THESIS

PHYTOREMEDIATION AND BIOFORTIFICATION POTENTIAL OF *CANNABIS SATIVA L.*

Submitted by

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ABSTRACT

PHYTOREMEDIATION AND BIOFORTIFICATION POTENTIAL OF *CANNABIS SATIVA L.*

Selenium (Se) is a micronutrient, but toxic at high levels. Both Se deficiency and toxicity are problems worldwide. I studied the potential of hemp (*Cannabis sativa* L.) for Se environmental cleanup (phytoremediation) and for accumulating elevated levels of this healthy micronutrient (Se biofortification). Hemp properties attractive for phytoremediation are fast growth, high biomass, hardiness and economic value. Furthermore, hemp produces highly nutritious seeds, of interest for Se biofortification.

The first Chapter of this thesis reviews *Cannabis sativa's* history, biological attributes and applications, as well as the technologies of phytoremediation and biofortification, and plant Se metabolism. The second Chapter presents experimental data on two hemp studies. The first was a field survey of Se accumulation in hemp grown across Colorado, and in commercial hemp products. The second study involved controlled greenhouse experiments to study hemp Se tolerance, accumulation and metabolism.

Hemp field surveys in four naturally seleniferous (Se-rich) agricultural areas in Colorado, U.S.A. found 15-25 μg Se/g in seed (intact or dehulled) and 5-10 μg Se/g dry weight in flowers and leaves. Hemp beer contained 42 μg Se/L. Considering the U. S. recommended daily allowance (RDA) of 55-75 μg Se, one bottle of hemp beer provides 25%, and 4 gram hemp seed (a half tablespoon) provides 100% of the RDA. In controlled greenhouse experiments, hemp was further characterized for Se tolerance, accumulation and Se speciation. Effects of Se on photosynthesis and cannabinoid and terpenoid levels were also analyzed. At the seedling level, hemp showed high selenate tolerance (up to 160 µM) and accumulation (up to 1,400 mg Se/kg shoot dry weight). Mature hemp was completely tolerant up to 40 µM selenate and accumulated up to 200 mg Se/kg DW in leaves, flowers and seeds. Seeds were found to contain free (water-extractable) selenomethionine and methyl-selenocysteine, superior forms for Se biofortification, reported to have anticarcinogenic properties for consumers. Hemp production of medicinal cannabidiol (CBD) and terpenoids was not affected by Se. Selenium enhanced potassium levels in seeds, and thus their nutritional value; other nutrient levels were unaffected.

It can be concluded from these studies that hemp shows promise for Se phytoremediation and can produce Se-biofortified dietary products; Se does not affect levels of valuable secondary plant compounds, nor does it negatively affect nutritional quality of seeds. These findings are of significance in view of the widespread and rapidly expanding cultivation of hemp in seleniferous areas across the U.S.A. and Canada.

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CHAPTER 1: INTRODUCTION TO CANNABIS, SELENIUM, PHYTOREMEDIATION, AND BIOFORTIFICATION

1.1 Cannabis history and roles in civilizations

The herbaceous annual plant *Cannabis sativa* L., common names hemp or marijuana, has a diverse and long history with human civilization throughout the world. Most likely hemp was first recognized for its versatility in producing a plethora of textile-based products including rope, clothing, paper, ship sails, etc. Its uses by human civilizations can be dated back to many ancient societies in the Middle East and Asian kingdoms. Early records of *C. sativa* use date back from 13,000 - 2,000 B.P., e.g. in excavation sites in the Yellow River basin in China, and on the island of Taiwan in areas associated with the Tapenkeng culture (Booth 2005). *Cannabis* uses is this area was usually associated with cordage in pottery art, rope, and clothing (Booth 2005; Hancock 2012). There is evidence that *C. sativa* was dispersed by nomadic tribes across Asia to western areas of Neolithic sites in Europe (Booth 2005). In ancient civilizations, *C. sativa* was likely harvested from wild-growing sources, but later it was domesticated; *C. sativa* may have been an agricultural crop for the past 8500 years (Fleming & Clarke 1998). Hemp was cultivated as a grain and fiber source in Europe during the Roman and Moorish empires; the first European hemp paper factory was in 1150 Jativa, near Valencia in Spain (Booth 2005; Dark 2000). *Cannabis* subsequently enjoyed worldwide geographic expansion together with human migrations. Hemp has also played prominent roles in the history of the Americas, e.g. the sails and ropes of the ships that sailed the Atlantic in 1492 were made of hemp, and early settlers widely grew hemp for their fiber needs (Booth 2005). The uses of *Cannabis* have diversified, from the textile products mentioned previously to nutritional and medicinal products.

Cannabis sativa has long been recognized for its medicinal properties in ancient Chinese medicine, where it is thought to have been harvested from the wild since ~ 8,500 years ago (Schultes & Hofmann 1980). One of the earliest definitive evidence for pharmaceutical or shamanistic use of *C. sativa* was found in Central Asia's archeological cave sites (dating to ca. 1300): artifacts containing large amounts of stored *C. sativa* that contained cannabitriol (CBO), a degradation product from the known psychoactive compound Δ^9-tetrahydrocannabinol, THC (Russo et al., 2008). *Cannabis sativa* has even been associated with ancient religious scripts (possibly as a religious sacrament or in shamanistic practices) from the Old Testament, in the word "Kaneh loosm" found in Exodus 30, ver. 22-33, which translates to aromatic hemp/reed. In summary, it is clear that *C. sativa* played roles in many ancient societies in a variety of applications. This versatility allowed *C. sativa* to be carried through generations and civilizