by selenite and effects can be rescued by the addition of radical scavengers, however, other researchers have suggested these effects cannot be rescued by the addition of radical scavengers. Therefore, we assessed whether the incubation of HepG2 cells treated with selenite ± GSH alongside scavengers of the thyl, hydroxyl, peroxyl radical and stable O_2, N_2 and H_2O_2 such as ascorbic acid, N-acetyl cysteine, uric acid, sodium hydrosulphide, cytochrome c, trolox and catalase would rescue the cytotoxic effects of selenite + GSH. In our hands, the co-treatment of cells with radical scavengers did not significantly increase cell survival in cells treated with either selenite or selenite+GSH ($p>0.05$) (Figure 5.11). This suggests that although ROS is produced, incubation with ROS scavenging compounds does not prevent cell death. A dose response and time course, along with analysis of ROS production via DCFDA would further help to elucidate the role of ROS production within the selenite + GSH mix, unfortunately time pressures and limitations on availability of cell culture reagents meant this was not possible as part of this study. However, there is still reason to suggest that an alternative short lived reaction intermediate plays a role in selenite + GSH mediated cell death. However, the co-treatment of HepG2 cells with selenite in the presence of sodium hydrosulphide (NaHS), resulted in a significant decrease ($p<0.029$) in cell survival compared to selenite only treated cells, this can

be explained by the presence of a thiol group within NaHS which reacts with selenite in the same way as GSH.

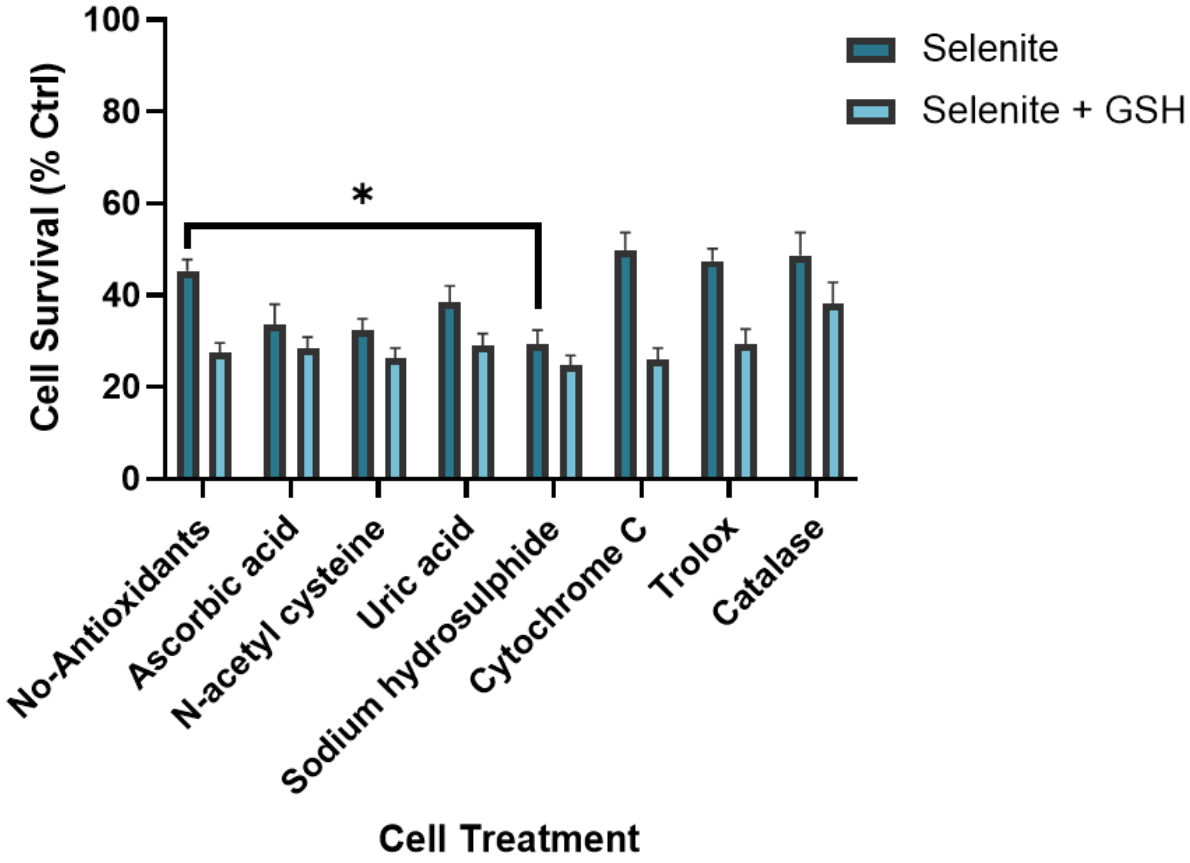

Figure 5.11- Impact of radical scavenging antioxidants, 500µM of ascorbic acid, N-acetyl cysteine, uric acid, sodium hydrosulphide, cytochrome C, trolox and catalase on cells treated with 75µM of Selenite +/- 500µM GSH as determined at 24 hours using the crystal violet cell viability assay. Data is representative of three separate experiments and expressed as percentage survival compared with control (n = 12) (mean±SE). * indicated significance, $p<0.05$ as determined via ANOVA and Tukey post hoc test.

5.5 Discussion

In this study, we further validated a human hepatoma HepG2 cellular model to assess cytotoxicity of Se compounds in mammalian cells. HepG2 cells are routinely used in apoptotic studies as an anti-cancer model (Fan et al., 2010, Piret et al., 2006). Increased Se status in humans has been linked to reduced risk of various diseases particularly cancers of the breast, lung, oesophagus, gastric and prostate in a meta-analysis study (Cai et al., 2016). Currently, the mechanisms for this are not widely understood but appear to be associated with the induction of various forms of cell death (Sanmartín et al., 2012), autophagy (Králová et al., 2012), redox mechanisms (Kuršvietienė et al., 2020) and the induction of cellular cytoprotection (Bartolini et al., 2020). In Chapter 4 several Se compounds, namely SeMet, $SeCys_2$, SeMeSeCys and Selenate were shown to accumulate in plant tissues grown in the presence of a Se source. Therefore, I assessed whether these compounds and selenite, found in other crop Se enrichment studies such as Peanut (Luo et al., 2021) could induce apoptosis in our *in vitro* HepG2 cell model. This research builds on past studies and opens up the possibility that reactive Se intermediates may be important in the response of mammalian cells to Se compounds. This information serves to, firstly to validate the current *in vitro* model and allow for comparison of data with that of other researchers, and secondly provides some information on the potential anti-cancer properties of known dietary Se compounds. I elected to measure the effects of Se compounds on cell viability and interaction with cellular antioxidants like GSH. Further analysis using cellular markers of apoptosis including DNA damage, membrane leakage, caspase apoptosis and ROS production were also assessed to explore the mechanism of cell death in our laboratory. These studies may go some

way to explaining the reported effects of Se and associated compounds on cancer cells and on cancer cell proliferation. Significant increases in cell proliferation were seen in cells treated with very low concentrations of Selenite and $SeCys_2$, this is supported by increased cell proliferation in bovine luteal cells and human leukaemia cells (HL-60) treated with nanomolar concentrations of Se species (Kamada and Ikumo, 1997, Zeng, 2002) as well as increases in the expression of cell cycle related genes and leading to the premotion of the G2/M transition in HL-60 cells. Of the Se compounds analysed, only $SeCys_2$ and selenite resulted in a significant decrease in cell survival at higher concentrations. All other Se species at the concentration ranges used had no appreciable effects on cell viability in our hands. Our results parallelled that of other researchers who have shown that exposure to selenite and $SeCys_2$ in a multitude of cancer cell lines such as LNCaP prostate cancer cells (Zhong and Oberley, 2001), HCT-16, Colo201 and SW620 human colon cancer cells (Chen and Wong, 2009, Králová et al., 2009, Li et al., 2013), A375 human melanoma cells (Chen and Wong, 2009), CNE2 nasopharyngeal carcinoma (Chen and Wong, 2009), MCF7 and MDA-MB-231 breast adenocarcinoma (Chen and Wong, 2009), HC60 acute myeloid leukaemia (Chen and Wong, 2009) as well as HeLa cervical cancer cells (Wallenberg et al., 2014) results in a significant decrease in cell viability. Data from the available literature indicates that the IC_{50} of selenite ranges from 1µM-50µM and the IC_{50} for $SeCys_2$ ranges from 3µM-50µM. Variations in IC_{50} are reported between different cell lines and exposure time and this potentially explains why the IC_{50} values noted in the literature are lower than what we found in our experiments. Compounds including selenate, SeMet and SeMeSeCys had no appreciable effects on

cell viability mirroring other studies (Chen and Wong, 2009, Estevez et al., 2014, Weiller et al., 2004), (Figure 5.1).

The cellular thiol glutathione (GSH) is one of the main antioxidant species in mammalian cells, and plays an important role in maintaining a redox environment of multiple organelles and cell signalling (Franco and Cidlowski, 2009). Interestingly, many cancer cells are reported to contain higher levels of GSH than healthy cells and this causes cancer cell resistance to various chemotherapeutic drugs (Kennedy et al., 2020). Previous research has suggested that increasing levels of intracellular GSH protects cells from oxidative damage (Lash et al., 1986), therefore I tested if the incubation of cells with GSH would rescue the cytotoxic effect of Se compounds. Interestingly, our results show that in the presence of the cellular thiol GSH cytotoxicity of selenite was enhanced but cytotoxicity of other Se containing species was not affected (as shown in Figure 5.2). This enhancement of selenite cytotoxicity has been seen in the work of Shen et al. (2000) in HepG2 as well as HeLa cells (Caffrey and Frenkel, 1991), human mammary tumour cell line (Yan et al., 1991) and in yeast, *Saccharomyces cerevisiae* (Tarze et al., 2007). Previous studies suggest that GSH plays a pro-oxidant role in the presence of selenite. As such, I elected to manipulate the levels of cellular GSH in order to assess the impact on cell survival in the presence of selenite by driving or suppressing GSH synthesis via pre- or co-treatment with N-acetylcysteine (NAC) or Buthionine sulphoximine (BSO) a known inhibitor of glutathione synthesis. We found that co-treatment with NAC significantly increases cytotoxicity of selenite (Figure 5.3B). However inhibiting GSH synthesis did not prevent this effect similarly to the findings of Shen et al. (2000). Since only selenite promoted a significant loss in cell viability, we explored the molecular

mechanisms for this effect in the current chapter. Our results suggest that the cytotoxic effect seen in the interaction between selenite and GSH is due to the presence of the thiol group (R-SH). To confirm this observation, we assessed the impacts of other thiol species and their associated disulfides (R-SS-R). We report that L-cysteine when incubated with selenite caused a loss in cell viability, however disulfide species failed to enhance this effect (figure 5.4). This finding is supported by the work of Tarze et al. (2007) who found that L-cysteine and DTT (both thiol containing compounds) reduced cell viability in yeast. This increase in cytotoxicity provides an element of pharmacological interest as cancer cells typically contain higher levels of GSH (Ding et al., 2021). Future research may show selenite or derivatives thereof, may be useful as chemotherapeutic agents when targeted to cancer cells (Reviewed in (Kim et al., 2021, Lipinski, 2017), or when added as a co-treatment with existing chemotherapy drugs such as doxorubicin/adanamycin (Wu et al., 2019) and radiotherapy (Knox et al., 2019). Following the confirmation of selenite interaction with thiol groups I carried out an assessment on latter stage apoptosis mechanisms to understand their involvement in selenite ± GSH mediated cell death.

Further to the impacts of GSH, we also report that pan caspase inhibitors Z-DEVD-FMK, prevented selenite mediated loss in cell viability, this result suggesting that selenite mediated apoptosis occurs via caspase driven pathways (Figure 5.8A). However, we anticipate that as indicated by Shen et al. (2001), caspase 3 mediated mechanisms are likely involved in HepG2 cells, which has also been seen in promyelocytic leukaemia cancer cells (NB4) (Zuo et al., 2004) and human prostate cancer cells (LNCaP)(Jiang et al., 2004). However, inhibition of caspase 3 or pan

caspases prior to treatment with selenite and GSH, did not rescue the cytotoxic effect of the selenite + GSH reaction mixture (Figure 5.8A), suggesting a caspase independent apoptosis. Caspase independent apoptosis has also been seen in cervical carcinoma cell line (HeLa Hep-2) treated with sodium selenite alone (5-50µmol/L)(Rudolf et al., 2008b). A significant increase in PARP cleavage was also seen in HepG2 cells treated with selenite + GSH as compared to control (Figure 5.8B). PARP cleavage can occur independent of caspases via other suicidal proteases such as calpains, cathepsins, granzymes and matric metalloproteinases (reviewed in Chaitanya et al. (2010)). Although the specific suicidal protease responsible for PARP cleavage in the presence of selenite and GSH is still not fully understood. Further experiments over varying timescales and concentrations would help to further clarify the roles of caspases in selenite ± GSH mediated cell death.

We observed an age dependent cytotoxic effect of the selenite + GSH mix (Figure 5.5, 5.6 and 5.10), suggesting that the cytotoxic effects seen such as DNA condensation and lipid peroxidation are due to the generation of short-lived reaction intermediates. During the reaction of selenite and GSH, several reactive intermediates have been proposed. Some studies suggest that the generation of the superoxide anion and subsequent hydroxyl radicals causes DNA damage and fragmentation and leads to cell death (Saito et al., 2008, Shen et al., 2001).

Using the ROS fluorescent probe DCFDA, a chemical probe widely used to measure cellular ROS production in mammalian cells, we next measured ROS production in cells exposed to selenite + GSH. Our results indicate that HepG2 cells treated with fresh selenite + GSH have increased ROS production and lipid peroxidation as

compared to control (Figure 5.9 and 5.10). Although it is noteworthy that lipid peroxidation peaked at the 30 minute time point and subsequently decreased up to 120 minutes, ROS levels continued to increase over time using the DCFDA probe. This may suggest that ROS is not solely responsible for the cytotoxic effects seen for selenite in our hands. Therefore, to confirm this observation, we incubated cells in the presence or absence of the selenite + GSH mix with multiple radical scavenging compounds such as ascorbic acid (stable O_2, N_2 and thyl radical), NAC (hydroxyl radical, H_2O_2 and peroxyl radical), uric acid (hydroxyl radical, O_2 singlet, oxo-heme oxidants), NaHS (hydroxyl radical), cytochrome c (mitochondrial H_2O_2), trolox (peroxyl radical) and catalase (H_2O_2) as used in various studies (Alberto et al., 2013, Ates et al., 2008, Niki, 1991, Stinefelt et al., 2005, Yu et al., 2015, Peyroche et al., 2012). These antioxidant species failed to prevent loss of cell viability in the current cell model (Figure 5.11). While in the present study the generation of the superoxide anion nor pharmacological inhibitors were assessed, past studies have shown that incubation with radical scavengers such as SOD ($O_2^{\cdot -}$), catalase (H_2O_2) and mannitol (.OH scavenger) did not prevent the loss in cell viability in cells treated with selenite + GSH (Tarze et al., 2007). Moreover, Saito et al. (2008) observed that incubation of DNA in a cell free system with SOD and catalase in the presence of selenite + GSH did not prevent single stranded breaks of Col E1 DNA, suggesting that neither the superoxide anion or H_2O_2 were solely responsible for selenite + GSH DNA damage. However, in the presence of mannitol, selenite + GSH single stranded breaks were reduced, suggesting that the hydroxyl radical may be involved in DNA damage (Saito et al., 2008). In contrast to this, other studies have found that incubation of the selenite + GSH mix with $O_2^{\cdot -}$ scavengers prevents mitochondrial changes and

apoptotic signalling pathways (Kim et al., 2003), and mitochondrial membrane depolarization and release of cytochrome c (Shen et al., 2000). These cellular events are critical for the initiation of apoptotic cascades. Tarze et al. (2007) reported that yeast cell survival remained at nearly 100% when exposed to ROS levels exceeding that generated in the reaction between selenite + GSH, again suggesting that ROS is not wholly responsible for the toxicity between selenite and GSH.

The biological chemistry of Se and cellular reactions is complex. It has recently been proposed that selenite catabolism in the presence of excess GSH promotes the generation of several reaction intermediates (Tarze et al., 2007). The first being selenodiglutathione (GS-Se-SG)(Reaction 1), which in the presence of GSH is reduced to glutathioselenol (GS-SeH)(an unstable intermediate)(Reaction 2) which either spontaneously dismutates to elemental Se (Se^0)and GSH (Reaction 3) or is further reduced in the presence of GSH to form hydrogen selenide (H_2Se)(HSe^- at physiological pH (Cupp-Sutton and Ashby, 2016)) (Reaction 4). Hydrogen selenide can then be oxidised to form elemental Se and water (Reaction 5). Although excess GSH can prevent H_2Se from being oxidised and drives further production of hydrogen selenide (Reaction 6).

Reaction 1 $SeO_3^{2-} + 4GSH + 2H^+ \rightarrow GS\text{-}Se\text{-}SG + GSSG + 3H_2O$

Reaction 2 $GS\text{-}Se\text{-}SG + GSH \rightarrow GS\text{-}SeH + GSSG$

Reaction 3 $GS\text{-}SeH \rightarrow Se^0 + GSH$

Reaction 4 $GS\text{-}SeH + GSH \rightarrow H_2Se + GSSG$

Reaction 5 $H_2Se + 1/2O_2 \rightarrow Se^0 + H_2O$

Reaction 6 $H_2Se + 1/2O_2 \rightarrow 2GSH \rightarrow H_2Se + GSSG + H_2O$

Other researchers have attempted to explore the toxicity of several of these reaction intermediates. The intermediates selenodiglutathione (Reaction 1) and elemental Se (Reaction 3) were found to cause no significant impact on cell survival (Tarze et al., 2007). Interestingly, glutathioselenol is unstable and upon decomposition produces hydrogen selenide (Reaction 4)(Liu et al., 2021) with some researchers indicating that this gas could potentially be the fourth gaseous mediator in mammalian cells alongside nitric oxide (Nowaczyk et al., 2021), hydrogen sulphide (Rose et al., 2021, Wang, 2002) and carbon monoxide (Yang et al., 2021b), (reviewed in Kuganesan et al. (2019)).

Hydrogen selenide (H_2Se) gas is generated in the reaction between selenite + GSH (Reaction 4), at physiological pH H_2Se is mainly in the form of HSe^- (H_2Se (aq) $\leftrightarrow$ HSe^- (aq) + H^+ (aq)) a strong nucleophilic species (Luo, 1990, Cupp-Sutton and Ashby, 2016, Newton and Pluth, 2019). H_2Se producing compounds such as Na_2Se, when applied to yeast cells cause double stranded DNA breaks, cell cycle arrest in G2/M phase and correlate with increased rate of cell death (Peyroche et al., 2012). Other studies have also postulated that the continuous consumption of cellular antioxidants such as GSH and NADPH may be the mechanism of H_2Se toxicity (Tarze et al., 2007). H_2S is the S analogue of H_2Se and is already deemed to be a gaseous mediator capable of generating cell signalling effects (reviewed in Li et al. (2011)). Few studies have considered H_2Se as important in Se metabolism or in relation to its cellular effects (Kuganesan et al., 2019). Increased Se accumulation has been found in cells supplemented with selenite and GSH (Leblondel et al., 2001, Tarze et al.,

2007). This leads us to hypothesise that H_2Se may play an important role in the biological effects of Se compounds (Newton et al., 2021).

While we cannot conclusively confirm H_2Se production in our model since additional work is needed viz. biomarker analysis it would be interesting for future investigations to build on the current evidence. One area of importance would be to develop methodologies to manipulate H_2Se levels in cells and to determine production rates in vitro and in vivo. Recently, researchers have developed H_2Se specific fluorescent probes for the detection of H_2Se in mammalian cells, mito-N-D-MSN (Cheng et al., 2018), NIR-H_2Se (Hu et al., 2018, Kong et al., 2016, Kong et al., 2017). Although these compounds are not currently commercially available, the probes have allowed for the imaging of H_2Se production in several cells and tissue types. Another area of research would be to develop molecules that can release H_2Se into cells at rates that could mirror endogenous production.

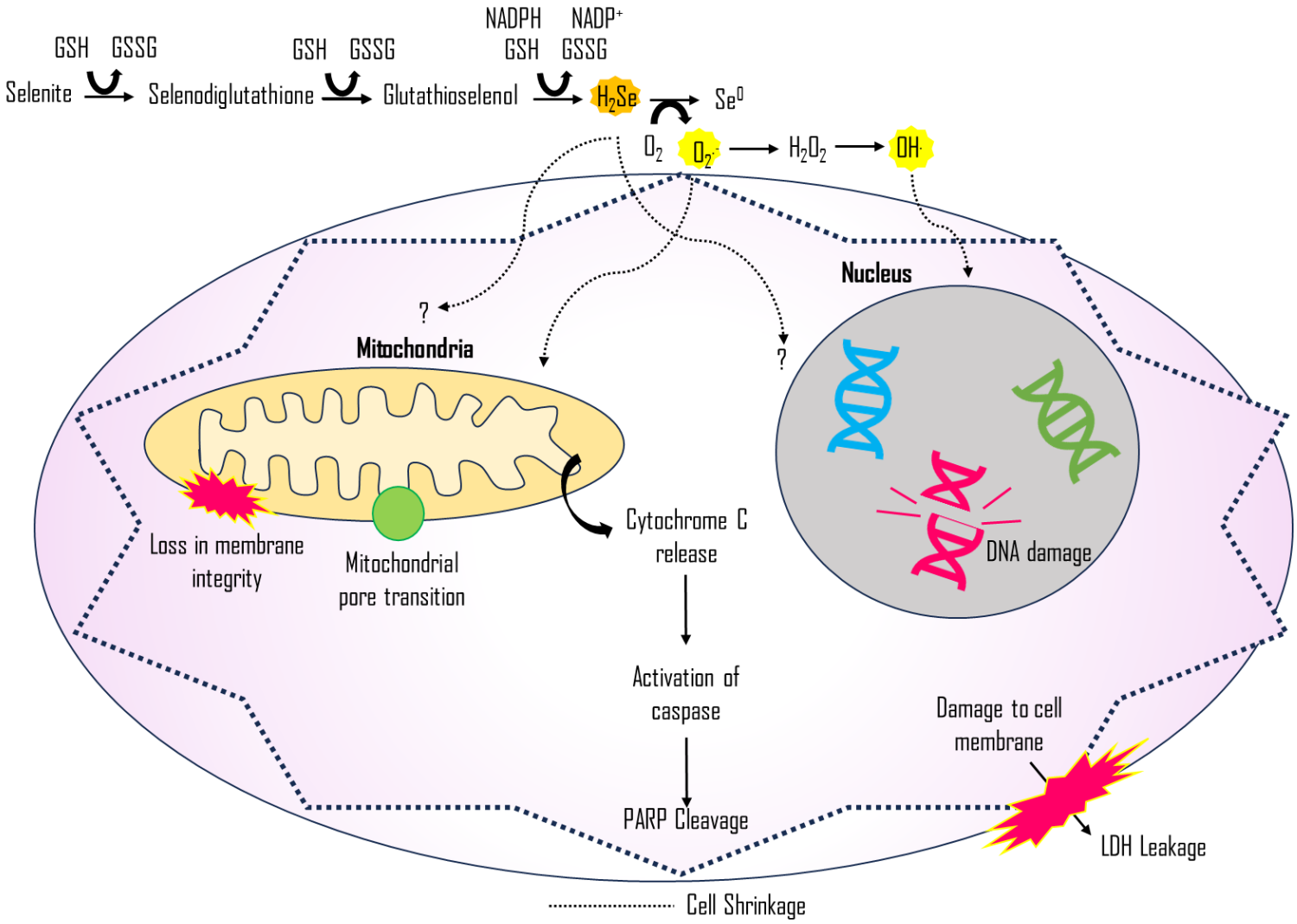

Figure 5.12- Summary figure of apoptotic cascades leading to cell death in the presence of Selenite + GSH. Equation adapted from (Letavayová et al., 2006)

5.6 Conclusion

$SeCys_2$ and selenite were found to be the most toxic Se species when applied to HepG2 cells. In the presence of GSH, the toxicity of Selenite is significantly increased unlike the other Se species examined. Toxicity of the selenite and GSH mixture appears to be dependent on short lived reaction intermediates that dissipate over time. HepG2 cells incubated with 'fresh' selenite + GSH mixture show an increased lipid peroxidation and DNA damage resulting in decreased cell viability compared to an 'aged' mixture. The mechanism of selenite and GSH cell death is still unclear but appears not solely to be due to the presence of ROS and could potentially be independent of caspases, although not PARP cleavage. H_2Se is postulated to have a significant role in selenite + GSH mediated cell death, although the mechanism of action is unclear. I hope that the role of H_2Se can be further studied with the development of commercially available of H_2Se targeted probes.

6 Characterisation of a Se containing analogue (SeGYY) of the prototypic H_2S releasing molecule, GYY4137. Development of a novel chemical tool for Se research.

6.1 Abstract

Dietary selenium is obtained in both organic and inorganic forms from foods, but one common theme during mammalian metabolism is the production of hydrogen selenide prior to incorporation into Se containing amino acids. Currently, few studies have explored the role of this molecule in biological systems. Therefore, we synthesised a novel selenium containing analogue TDN1042 (SeGYY) of the drug GYY4137, a compound known to release the allied compound hydrogen sulphide. We characterised this new Se containing analogue to assess the impact of this molecule on cell apoptosis. SeGYY caused concentration-dependent loss in cell viability of human hepatoma HepG2 cancer cell lines as determined by crystal violet ($p<0.05$) and leakage of lactate dehydrogenase ($p=0.032$). SeGYY had an IC_{50} of 640µM as compared to 73µM for $SeCys_2$ and 75µM for selenite, suggesting it to be less toxic as compared to other Se compounds. Mechanistic studies revealed that HepG2 cells exposed to 1000µM of SeGYY had significantly increased ROS production over time ($p<0.05$) and increased lipid peroxidation as determined at 0-, 30-, 60- and 120-mins post treatment. Parallel studies failed to note any induction of caspase 3

and 8 as measured 24 hrs post treatment ($p>0.05$). However, pre-treatment of cells with 75µM of the pan-caspase inhibitor, Z-VAD-FMK, or caspase-3 inhibitor, Z-DEVD-FMK, prior to exposure to 640µM SeGYY (IC_{50}), significantly increased cell viability by 40% and 20% respectively ($p<0.001$ and $p=0.057$) as compared to unpretreated SeGYY control cells. Significantly increased PARP cleavage was also seen when HepG2 cells were treated with 500µM SeGYY ($p<0.001$). This suggests a pro-apoptotic effect, involving ROS production, lipid peroxidation, caspases and PARP cleavage. At lower non-cytotoxic concentrations, namely 15µM and 30µM SeGYY, this compound was cytoprotective and significantly reduced SIN-1 ($p=0.004$ and $p=0.038$) mediated cell death. A trend in increased cell survival, although not significant ($p>0.05$), was also seen in cells pre-treated with SeGYY before exposure to H_2O_2 or diethylamine NONOate. While only pilot data, we conclude that SeGYY exhibits anti-cancer activity, likely via its capacity to release H_2Se. We also propose that H_2Se donors should be investigated further as potential tools for use in selenium focused research.

Key words: Hydrogen selenide; Apoptosis; Gaseous mediators

6.2 Introduction

Selenium (Se) metabolism is a highly regulated process that involves a convergent metabolic step needed for the incorporation of inorganic and organic Se forms into biological molecules. Initially, dietary Se is absorbed in the lower part of small intestine in the form of selenomethionine (SeMet) and selenocysteine (SeCys) derivatives or as selenite ($Se0_3^{2-}$) and selenate ($Se0_4^{2-}$) (Roman et al., 2014). These sources are then rapidly converted into hydrogen selenide (H_2Se) via the metabolic action of the enzymes, glutathione peroxidase (GPx; EC. 1.11.1.9) and glutathione (GSH) or via an enzyme coupled reactions mediated by the thioredoxin (TRx; EC 1.11.1.24)/ thioredoxin reductase (TRxR; EC 1.8.1.9) system (Roman et al., 2014). Alternatively, organic forms are substrates for the catabolic action of selenocysteine lyase (SCLY; EC 4.4.1.16), again forming the metabolic intermediate H_2Se (Suzuki et al., 2007). Some of the H_2Se generated is then combined with ATP via the enzyme selenophosphate synthase 2 (SEPSH2; EC 2.7.9.3) to form selenophosphate (SeP) (Xu et al., 2007a). This metabolite is needed for the formation of Se - tRNA derivatives that aid in the incorporation of SeCys into proteins (Xu et al., 2007b). Consequently, researchers have made significant advances in our understanding of Se incorporation into RNA and proteins. However, much less is known about the regulatory routes of catabolism and detoxification in mammalian systems or whether chemical intermediates of Se metabolism have other biological roles in cells and tissues.

Importantly, in Chapters 3-5, we have shown that Se biology is complex, that Se is incorporated into a spectrum of compounds and that this incorporation varies between cultivars or plant tissues. We therefore began to consider whether new

chemical tools were needed to further this research field. To this end, in the last two decades, there has been increased interest in the role of gaseous mediators in mammalian systems. To date, the best characterized of these are nitric oxide (NO), hydrogen sulfide (H_2S) and carbon monoxide (CO). These three molecules play important regulatory roles in cell signalling systems in the animal, bacterial, fungal, and plant kingdoms (Giuffrè and Vicente, 2018, Hichri et al., 2015, Hopper et al., 2020, Huang and Xie, 2023, Martínez-Medina et al., 2019). All three mediators are produced enzymatically in cells and tissues and have short half-lives but are freely diffusible across cell membranes (Wang, 2002). While no specific cellular receptors have been identified these molecules readily interact with proteins and genes to influence biochemical and physiological responses in biological systems. Interestingly, H_2Se is produced during Se metabolism and shares many chemical properties to that of H_2S, a widely studied gasomediator. Hydrogen selenide gas possess several characteristics of other gaseous signalling molecules in that it is a small molecule of gas, freely diffusible across cell membranes and produced enzymatically (Wang, 2002). However, currently there are few tools available to allow for the manipulation of H_2Se levels in mammalian systems.

The development of fluorescent NIR-H_2Se detecting probes first suggested the involvement of H_2Se in cell death in a cancer therapy model (Kong et al., 2016, Kong et al., 2017). More recently Newton and Pluth (2019) developed TDN1042, a slow release H_2Se donor, however its biological role was not characterised. Here we present the bioactivity of a potential slow release H_2Se donor in human hepatoma HepG2 cells.

We hypothesize that hydrogen selenide is a biologically active molecule in mammalian cells. To explore this hypothesis, we:

1. Synthesised and characterised a Se containing analogue TDN1042 (SeGYY) of the prototyptic slow release H_2S donor molecule, GYY4137.
2. Assess the cytoprotective and anti-cancer properties of the novel Se containing GYY4137 derivative in a human hepatoma HepG2 model. This work to determine the capacity of this compound to include programmed cell death and mechanism of action.
3. To determine whether SeGYY can induce the expression of Cystathionine-β-synthase (CBS) and Cystathionine-γ-lyase (CSE) in mammalian cells, that could point to an interplay between H_2S production and H_2Se status.

6.3 Materials and Methods

6.3.1 Chemicals and reagents

Sodium hydrosulfide (NaHS), Diethylamine NONOate sodium salt hydrate (NONOate), Woolins' reagent, Morpholine and Dichloromethane anhydrous were all from Sigma-Aldrich (St Louis, USA). SIN-1 hydrochloride was from EMD Millipore Corp (Burlington, USA).

6.3.2 Synthesis of GYY4137 analogue containing Se

SeGYY was prepared in an overall yield of 69.6% following the reaction of Woollins' reagent with morpholine in a one-step reaction process as detailed in Newton and Pluth (2019). In brief, 546mg of Woollins' reagent was added to 10ml of dichloromethane anhydrous under argon. 440µl of Morpholine (5.1mmol) was added and the reaction mixture stirred for 5 hours at room temperature. The reaction mixture was then filtered under vacuum through filter paper resulting in a black precipitate and golden-yellow filtrate. The filtrate was concentrated under reduced pressure and cooled to promote crystallisation. The resulting precipitate was isolated via filtration and washed with dichloromethane and dried overnight under reduced pressure to produce a white solid.

6.3.3 Mass spectral and NMR analysis to confirm structure

Mass Spec and NMR structural confirmation was carried out by Dr Fran Smith based in Chemistry, University of Nottingham under the guidance of Dr Nicholas Mitchell.

6.3.4 Measures of H_2Se production, Lead acetate

Lead acetate paper (Johnson Test Papers, Oldbury, UK) was used to attempt to assess H_2Se generation based on the H_2S detection work of Bethea (1973). Lead

acetate paper was cut into small squares of 8mmx8mm dimension and fitted into the lid on 1.5ml Eppendorf's. Sodium hydrosulphide (NaHS) was diluted into a range of concentrations from 0, 1.5, 3, 7, 15, 30, 60, 125, 250, 500 and 1mM in PBS as standards. Similarly, multiple dilutions of SeGYY were prepared 0µM, 15.63µM, 31.25µM, 62.5µM, 125µM, 250µM, 500µM to 1000µM SeGYY. 500µl of each standard and sample was placed into separate Eppendorfs containing lead acetate paper in the lid, the lids were closed and the Eppendorfs were allowed to incubate for an hour at room temperature.

6.3.5 Cell culture

HepG2 cells were cultured as detailed in 2.4.1-2.4.4. Cells were seeded at a seeding density of 10,000 cells/well in clear 96 well plates (Costar Flat Bottom with Lid) for the assays of SeGYY concentration dependent cell viability curves, caspase inhibition, SIN1, H_2O_2 and NONOate assays. Cells were seeded at 20,000 cells/well in clear 96 well plates for the lactate dehydrogenase assay. Cells were seeded at 10,000 cells/well in black (with clear bottoms) (µCLEAR®, BLACK CellStar® F-Bottom) plates for DCFDA. Cells were seeded at 400,000 cells/well in clear 6 well plates for the detection of cleaved PARP via ELISA. For western blot analysis cells were seeded in 100mm x 20mm petri dishes at a seeding density of $2.2x10^6$/petri dish.

The Se containing GYY4137 analogue was assessed in the current work and was dissolved in 10% DMSO to a final concentration of 100µM, before being adjusted to the required concentration by dilution in cell culture medium. All reagents were prepared fresh for each individual experiment.

6.3.6 Cell viability assessment, crystal violet and LDH analysis

Cells were treated with a range of concentrations of SeGYY 0, 15, 30, 60, 125, 250, 500 and 1000µM ± 500µM of GSH for 24 hours. Cell viability was determined using the crystal violet viability assay as previously detailed in 2.5.1.

For assessment of Lactate dehydrogenase (LDH) leakage, cells were treated with 0, 15, 30, 60, 125, 250, 500 and 1000µM of SeGYY for 24 hours prior to detection of LDH in the medium via the *CyQUANT* ™ LDH cytotoxicity assay kit (C20301 Invitrogen by Thermo Fisher Scientific) was measured using an Biorad microplate reader (Model 680 XR) at 415nm and 655nm following the manufacturer's instructions as detailed 2.5.3.

6.3.7 Indices of apoptosis, DAPI staining

To assess apoptotic cell death morphological changes in chromatin structure were detected by DAPI staining as detailed in 2.5.2. Cells were treated with 0, 125, 250, 500 and 1000µM SeGYY for 24 hours. 1mM H_2O_2 was used as a positive control. Images were taken on the EVOS fluorescent microscope. Apoptotic cells were identified by their morphology and by the condensation and fragmentation of their nuclei.

6.3.8 Measures of ROS, DCFDA and Lipid peroxidation

Measures of intracellular ROS production was determined using DCFDA and lipid peroxidation using a commercially available Image-It™ Lipid Peroxidation Kit (C10445 -Invitrogen by Thermo Fisher Scientific) both methods are detailed in chapter 2. Cells had were incubated with DCFDA for 1 hour prior to cell treatment as detailed in 2.5.6. After this the cells were treated with control (HBSS), 1000µM

SeGYY or 1mM of H_2O_2 (positive control). 100µl of HBSS was added to cell free wells this was referred to as 'background'. Each treatment was replicated 6 times per plate with 3 independent replicates performed on separate days with freshly prepared reagents. Fluorescence was measured using the FluoStar Omega fluorescent plate reader (485/520nm). Data was exported to Microsoft excel, where background fluorescence was deducted from all measurements of fluorescence.

Lipid peroxidation was determined using a commercial kit Image-It™ Lipid Peroxidation Kit (C10455-Invitrogen by Thermo Fisher Scientific) following the manufacturers instructions as detailed in 2.5.7. Cells were treated with 0, 250, 500 and 1000µM SeGYY, 100µM of cumene hydroperoxide (positive control) for 2 hours, or 1000µM for 0, 30, 60 and 120 mins. Images were taken on the EVOS Fluorescent microscope.

6.3.9 Western blot analysis

For western blot analysis of caspases, cells were treated with 0, 30, 125 and 500µM of SeGYY for 24 hours prior to cell scraping and protein extraction. In the assessment of SeGYY on markers of Se status, cells were treated with 0, 15, 30, 60, 125, 250, 500 and 1000µM SeGYY for 24 hours prior to cell scraping and protein extraction. Protein extraction, normalisation and western blotting was carried out as detailed in 2.6.1-2.6.3.

6.3.10 Caspase Inhibition

To assess a role of caspase in the cytotoxic response of cells to SeGYY, cells were first pretreated with caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK as detailed in 2.5.4

prior to 24 hour treatment with 640µM (IC_{50} concentration) of SeGYY. Cell viability was assessed via crystal violet (2.5.1).

6.3.11 PARP ELISA

For the determination of cleaved PARP, cells were treated with control (FBS free media) or 500µM of SeGYY for 24 hours. Cleaved PARP was assessed in cell lysates using a commercial ELISA kit (Thermo fisher) following the manufacturer's instructions detailed in 2.5.5. Cleaved PARP was then normalised to protein levels via the Lowry assay also detailed in 2.6.2.

6.3.12 SeGYY as a protective molecule

Cells were pre-treated for 1 hour with 0µM, 15µM or 30µM SeGYY diluted in FBS free media, prior to the addition of oxidant species, SIN-1 (1mM), Diethylamine NONOate (2mM) and H_2O_2 (100µM) adapted from Le Trionnaire et al. (2014). For the H_2O_2 treatment, cells were exposed to H_2O_2 diluted in HBSS for 30 mins at 37°C and 5% CO_2, before H_2O_2 was removed and SeGYY treatments were added again. Cell viability was measured via crystal violet assay after 24 hours of treatment as detailed in 2.5.1.

6.3.13 Statistical analysis

All data are represented by three separate experiments. All experimental data consists of mean±SE unless otherwise stated and were analysed by one-way ANOVA with post-hoc Tukey tests for 3 or more groups or Independent t-test for comparing 2 groups.

6.4 Results

6.4.1 Synthesis of SeGYY

SeGYY was prepared in an overall yield of 69.6% following the reaction of Woollins' reagent with morpholine in a one-step reaction process as detailed in Newton and Pluth (2019)(Figure 6.1). Structural confirmation was obtained flowing mass spectral analysis and ^{1}H, ^{13}C, ^{31}P NMR analysis as shown in (Supplementary Figure 1-3). Mass spectral analysis conformed a product having a molecular weight of 353.9.

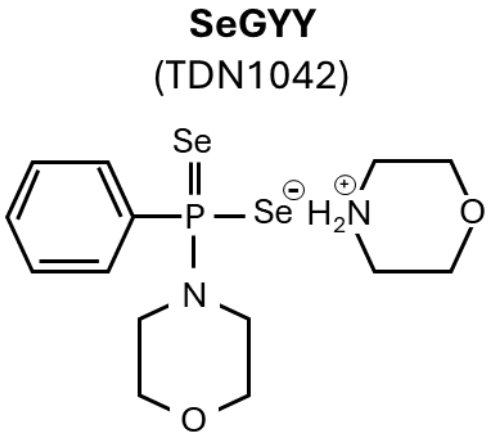

Figure 6.1-Chemical structure of SeGYY (TDN1042) (Newton and Pluth, 2019).

6.4.2 Evaluation of H_2Se production from SeGYY

We attempted to determine the presence of H_2Se release from SeGYY using the H_2S capturing system of lead-acetate paper as there are currently no commercially available H_2Se probes for detection. In the presence of H_2S, H_2S reacts with lead ions on the acetate paper forming solid lead sulfide, a black solid. A standard curve 0-1mM of H_2S NaHS was prepared and images were taken of the squared of lead-acetate paper after 1 hour, H_2Se was unsuccessfully detected using this method, even after 24 hours (Figure 6.2).

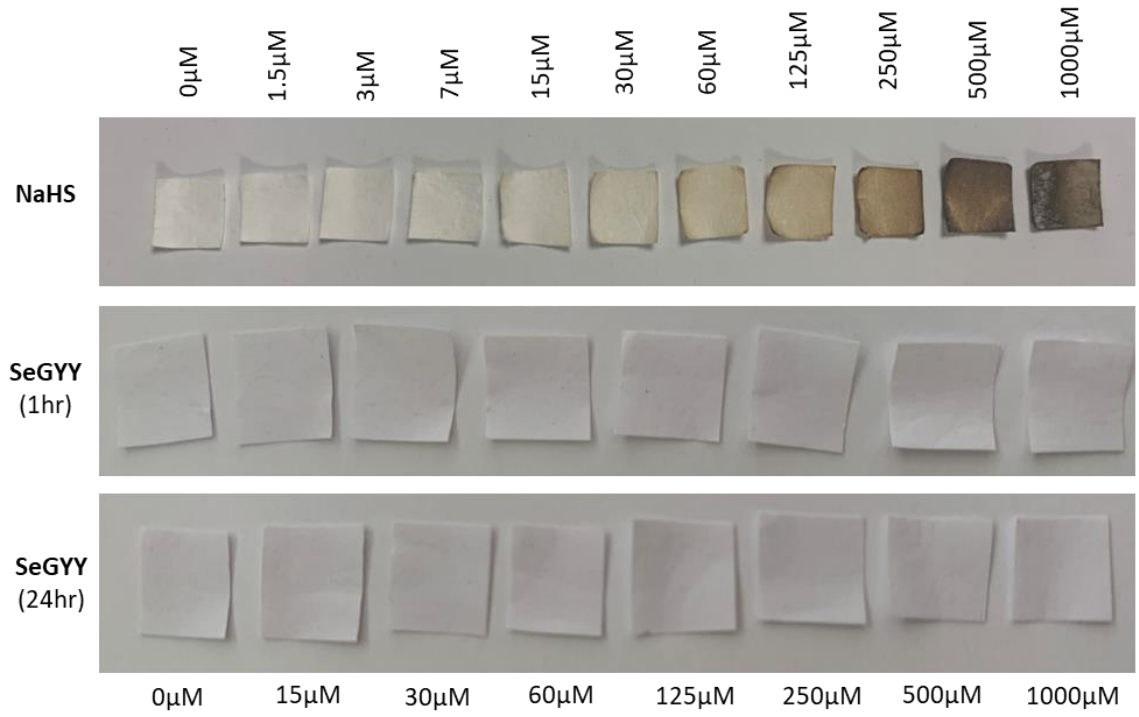

Figure 6.2- Attempt to detect H_2Se via Lead-acetate paper. Standard curve generated using H_2S releasing NaHS after 1 hour incubation with lead-acetate paper. SeGYY prepared in concentration range, 0, 15, 30, 60, 125, 500 and 1000µM, solutions were exposed to lead-acetate paper for 1 hour and 24 hours.

6.4.3 SeGYY promotes cell death in HepG2 cells

Preliminary control experiments were conducted to determine the cytotoxic profile of SeGYY. The addition of ≥500µM of SeGYY for 24 h caused a significant induction of cytotoxicity as compared with the vehicle controls ($p=0.032$) as determined via one-way ANOVA (Figure 6.3A). The IC_{50} for SeGYY compound was 640 µM as compared to naturally occurring Se containing species viz. selenite (IC_{50}, 75µM) and $SeCys_2$ (IC_{50}, 73 µM), respectively. This suggests that the slow release molecule SeGYY was less toxic than Se compounds $SeCys_2$ and Selenite. Incubation of SeGYY in the presence of GSH had no significant negative effect on cell cytotoxicity ($p>0.05$) suggesting SeGYY

does not interact with thiols in the same way as selenite (IC_{50} 15µM in presence of 500µM GSH). Indeed, incubation of 1mM of SeGYY with GSH significantly increased cell survival as compared to SeGYY alone (p=0.018), (Figure 6.3A). Additional indices of apoptosis were also evaluated using 4',6-diamidino-2-phenylindole (DAPI) staining and LDH leakage. DAPI staining revealed a concentration dependent increase in nuclear condensation and blebbing in cells treated with SeGYY (Figure 6.4). This suggests that a possible mechanism of cell death was via DNA damage. LDH is an cytosolic enzyme present in almost all cells that is released into the extracellular space when membranes are damaged. In cells treated with GYY, it was found that LDH leakage was only significantly increased at the 1mM concentration as compared to control (p=0.032)(Figure 6.3B). This suggests that treatment of cells with a high concentration of GYY results in membrane damage prior to cell death. For reference, all subsequent studies focused on determining the mechanisms by which SeGYY caused a loss in cell viability.

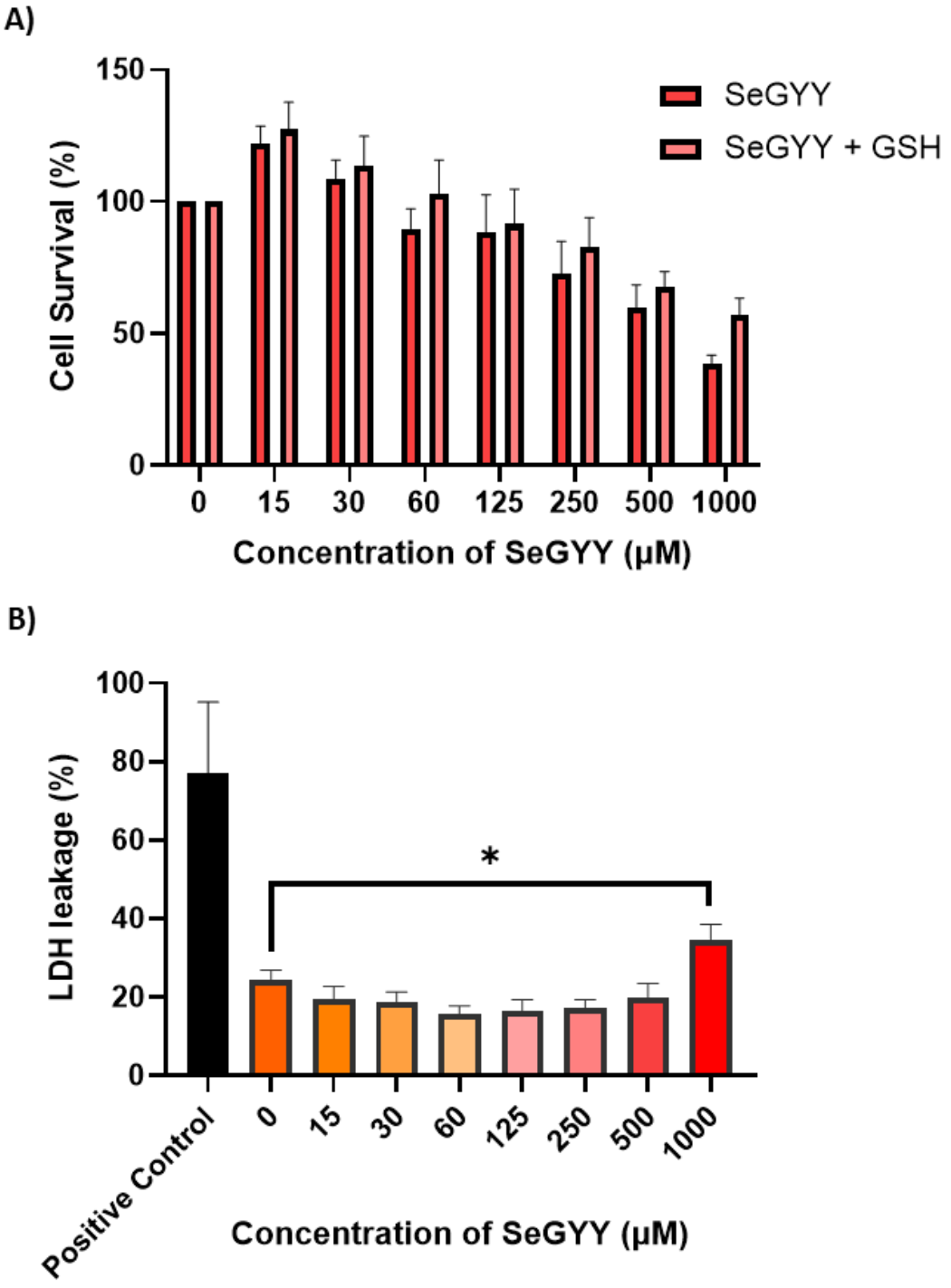

Figure 6.3- A concentration dependent loss of cell viability induced by SeGYY determined at 24 hours using the crystal violet viability assay. Data is representative of three or more separate experiments and expressed as percentage survival compared with control (mean±SE). B) Effects of SeGYY 0-1000μM on LDH leakage determined at 24 hours via the *CyQUANT* ™ LDH Cytotoxicity Assay Kit. Data is expressed as percentage of maximum LDH activity as mean ±SE, and is representative of 3 or more separate experiments (n=9), * signifies significance $p<0.05$ as determined via ANOVA and Tukey post-hoc testing.

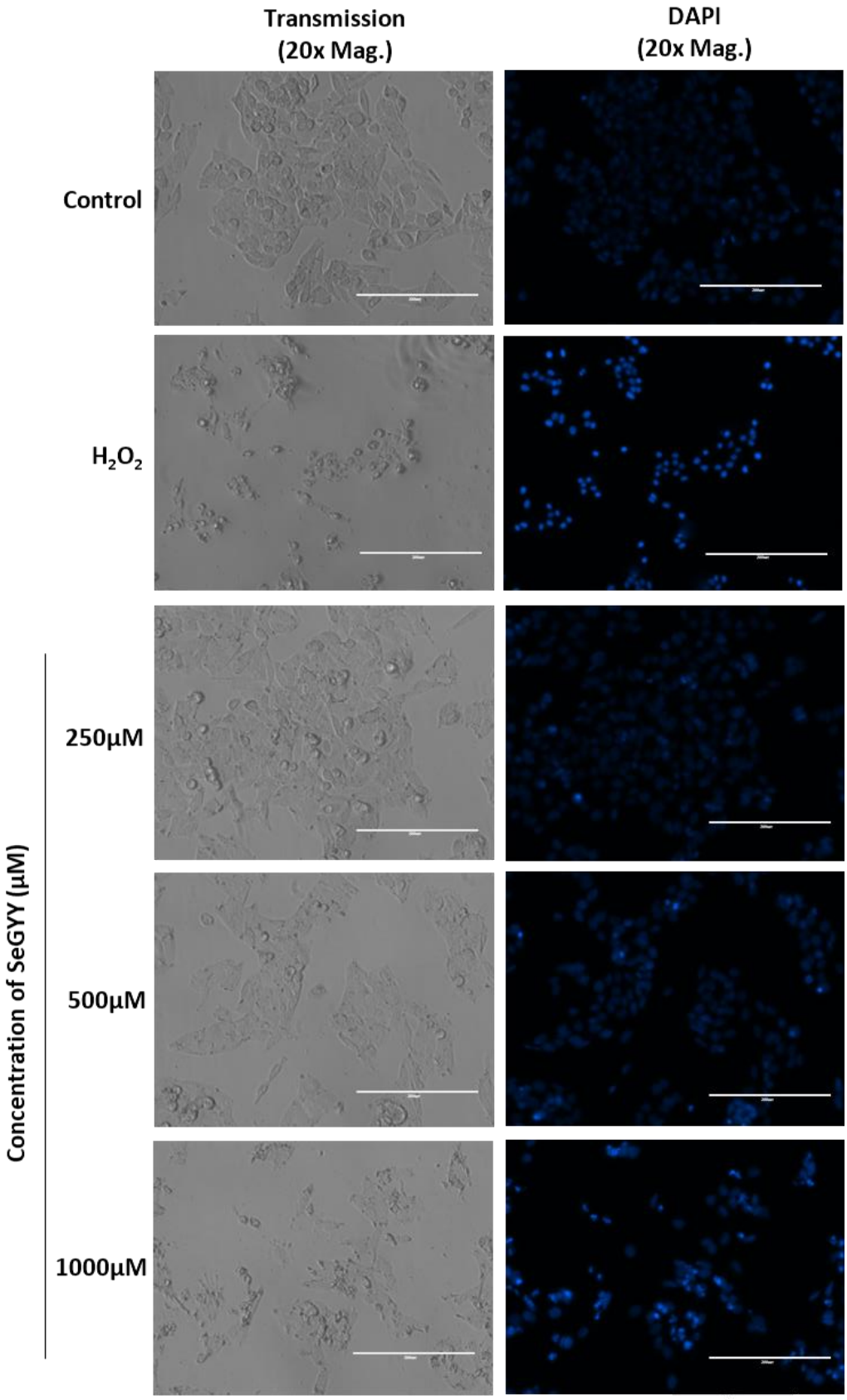

Figure 6.4-Morphological observation of HepG2 cells treated with concentration range of SeGYY using DAPI staining (20x) : a)control group; b) positive control (1mM

H_2O_2); The cells morphology was observed under the fluorescent microscope after DAPI staining. Scale bar represents 200µm.

6.4.4 SeGYY promotes ROS production in HepG2 cells

ROS has been widely reported to initiate the apoptotic cascade in mammalian cells. We used DCFDA to investigate ROS production in HepG2 cells when incubated with SeGYY for different time periods 0 – 120 minutes. Results showed that ROS generation was stimulated, and this followed a concentration and time dependant pattern (Figure 6.5). Treatment of HepG2 cells with 1000µM SeGYY resulted in significantly increased ROS production at every time point measured as compared to control (p= 0.001, respectively), (Figure 6.5). In parallel experiments, lipid peroxidation was also determined using the Image-iT® Lipid Peroxidation Kit (Thermo Fisher Scientific). The Image-iT® Lipid Peroxidation Kit is based on BODIPY™(581/591) C11 reagent and is a fluorescent lipid peroxidation reporter molecule that shifts its fluorescence from red (590nm) to green (510nm) upon oxidation. Images suggest that there is a marginal time and concentration dependent increase in lipid peroxidation (Figure 6.6 and 6.7).

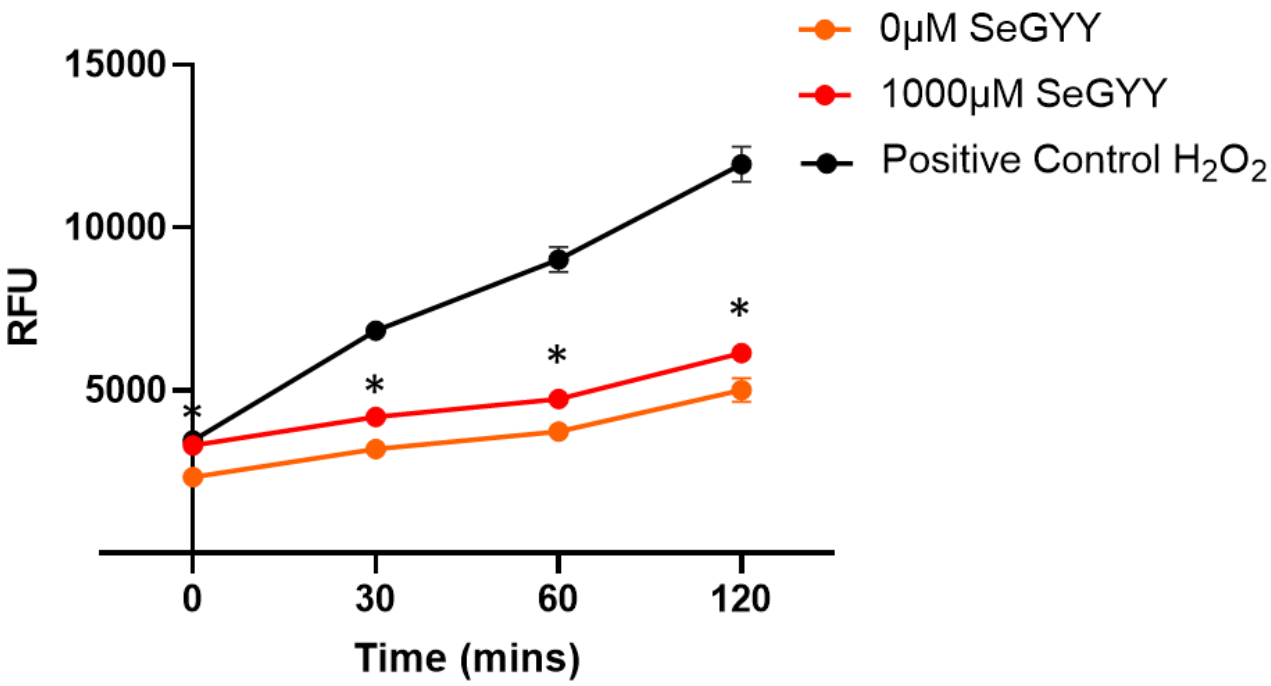

Figure 6.5-Time dependent increase in RFU after staining with DCFDA for 1 hour prior to treatment with control FBS free media , 1mM H_2O_2 and 1000μM SeGYY. Plates were read at 485/520nm at 0-, 30-, 60- and 120-minute time points. Data is representative of three or more separate experiments (mean ±SE) n=9, significance is represented as * $p<0.05$ as determined by independent sample t-test.

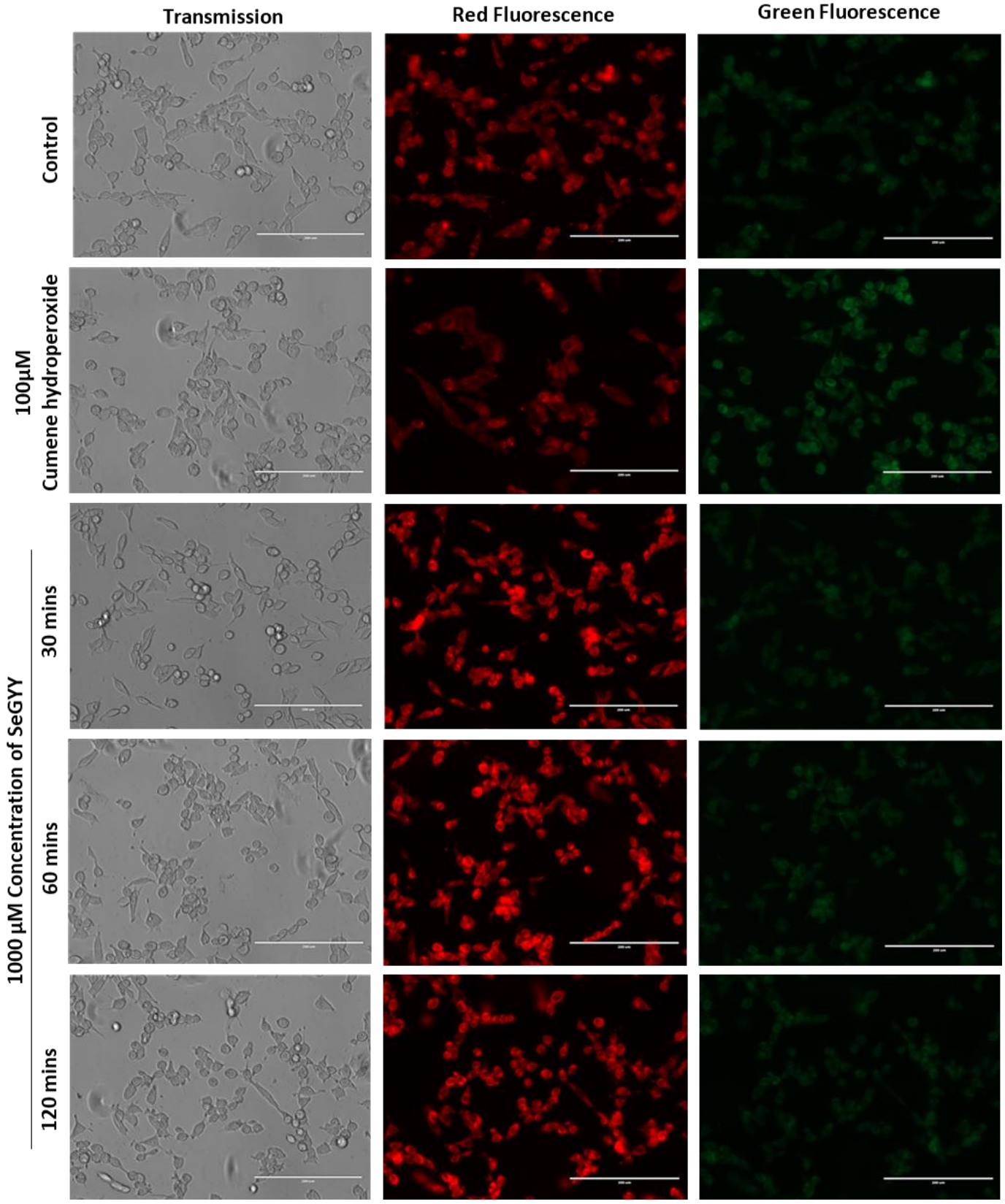

Figure 6.6- Fluorescent images of HepG2 cells stained with lipid (red)/lipid peroxide detection (green) reagent after 30, 60, 120 minutes incubation with SeGYY . Scale bar represents 200µm.

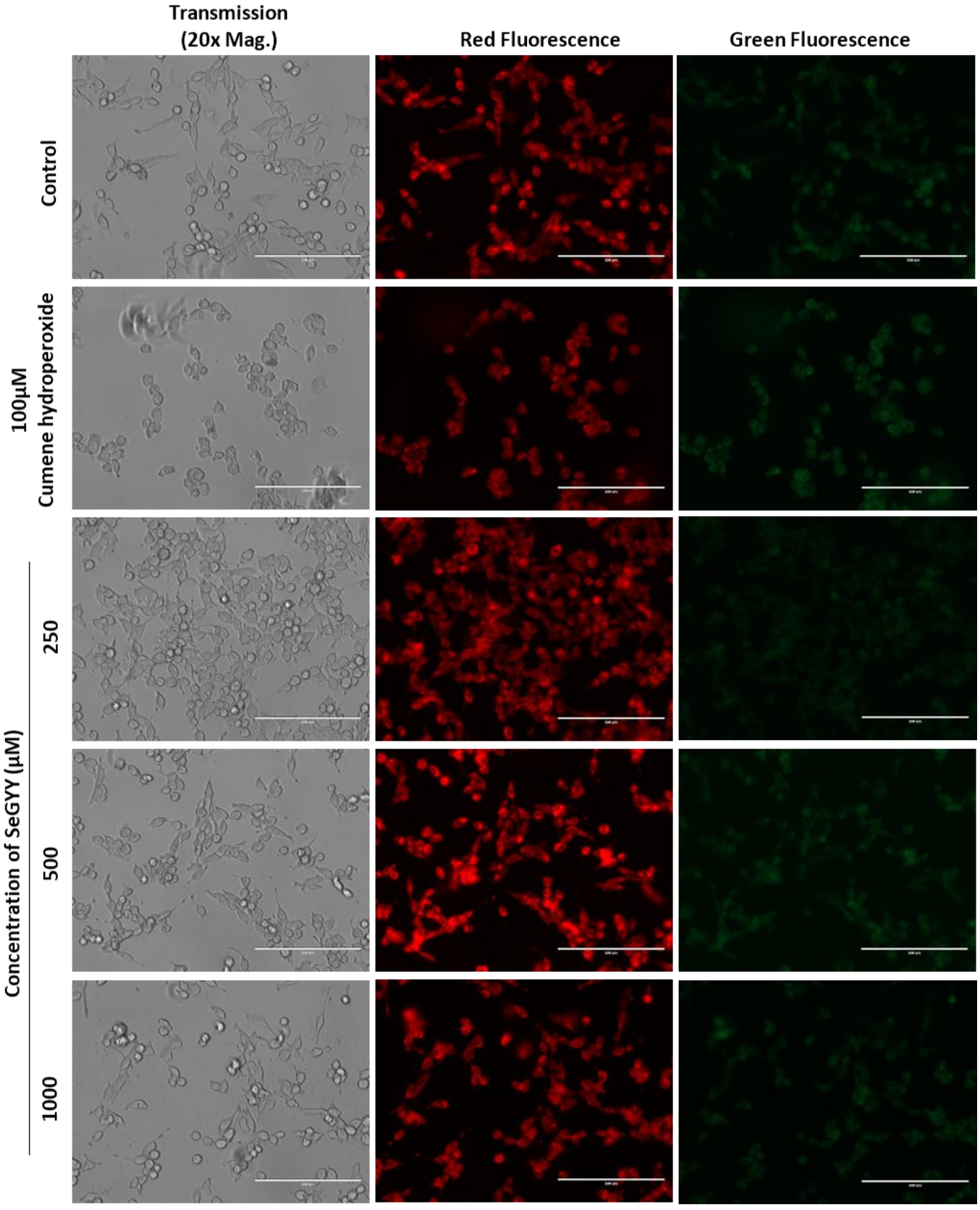

Figure 6.7- Fluorescent images of HepG2 cells stained with lipid (red)/lipid peroxide detection reagent (green) with a range of concentrations, 250µM, 500µM and 1000µM of SeGYY after 120 minute incubation. Scale bar represents 200µm.

6.4.5 Involvement of Caspases in SeGYY mediated apoptosis.

We next assessed the inhibitory effects of the pan caspase inhibitor Z-VAD-FMK and the capsase-3 specific inhibitor Z-DEVD-FMK on SeGYY mediated loss of cell viability, to further understand the mechanisms involved in SeGYY mediated cell death. In our hands, pre-incubation with individual caspase inhibitors for 1hr prior to exposure to SeGYY (IC_{50} 640µM), prevented cell death by up to 40% and 20% respectively (Table 6.1). A significant increase in cell survival was seen in cells pre-treated with the pan caspase inhibitor Z-VAD-FMK ($p<0.001$) prior to SeGYY exposure as compared to SeGYY treated alone. Although a near significance ($p=0.057$) increase in cell survival was also observed in cells pre-treated with the caspase 3 inhibitor Z-DEVD-FMK prior to SeGYY exposure as compared to SeGYY treated alone. This finding being reflective of a potential role of caspases in the apoptotic cascade, and potentially caspase 3 involvement. Treatment of HepG2 cells with 500µM of SeGYY for 24 hours resulted in a significant increase ($p<0.001$) in cleaved PARP as compared to control (Figure 6.8). This suggests that PARP cleavage is part of the apoptotic cascade in SeGYY mediated cell death. Western blot analysis was used to further confirm the involvement of caspase -3 and -8 in SeGYY mediated apoptosis. However, analysis of the pan-caspases proteins for caspase -3 and -8 showed no significant reductions ($p=0.145$, 0.238 respectively) in this protein in cell extracts treated with SeGYY for 24hrs (Figure 6.9). This negative result could be reflective of the need to conduct a full-time course analysis in cells exposed to this molecule. Sadly, time constraints prevented this set of experiments.

Table 6-1- Assessing the role of caspases within SeGYY mediated cell death . HepG2 cells were pre-treated for 1 hour with 75uM of caspase inhibitors Z-VAD-FMK/Z-DEVD-FMK prior to cell treatment with 640µM SeGYY. Cell viability was determined after 24 hours using the crystal violet cell viability assay. Data is representative of three separate experiments and expressed as percentage survival compared with control (n=9) (mean±SE). Mean ±SE followed by different letters indicates significance $p<0.05$ as determined via ANOVA and Tukey post hoc test.

Pre-treatment of HepG2 cells (75µM)	640 µM SeGYY Cell survival (% ctrl ±SE)
None	**56.54** ±4.38[a]
Z-VAD-FMK	**110.65** ±6.50[b]
Z-DEVD-FMK	**81.80** ±7.11[a]

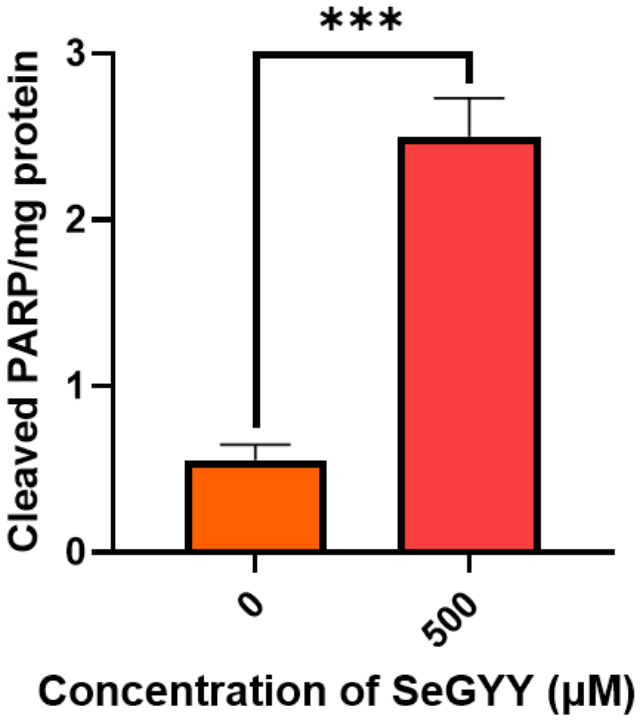

Figure 6.8- Cleaved PARP/mg protein as determined via ELISA and normalisation to Lowry protein assay, after treatment of HepG2 cells with a range of concentration of SeGYY for 24 hours. Data is represented at mean ± standard deviation (n=3). Statistical significance calculated by independent t-test, *** indicated significance, $p<0.001$.

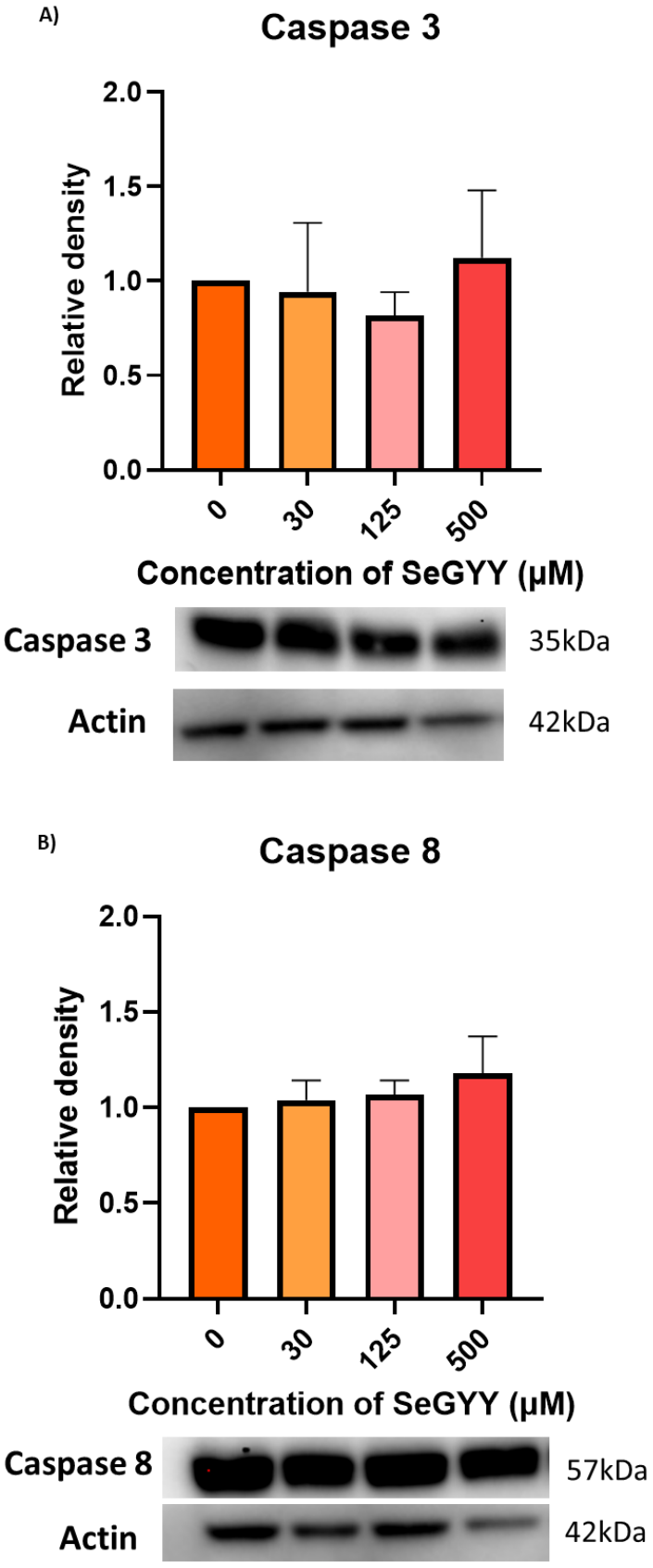

Figure 6.9- Expression of A) Caspase 3 and B) Caspase 8 as determined via western blot analysis and subsequent densitometry. Data expressed as mean ± standard deviation (n=3). Western blots were normalised to actin as loading control.

6.4.6 Effects of SeGYY on oxidative stress induced cytotoxicity

While higher concentrations of SeGYY were found to promote cytotoxicity in HepG2 cells (Figure 6.3), we also questioned whether non-toxic levels could promote cytoprotection. It has previously been shown that slow release H_2S donor GYY4137 protected human cells in culture from oxidative stress-induced toxicity, induced by a range of oxidant species such as 4-hydroxynonenal, hydrogen peroxide and NO donors such as SIN-1. No evaluation had yet been made for SeGYY. Therefore, in order to determine whether SeGYY had cytoprotective properties at lower concentrations we exposed human hepatoma HepG2 cells to SIN-1 (1mM), NONOate (2mM) and H_2O_2 (100µM) in the presence or absence of non-toxic doses of SeGYY (15-30µM) for 24 hrs. Cellular viability was then determined after 24 h. As shown in Figure 6.10, incubation of HepG2 cells with 15µM or 30µM of SeGYY significantly inhibited SIN-1 oxidative stress-induced cell death ($p=0.004$, 0.038 respectively). Although not significant, there was also a concentration dependent trend in SeGYY protection to oxidative stress of NONOate and H_2O_2.

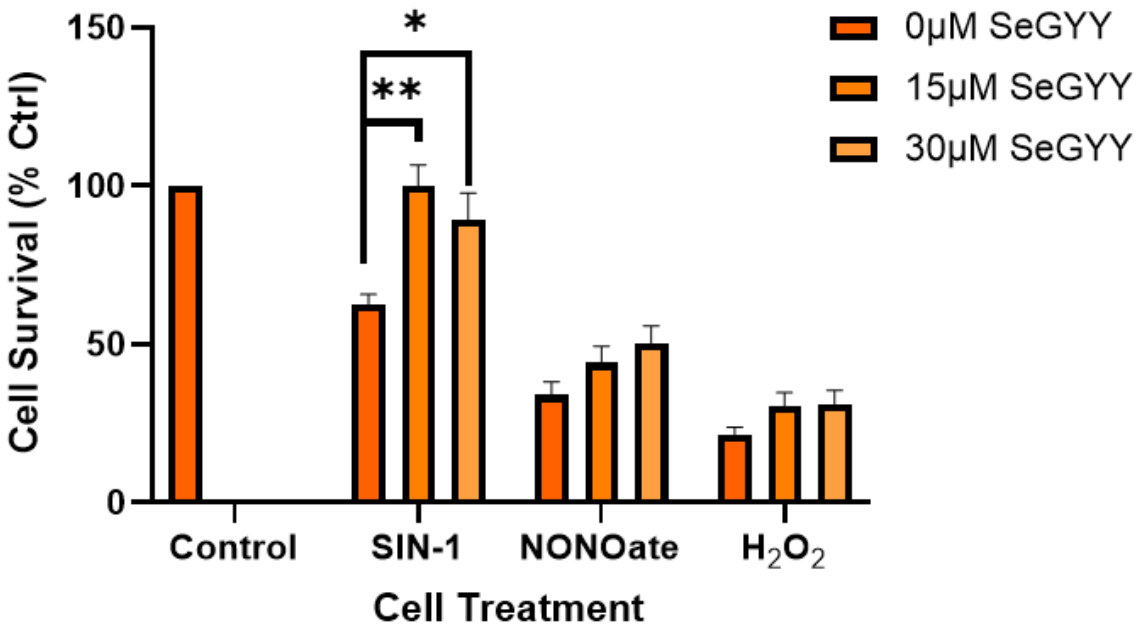

Figure 6.10- Cell survival (% of ctrl) of HepG2 cells pretreated with 0, 15 or 30μM SeGYY for an hour prior to the addition of oxidant species, SIN-1 (1mM), NONOate (2mM) and H_2O_2 (100μM). Cell survival determined after 24 hours using the crystal violet cell viability assay. Data expressed as mean ± standard error (n=9). Statistical analysis determined via ANOVA, * signifies significance of $p<0.05$ and ** $p<0.01$.

6.4.7 Impact of SeGYY on CBS, CSE and SCLY expression in hepG2 cells

Another key question relating to the biological function of SeGYY was whether it induced Se metabolism enzymes and proteins involved with other gaseous mediators. To test these effects, we used western blot analysis to determine the expression levels of Selenocysteine lyase (SCLY), Cystathionine-β-synthase (CBS) and Cystathionine-γ-lyase (CSE) in HepG2 cells treated with SeGYY. Results from this analysis would firstly provide evidence the induction of Se metabolic systems and, secondly cross talk between other gaseous mediator systems. Treatment of HepG2 cells with a range of SeGYY concentrations (0-1000μM) for 24 hrs was found to not significantly induce SCLY ($p=0.495$), however a trend in increased SCLY expression

was seen at SeGYY concentrations of 500µM and 1000µM (Figure 6.11). This enzyme is critical in the catabolism of organic Se forms and likely contributes to the production of endogenous levels of H_2Se.

In addition to Se related protein targets, we also explored whether SeGYY had the capacity to induce the expression of other enzymes involved in gaseous mediator production. Previous studies have shown coordinated interplay between NO and H_2S biosynthetic pathways, this can be used to compensate for any loss of capacity to produce either of the aforementioned gaseous mediators. To this end, we tested if SeGYY could induce the expression of either CBS or CSE in HepG2 cells. We report that SeGYY had no significant impact on the expression of CBS and CSE at all concentrations tested (15-1000µM) ($p=0.946, 0.082$) after 24 hrs treatment (Figure 6.12). However, despite the lack of significant difference there appeared to be a concentration dependent trend of increased CSE protein levels (Figure 6.12B). This data is intriguing since it indicates that SeGYY could potentially promote H_2S biosynthesis, although further work is needed to confirm this. This finding parallels the known action of other H_2S donor molecules that also induce these enzymatic systems in mammalian cells and tissues.

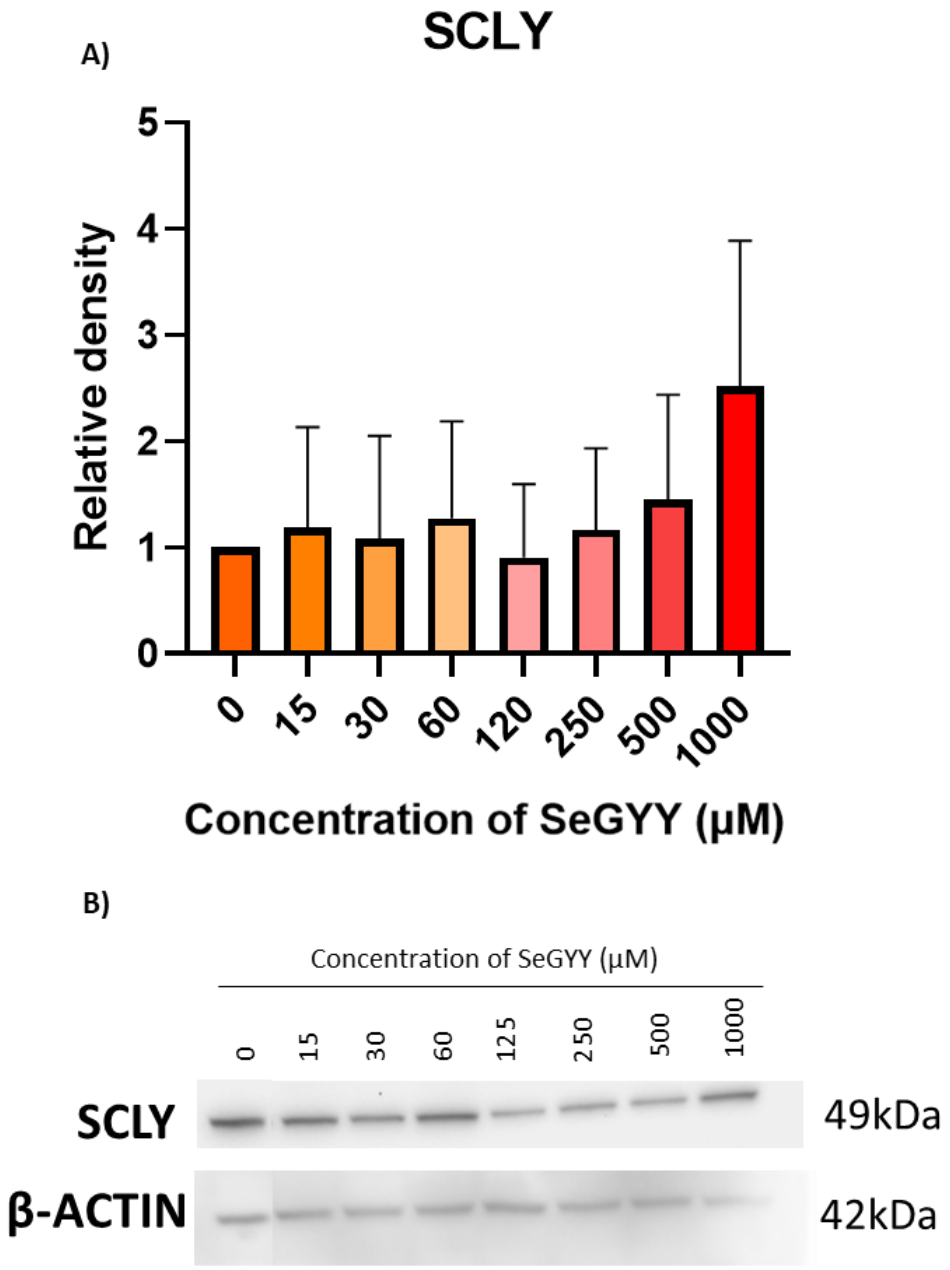

Figure 6.11- A) Expression of SCLY as determined via western blot analysis. Data expressed as mean ± standard deviation (n=3). B) Example of western blots generated with SCLY and Actin (for normalisation purposes), densitometry was calculated from these blot to generate A).

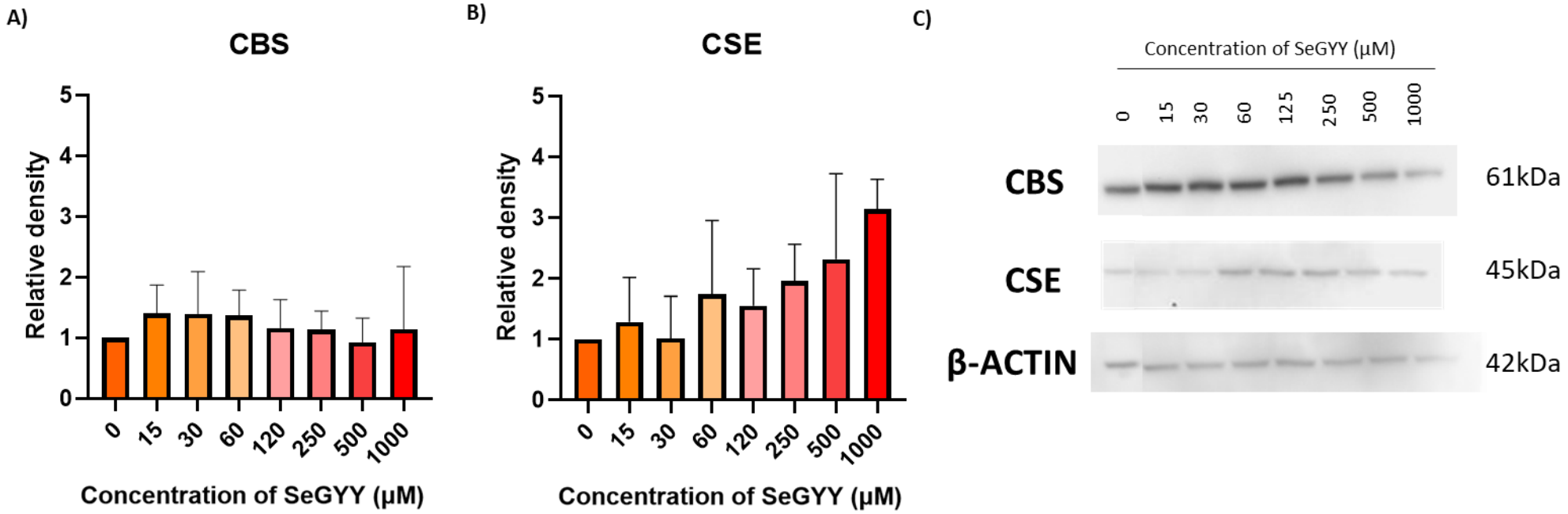

Figure 6.12- Expression of A) CBS and B) CSE as determined via western blot analysis. Data expressed as mean ± standard deviation (n=3). C) Western blots generated with CBS, CSE and Actin (for normalisation purposes), densitometry was calculated from these blot to generate A) and B).

6.5 Discussion

In chapter 5, we showed that Se compounds promote loss in cell viability in hepatoma HepG2 cells. We also provided evidence that the interaction of selenite with GSH enhanced toxicity and postulate the involvement of H_2Se. Currently there are no commercially available probes for detection of H_2Se, because of this we attempted to use the H_2S measuring system lead-acetate paper to detect H_2Se generation due to the similarity in H_2S/H_2Se, unfortunately this method failed to detect H_2Se production. However, slow and controlled H_2Se production from SeGYY when in solution was detected by the developers of SeGYY (Newton and Pluth, 2019). Building on the previous chapter we report here that SeGYY causes a concentration dependent reduction in HepG2 cell survival as well as DNA damage and cell membrane damage as indicated by nuclear condensation and blebbing during DAPI staining and increased LDH leakage (Figure 6.3 and 6.4). Thus, the present data reveals for the first time the potential anticancer effect of slow releasing H_2Se donor TDN1042 (SeGYY). In a study by Lee et al. (2011) cell death and LDH leakage was observed in HepG2 cells treated with 400µM or 800µM of H_2Se releasing GYY4137 for 5 days. This suggests that SeGYY is more bioactive than GYY4137, as significant cytotoxicity occurs after 24 hours exposure to SeGYY. This could potentially be explained by the increased reducing activity of the Se atom compared to S atom (Krakowiak and Pietrasik, 2023). On the other hand, Lu et al. (2014) found a similar cell viability percentage to our study of approximately 60% cell survival after treatment with 400µM GYY4137 for 24 hours. Cell survival was further reduced to 40% and 20% after 48 and 72 hr exposure respectively. Lu et al. (2014) also demonstrated the ability of GYY4137 to inhibit interleukin-6 (IL-6) induced

STAT3 (signal transducer and activation transcription factor 3) activation, via blocking the phosphorylation of janus activated kinase (JAK2). Altered expression of STAT3-regulated downstream proteins such as cyclin D1, Mcl-1, Bcl-2 and survivin were also seen in GYY4137 treated HepG2 cells which was postulated to contribute to inhibition of the cell cycle and initiation of apoptosis in HepG2 cells (Lu et al., 2014). Further experiments are needed to assess whether SeGYY exerts apoptotic effects seen in HepG2 cells via similar molecular targets as S analogue GYY4137. Previous literature has shown the ability of Se to substitute for S in metabolism and transulfuration pathways due to their similarity in physical and chemical properties, therefore sharing molecular targets of apoptosis may be a possibility. Further to this, our observation of DNA damage via DAPI staining is supported by DNA phosphodiester bond breaks observed in yeast cells treated with H_2Se donor sodium selenide in work conducted by Peyroche et al. (2012). This DNA damage was demonstrated to occur in yeast cells by an O_2-dependent reaction involving radical generation (Peyroche et al., 2012).

We report here that pre-treating HepG2 cells with pan caspase inhibitor Z-VAD-FMK and Z-DEVD-FMK, prior to treatment with IC_{50} concentration of SeGYY causes significant increase (and near significant increase in the case of Z-DEVD-FMK) in cell survival suggesting that caspases are involved in SeGYY mediated cell death (Table 6.1). An increase in PARP cleavage was also found in HepG2 cells treated with 500µM SeGYY (Figure 6.8). Previous literature regarding GYY4137 has also shown the increase in cleaved PARP as well as increases in cleaved caspase 9, in HepG2 and MCF-7 cell types (Lee et al., 2011, Lu et al., 2014). An increase in cleaved caspase 3 was also shown in Lu et al. (2014) when HepG2 cells were incubated with 0-400µM

range of GYY4137 for 24 hours, suggesting the potential involvement of caspase 3 in H_2S mediated cell death. Further caspase inhibitor experiments, using a concentration range of SeGYY and time course would further elucidate the role of caspases in SeGYY mediated cell death, although time constraints prevented this from happening in the current study.

SeGYY caused the production of ROS in a concentration and time dependent manner as indicated by DCFDA staining (Figure 6.5). Lipid peroxidation also suggests the exposure to SeGYY causes a time and concentration dependent increase in lipid peroxidation (Figure 6.6 and 6.7), although this is not as stark as compared to selenite and glutathione treated cells in chapter 5. This suggests that H_2Se release from SeGYY is much slower, and at 2 hours endpoint the potential full effect of SeGYY on lipid peroxidation may not be fully understood. However, in smaller non-toxic doses SeGYY was capable of preventing oxidative damage caused by peroxynitrite donor SIN-1 as well as H_2O_2 and NONOate (Figure 6.10), although these were not significant. The cytoprotective effects of slow release H_2Se donors have not been evaluated, however incubation of HepG2 cells with H_2Se produced from sodium hydroselenide (NaHSe) was found to confer cytoprotection from 500µM of H_2O_2 in a concentration dependent manner, similar to the non-significant trend seen in the current study (Samra et al., 2021). The GYY4137 H_2S donor has also been found to inhibit SIN-1 oxidative stress induced cell death in human joint cells (mesenchymal progenitor cells) and human cerebral endothelial cells (hCMEC/D3)(Fox et al., 2012, Le Trionnaire et al., 2014). Fox et al. (2012) also showed that addition of GYY4137 addition to mesenchymal progenitor cells caused a time and concentration dependent increase in survival protein Akt, as well as

increases in ERK1/2 phosphorylation and inhibiting P13K, Akt and ERK significantly reduced the protective effect of GYY4137 when cells were exposed to SIN-1. Thus suggesting the potential for H_2S to activate the PI3K-Akt/ERK cytoprotective pathways. While not tested in the current work it is compelling to hypothesise that SeGYY at low concentrations works in a similar manner. Pretreatment of H9c2 rat cardiomyoblast cells with low concentrations (500nM) of sodium selenite was found to be protective against oxidative stress induced by H_2O_2 (Sun et al., 2020). Sun et al. (2020) also found that pre-treatment of H9c2 cells with selenite resulted in increased thioredoxin activities, glutathione peroxidase activity and SOD activity, decreased ROS and MDA production as compared to H_2O_2 treated cells, as well as ameliorating H_2O_2 induced inactivation of P13K/AKT signalling pathway. Other Se compounds such as selenite and selenocystine have been shown to interfere with the phosphorylation of Akt and activation of pro-survival PI3K/AKT and MAPK/ERK pathways respectively during apoptosis (Lazard et al., 2017, Sanmartín et al., 2012), therefore we postulate if H_2Se (a key intermediate during metabolism of Se compounds) can modulate phosphorylation of such pathways in a concentration dependent manner to switch between cytoprotection and cytotoxicity. It is also known that Se can induce Nrf-2 signalling as a method of protecting cells from heavy metal and toxin damage (Deng et al., 2023, Wang et al., 2023, Xue et al., 2021), therefore this would be a worthy target in future studies.

H_2S is synthesised from cysteine by several enzymes such as CSE, CBS and 3-mercaptosulfurtransferase (3-MST) not measured here (Lee et al., 2011).In previous literature Se deficiency has been shown to increase the expression of CSE and CBS and in turn increase H_2S production in liver tissue from Se deficient chickens as

compared to control (Wenzhong et al., 2017, Zheng et al., 2018). Although we report here that application of up to 1mM SeGYY does not significantly impact on the expression of CBS or CSE, a general trend in increased CSE expression is seen with increasing SeGYY especially at higher ≥500µM concentrations (Figure 6.12). Tentatively looking at these results on the whole, this potentially suggests that exogenous application of H_2Se via SeGYY could induce CSE to produce H_2S. This would further suggest that there is crosstalk between H_2Se and H_2S, and may even suggest that H_2Se and H_2S can compensate for each other. Although further work is needed to confirm this. This is a novel area of research for H_2S/H_2Se, however H_2S has been shown to upregulate the production of NO, in particular H_2S and NO have been found to interact with in each others catalysing enzymes (CSE and interleukin induced NOS production)(Altaany et al., 2013, Jeong et al., 2006, Zhao et al., 2001). Other gaseous mediators such as CO have also been shown to upregulate the production of NO (Choi and Kim, 2021). This has subsequently been found to be useful in delivering highly reactive NO to distant targets (Motterlini and Otterbein, 2010). This suggests that crosstalk between gaseous mediators is possible, and there is potential for H_2Se to influence the production of H_2S, although further work is needed to explore this.

H_2Se is a key central molecule in the assimilation, metabolism and detoxification of Se in the diet (Roman et al., 2014). The enzymes CBS and CSE are involved in the transelenation of selenomethionine to selenocysteine (CBS/CSE) and the cleavage of selenocysteine into H_2Se (SCLY)(Roman et al., 2014). The slight but not significant increase in CSE and SCLY protein expression seen in HepG2 cells exposed to higher concentrations of ≥500µM of SeGYY (Figure 6.12B and 6.11), could also be due to the

presence of H_2Se increasing the Se status of the cell. This has previously been found in yeast cells exposed to selenite and glutathione (a reaction known to generate H_2Se). Exogenous application of SeGYY and subsequent slow release of H_2Se may increase expression of enzymes involved in transelenation and subsequent breakdown of SeCys to form the central H_2Se as a potential method of detoxification. Further experiments assessing the accumulation and speciation of Se within HepG2 cells exposed to H_2Se may further elude to whether Se status of cells is increased in the presence of SeGYY.

In terms of impacts on health, H_2Se has already been shown to have therapeutic properties such as in the reversal of liver fibrosis via the uncoupling of the sulfilimine bond which induces collagen IV degradation (Luan et al., 2021), reducing reperfusion injury in mice via reduction in metabolic rate (Iwata et al., 2015), as well as exhibiting toxic effects in cancer cells such as cervical cancer cells (Krakowiak et al., 2022) although the mechanism of action still remains unclear. Not only is SeGYY a new tool for discovering the application of H_2Se within mammalian cells but it could also lend itself to discoveries about the role of H_2Se in the wider biological world such as plants. More recently H_2S has been discovered to play key biological roles in plants (Filippou et al., 2013) by participating in processes such as stomatal movement, senescence and lateral root formation which help the plants respond to environmental stimuli such as salinity (Pourebrahimi et al., 2023, Guo et al., 2016). Non-accumulator *Arabidopsis thaliana* exposed to exogenous levels of H_2S have been shown to negatively correlate to length of primary root and display decreased meristem cell division and length of meristematic zones (Jia et al., 2015, Zhang et al., 2017)(reviewed in Li et al. (2022a)). H_2S also appears to initiate auxin synthesis in a

range of plants such as sweet potato, soyabean and willow (Zhang et al., 2009). Nothing is currently understood about the role of H_2Se within plants, although *Arabidopsis thaliana* exposed to excess selenite also showed root growth inhibition and loss of cell viability at the root apex. These effects are thought to be mediated by the plant hormones auxin and ethylene (Lehotai et al., 2012). The phenotypic changes are remarkably similar to H_2S treated roots, this leads us to further postulate whether the presence of H_2Se either induces the production of H_2S resulting in these phenotypic effects, or whether H_2Se can also initiate plant responses to stimuli.

6.6 Conclusion

In conclusion, our study shows for the first time that SeGYY exerts an anticancer effect on HepG2 cells, the apoptotic mechanism may be partially due to the generation of ROS, involvement of caspases and cleavage of PARP. However, further experimentation is needed to fully understand the mechanism involved. Although H_2Se is seen as a toxic gas, at low concentrations we have shown it to have a protective effect in HepG2 cells when exposed to oxidant releasing chemicals, suggesting a dual role for H_2Se in both cytoprotection and cytotoxicity. Results from the current study also suggest that H_2Se production may have crosstalk with production of other gaseous mediators, although again further work is needed to elucidate this role.

7 Overall Discussion

Low Se intake in the general UK population is widespread and was thought to be as low as 30-40µg/day in 1997 (Rayman, 1997). More recently the national diet and nutritional survey (NDNS) for the period 2008/9-2010/11 found that 39% of adults (19-64 years old) and 44% of adults 65 and over had an intake of Se that was less than the LRNI of 40µg Se/day (NDNS, 2012). Elderly and vegetarian groups are at greater risk of Se deficiency due to having low dietary Se intakes (Jackson et al., 2003). Strategies to increase Se content within crops and subsequently the general population have previously included fertilization of soil (Alfthan et al., 2011) however, newer methods of Se biofortification such as foliar/fruit spraying, seed treatments and hydroponics systems are being explored in order to increase Se status of edible foods with the view of increasing intakes in consumers.

Prior to this work it has been widely accepted that several Allium species tolerate and readily absorb Se (González-Morales et al., 2017). However, little had been reported on the effects of Se accumulation in different cultivars nor the subsequent impacts on bioactivity or cell cytotoxicity. Since Se has a narrow therapeutic range it is important for researchers to consider the impacts of Se biofortification on plant bioactivity. As a model in the current research, we used *Allium sativum* (Garlic) a commercially important crop that is widely consumed globally with an estimated global market value of $19 billion (Anon, 2021). We assessed two methods to biofortify several garlic cultivars using foliar application (Chapter 3) and hydroponic manipulation (Chapter 4). In addition, we considered the impact of Se

biofortification on the bioactive nature of plant tissues, as assessed using measures of cytotoxicity (Chapter 4). We conducted pilot work to evaluate whether Se enrichment of plants altered cell cytotoxicity by measuring parameters linked to apoptosis, since Se is widely reported to have anticancer properties (reviewed in Kuršvietienė et al. (2020)). In addition, we further explored the biological role of Se compounds in mammalian cells (Chapter 5), testing the hypothesis that certain Se compounds differ in their capacity to promote cytotoxicity, and that some may promote the formation of a novel Se intermediate, hydrogen selenide (Chapter 6).

In chapter 3, we hypothesised that foliar application of Se would positively impact on growth and quality (as assessed by antioxidant capacity and accumulation of other minerals) of garlic plants and bulbs. Sodium selenate has been widely used to biofortify plants including species like rice (Delaqua et al., 2021), potato (Zhang et al., 2019a) and broccoli (Muñoz et al., 2021). Our results demonstrated that the foliar enrichment of the garlic cultivars with either 25µM or 50µM sodium selenate did not impact significantly on the majority of growth measures or negatively impact on the accumulation of other nutrients within the leaf and clove tissues. This finding is a promising result since it shows that this level of Se application does not hinder plant growth or lead to appreciable impacts on uptake of other minerals which is appealing to producers and consumers. We showed that the average bulb weights of Se treated plants were comparable to their commercial counterparts when grown under experimental conditions. Moreover, we showed that there are clear differences in growth traits between individual cultivars with some having greater bulb dimensions and clove weight compared to others i.e. Lyubasha vs Marco. However, the levels of Se accumulated via foliar application were low and not

significantly different as compared to the control. This suggesting that either additional optimisation of the application approach is needed or that foliar application is not an effective method of Se enrichment of garlic plants. Another concern reported in the current work, was the significant decrease in radical scavenging ability that clove tissues of all cultivars displayed following Se enrichment. Whilst we acknowledge that only one antioxidant assay was conducted this pilot data could open other avenues of research to assess the impacts of biofortification on other health quality traits of edible plants.

In chapter 4 we assessed whether Se supplied via hydroponic methods was accumulated differently in different cultivars of garlic and whether this impacts on toxicity of garlic tissues. Hydroponics are a soilless system, in which Se is supplied to the roots via liquid growing media. From a commercial aspect hydroponic systems have a high upfront cost resulting from the need for custom growing apparatus (Swain et al., 2021). Plants are often grown in controlled environments such as growth chambers or glasshouses, therefore there are additional costs of heating and lighting (McDonald, 2016). Hydroponics can however be more efficient in terms of water use efficiency compared to field grown crops (Verdoliva et al., 2021) and the soil-less system removes the risk of soil contamination. Hydroponic methods of Se enrichment have been shown to be a successful method of enriching garlic cloves (Tsuneyoshi et al., 2006) and are of commercial interest (https://selenoforce.com/index.html). In the UK garlic is currently grown commercially out in the fields, although climate change is having significant impacts on yields and quality due to losses in field and pathogen infections. Little research has focused on the assessment of the impacts of cultivar difference on accumulation

of Se using hydroponic methods. In Chapter 4, hydroponic methods were used to successfully enrich garlic cultivars Marco, Mersley, Solent and Lyubasha with Se in the range of 7.14-40.05 mg/kg DW depending on cultivar. Increases in the Se species SeMet, Selenate and $SeCys_2$ were also seen in the Se enriched clove tissues of the cultivars Mersley and Solent. Therefore, our hypothesis that the hydroponic application of Se will increase the Se content of garlic tissues and that the level of Se accumulated between cultivars will differ can be accepted. Initial rough calculations suggest that only half a clove of Se enriched garlic are needed to be consumed in order to meet the RDA of 60µg/day for women and 75µg/day for men. This illustrates that hydroponically Se enriched garlic tissues would deliver suitable amounts of Se that to provide health benefits. We also investigated whether Se enrichment impacted on the accumulation of other nutrients present in the hydroponic solution in all four cultivars. The results showed that Se enrichment did not result in any significant differences in other mineral accumulation within the clove tissues namely Na, Mg, P, S, K and Ca. This is preferable as significant decreases in other nutrients would impact on clove quality traits (Malagoli et al., 2015). Essentially, our study demonstrates hydroponics to be a successful technique for increasing Se content of garlic, without impacting on the accumulation of other nutrients. In addition, we also report that Se enriched garlic tissue extracts, when applied to a liver carcinoma HepG2 cell model show limited differences in cytotoxicity as compared to control. However, variation in cytotoxicity of garlic tissues between control and enriched tissues were seen in the cultivars Mersley and Solent at the IC_{50} value. Interestingly assessment of Se species present within these tissues did not explain the difference in cytotoxicity. This is an important finding as it

not only highlights the importance of assessing biofortified crops for toxicity but also how different cultivars of the same species can respond differently to Se enrichment which subsequently impacts on cytotoxicity.

Due to delays in receiving the standards and digestion enzyme lipase used for speciation experiments, we began investigating the impact of Se species known to occur within edible crops on cell cytotoxicity to explain the differences in cytotoxicity seen in chapter 4. In Chapter 5, we explored the differences in cytotoxicity between organic and inorganic Se forms found in crops by developing and validating a hepatoma HepG2 cell model. We hypothesised that inorganic species of Se would be more cytotoxic than organic species. We observed that $SeCys_2$ and selenite were the most toxic forms of Se to HepG2 cells whereas selenate, SeMeSeCys and SeMet had no significant impact on cell viability at the concentrations tested, this corresponding with other researchers (Chen and Wong, 2009, Shen et al., 1999). Therefore, we can partially accept our hypothesis that cytotoxicity differs between Se species although, it is not as clear cut as to whether inorganic species are more toxic than organic species. From our investigations, we also noted that the cytotoxicity of inorganic selenite could be significantly increased via the addition of the cellular thiol, glutathione (GSH). This chemical interaction is suggested to promote reactive oxygen species generation via the formation of the superoxide anion (Xiang et al., 2009). However, in our hands despite ROS production and lipid peroxidation occurring in cells, incubation of selenite and glutathione with a range of antioxidants failed to prevent cytotoxicity. Given the results of the current work, we propose that additional mechanisms could be important. Hydrogen selenide is another short-lived reaction intermediated produced during the reaction between selenite and GSH.

H_2Se is a toxic and highly soluble gas (Cupp-Sutton and Ashby, 2016), first reported in 1931 but there is limited information regarding its biological activity or toxicity. Importantly, this molecule is a convergent metabolic intermediate of Se metabolism for both organic and inorganic forms in mammals. Some researchers are proposing that H_2Se could be the fourth gaseous signalling molecule in mammalian cells and tissues alongside NO, CO and H_2S (Kuganesan et al., 2019).

Since there are currently no safe approaches to producing this molecule aside from using the gaseous form, we synthesised a slow release H_2Se donor molecule (SeGYY) as developed by Newton 2019. This slow-release molecule is an analogue of the prototypic H_2S releasing therapeutic GYY4137 (Rose et al., 2015). Using our existing HepG2 model we assessed the apoptotic effects and mechanism of action for SeGYY. We report that H_2Se releasing SeGYY is capable of concentration dependent cell death, LDH leakage, ROS generation, caspase activation and cleavage of PARP in mammalian cells similar to GYY4137 (Lee et al., 2011, Lazarević et al., 2018, Lu et al., 2014). Interestingly we found that at low concentrations, SeGYY is cytoprotective and prevented cell death induced by NO oxidants. We also noted that this compound had a slight, but non-significant trend in inducing the expression of CSE, a key enzyme involved in generation of H_2S (Lee et al., 2011), suggesting potential cross talk between these two systems although more work is needed to confirm this. Our data provides some of the first pilot data to show that SeGYY could be a valuable tool in Se research, since it offers the opportunity to manipulate the levels of an important metabolic intermediate in Se metabolism. It is compelling to predict that H_2Se is biologically active in mammalian cells and further research is needed to confirm this. Unfortunately, the measurement of H_2Se is very challenging and

currently there are no commercial methods to do this *in vitro*. We did attempt to assess H_2Se production using lead-acetate paper which is commonly used for the detection of H_2S, however, despite chemical similarities we were not able to detect H_2Se production directly. In the future, efforts should be focused on the development of probes similar to NIR-H_2Se (Kong et al., 2016) that has the capacity to react with H_2Se *in vivo* and *in vitro*. Sadly this probe is not currently commercially available from any source.

Overall, our overarching hypothesis *'Se enrichment will increase the bioactive properties of Allium sativum (garlic) and tissue extract by virtue of the accumulation of known Se compounds in plant tissues*.*'* Has been tested using several approaches and can be partially accepted for the cultivars Mersley and Solent. The cytotoxicity data (Chapter 5) demonstrates the differing cytotoxicity of Se species, however speciation, as reported for Mersley and Solent tissues, suggests that Se species and level of Se accumulated do not fully explain the cytotoxicity or bioactivity of plant extracts. Additional optimisation of extraction methods and further assessment of the bioactivity of Se enriched garlic compounds merits further work. It could be that incorporated Se is not liberated from protein forms or is poorly bioaccessible from plant tissue extracts. It is also likely that Se enrichment induces the accumulation of other molecules in plant tissues that will also contribute to cytotoxicity, and this also deserves further research.

7.1 Main conclusions from thesis:

This body of work illustrates the need for assessment of Se enriched crops if not solely to assess toxicity then also to select the best cultivar to enrich which will

accumulate the most important forms of Se species for human health. Further to this, more work is needed to understand the toxicity of different Se compounds and the role of H_2Se within this.

In summary the current thesis has added to the previous body of work by finding:

- Foliar application of 25µM and 50µM Se had no impact on any commercial growth parameters measured in five garlic cultivars. However, in our hands, we report that only very small amounts of Se accumulated in garlic tissues using this approach and increasing Se concentration diminished radical scavenging activity in both leaf and clove tissues. Additional work is needed to confirm this observation.
- Hydroponic methods of enrichment resulted in significant increases in Se accumulation and the Se species SeMet, $SeCys_2$, Selenate and SeMeSeCys within garlic tissues. Interestingly, Se accumulation and speciation varied between cultivars and different plant tissues i.e. clove, shoot and root tissues. Se application by this method also had no effect on the accumulation of other nutrients in clove tissues and only a minor impact on the accumulation of other nutrients within the shoot and root tissues of specific cultivars such as the accumulation of S, K and Ca in shoot tissues of Marco and Solent cultivars, and Na in root tissues of Mersley. Application of both control and Se enriched garlic extracts to cells resulted in a concentration dependent decrease in cell viability in the order of clove, root and shoot tissues. Se accumulation did not significantly affect the cell viability as compared to control for the majority of cultivars. Further to this, differences

in the Se species accumulated do not fully explain differences in cytotoxicity observed in Se enriched cultivars Mersley and Solent.

- The analysis of purified Se compounds, Selenite, Selenate, $SeCys_2$, SeMet and SeMeSeCys identified in the plant tissues experiments indicated differing cytotoxicity, with $SeCys_2$ and selenite being the most toxic. This toxicity being enhanced for selenite in the presence of the cellular thiol, GSH. Despite increased production of ROS and lipid peroxidation in the selenite + GSH reaction, antioxidants failed to prevent the loss in cell viability. This finding suggesting that another 'ROS' independent or complementary mechanism could be important in the toxicity of this compound. We and others postulate that hydrogen selenide, another short-lived reaction intermediate of selenite + GSH reaction may be partly responsible for mediating cell death.
- To assess the cytotoxic effects of H_2Se we synthesized a known slow release H_2Se donor (SeGYY). In our hands, SeGYY caused a concentration dependent decrease in cell viability but at much higher concentrations as compared to $SeCys_2$ and selenite. This pointing to the fact that this compound is less toxic than other Se molecules. However, at supraphysiological concentrations it could induce apoptosis via DNA condensation, ROS generation, lipid peroxidation, PARP cleavage (potentially independent of caspase). In contrast, at lower concentrations *viz* 15µM and 30µM this molecule has cytoprotective effects against oxidants and may also have other impacts on cell signalling cascades.

7.2 Potential Future Work:

Future work that should be considered to improve knowledge in this area:

- Further investigation is needed into the effect of Se enrichment via both foliar and hydroponic methods on the antioxidant capacity of Se enriched tissues. This is needed to determine whether Se enrichment reduce the nutritional quality of the crop for consumers, and also the impact this could have on the ability of plants to tolerate climatic changes or pest or pathogen attack. This could combine additional antioxidant assays to assess phenolic and allicin content, as well as assessing of molecular targets in plants linked to antioxidant production of ascorbic acid, such as GDP-mannose pyrophosphorylase, a rate limiting enzyme in the L-galactose pathway of ascorbic acid synthesis.
- To assess the other metabolites present in Se enriched garlic in order to build a more complete understanding of the impacts of Se enrichment on the nutritional profile of garlic tissues and how this may relate to differences in cytotoxicity.
- Continued exploration into the bioaccessibility and bioavailability of Se enriched plant tissues through in vitro digestions and further application of these digested extracts onto cells to assess impact on Se status. This could lead to a human intervention study to show that Se in biofortified garlic is absorbed and incorporated into humans with/without side effects or used in trials to assess anti-inflammatory and anti-cancer properties.

- Assessment of selenite + GSH on cytochrome C release and interaction with cellular signalling targets such as Nrf2 signalling and Nf-κB. As well as a full time and concentration dependent evaluation of caspase and PARP involvement. This would provide a more complete picture of the mechanism involved in selenite + GSH mediated cell death.
- Further development of H_2Se releasing molecules and methods to detect this molecule in biological systems would be useful. To help elucidate the production and localisation of H_2Se, whether endogenously or induced by Se compounds such as the reaction of selenite + GSH, and exogenous SeGYY application to mammalian cells. These developments would aid the confirmation of the suggested role of H_2Se as a gaseous mediator in mammalian or other species by meeting the defined list of requirements set out in Kuganesan et al. (2019). Such developments would also provide new tools to explore Se metabolism to greater depth in living organisms.

7.3 Final conclusion

Garlic is most successfully enriched via hydroponic methods. The application of both control and Se enriched tissues to HepG2 cells showed that Se enrichment increases the bioactive properties especially in the cultivars Mersley and Solent. Although we have evaluated that Se species present in Se enriched garlic have different cytotoxicity when applied to HepG2 cells, Se speciation reveals that the accumulation of these Se species may not be solely responsible for the differences observed in cytotoxicity between control and Se enriched tissues. Through our work in characterising the cytotoxicity of different Se species, we have identified that H_2Se

may be responsible for the decrease in cell survival seen in the presence of selenite and GSH. Our work shows for the first time the anticancer and cytoprotective effects of slow release H_2Se donor SeGYY that might begin to elucidate the role of H_2Se as a gaseous mediator. Furthermore, the work in this thesis suggests that more research is needed in assessing bioactivity and antioxidant capacity of Se enriched plant tissues as well as the role of H_2Se within apoptosis.

8 Supplementary

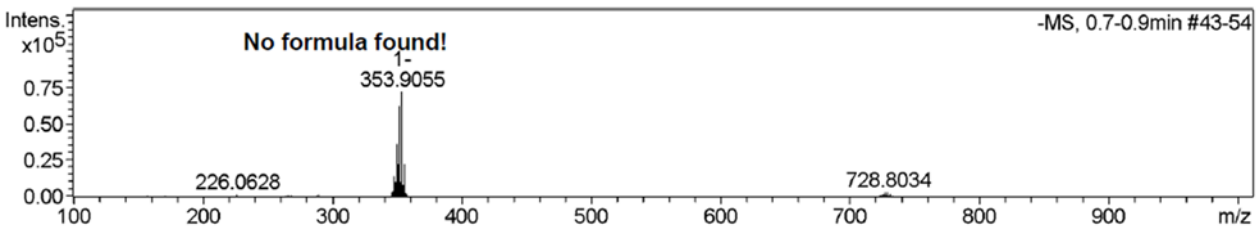

Figure 8.1 Mass Spectrometry of SeGYY (TDN1042). Molecular weight was found to be 353.9.

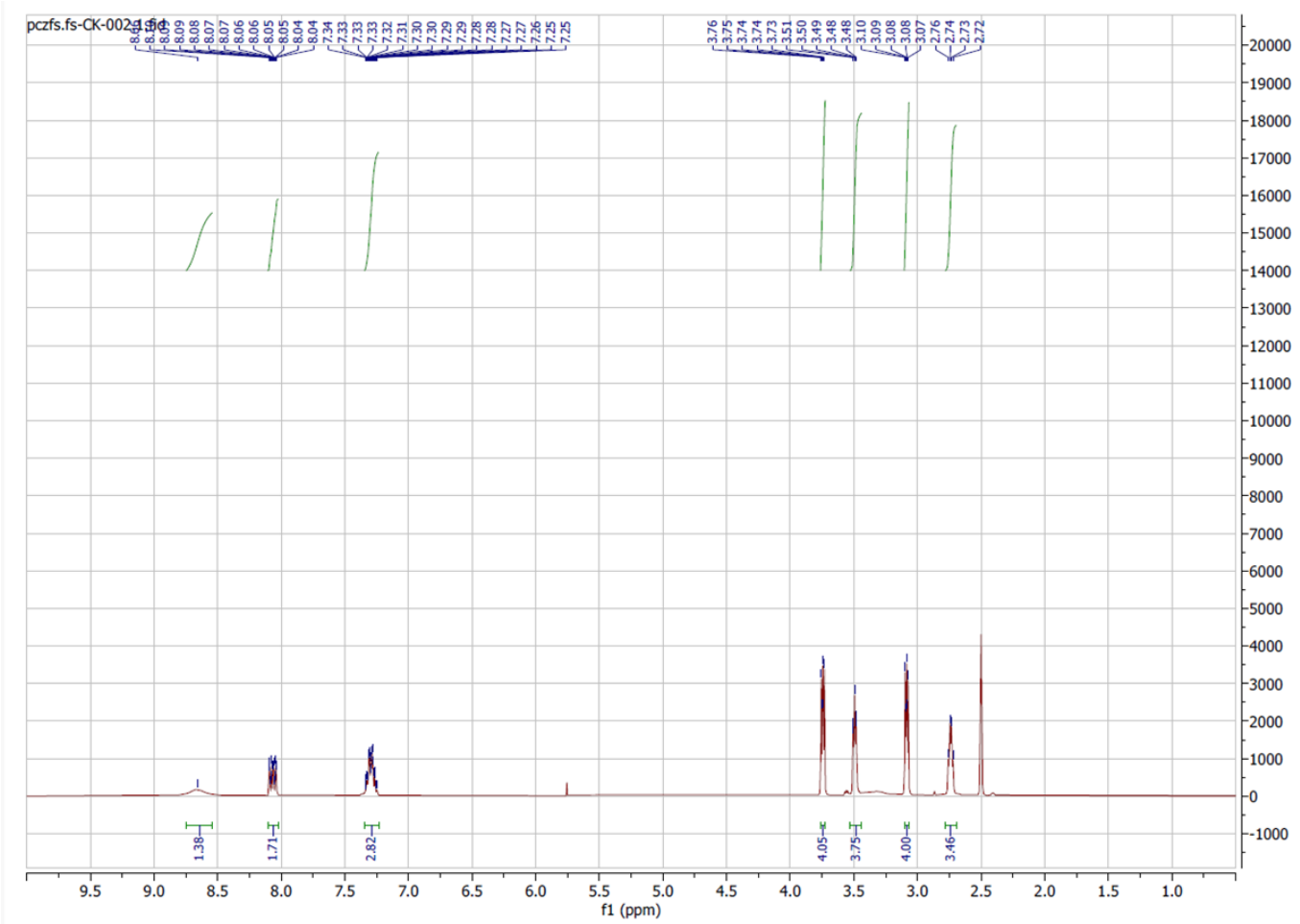

Figure 8.2- ^{1}H NMR of SeGYY compound (TDN1042).

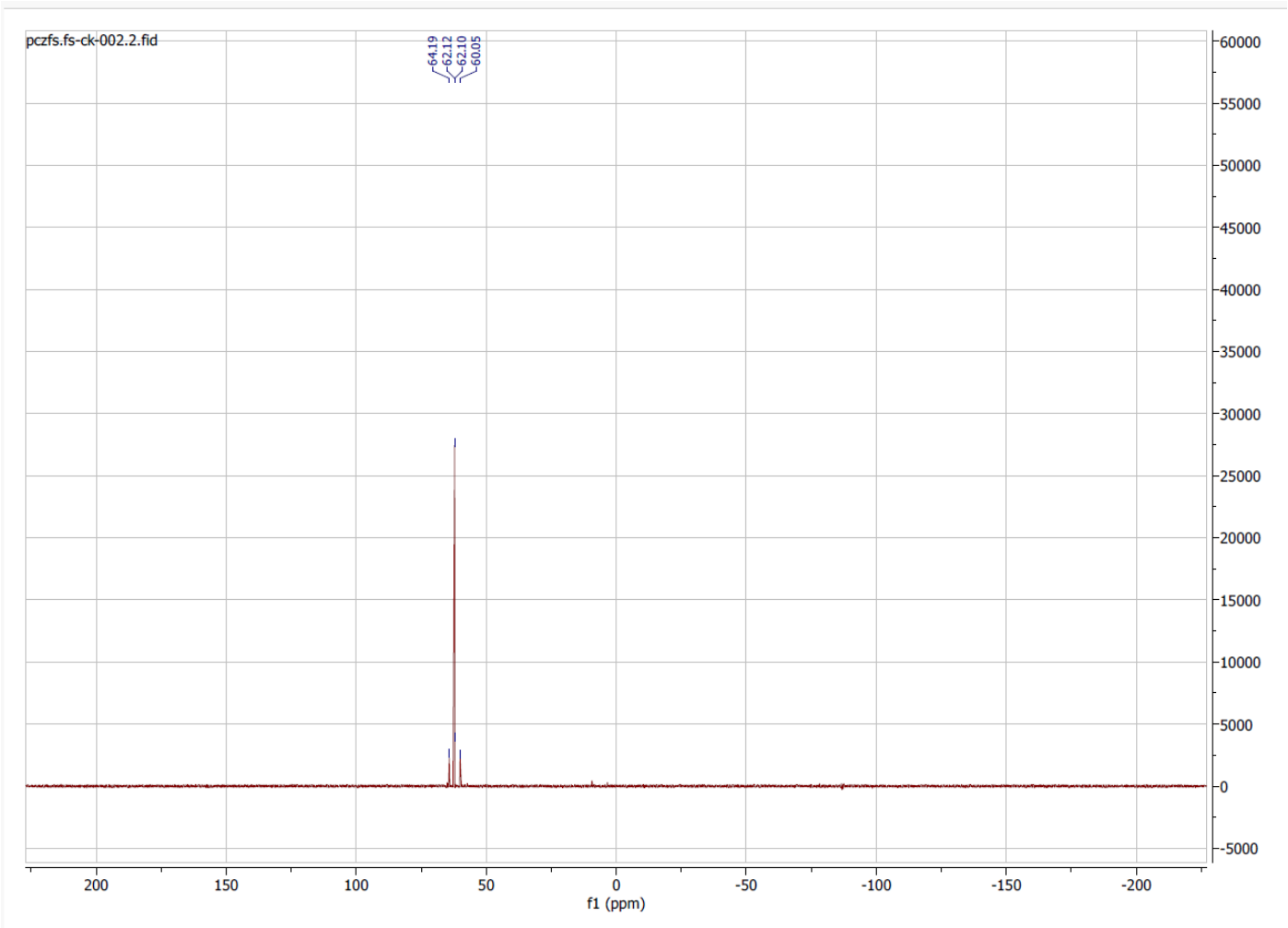

Figure 8.3-^{31}P NMR spectrum of SeGYY compound (TDN1042).

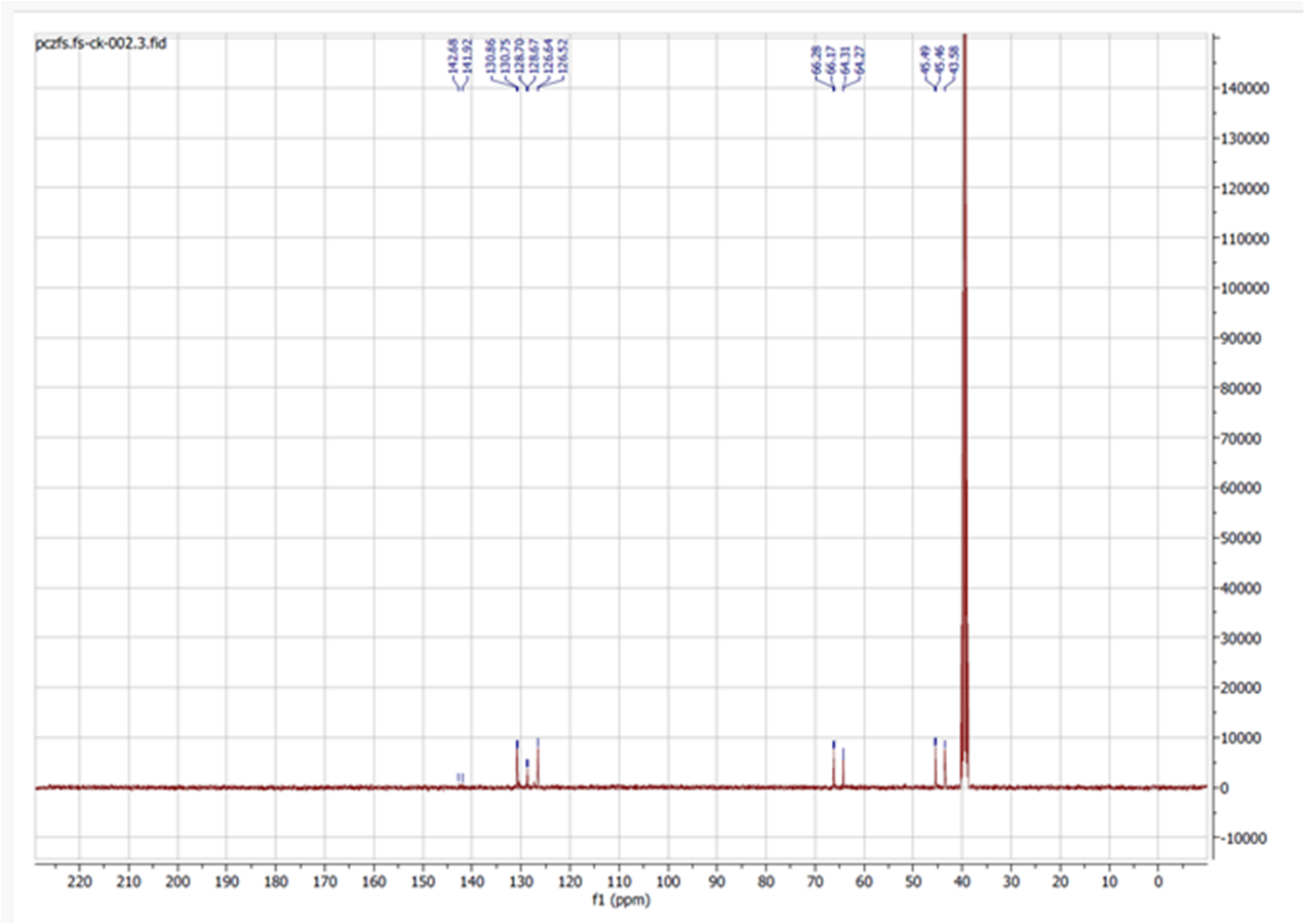

Figure 8.4- ^{13}C NMR spectrum of SeGYY compound (TDN1042).

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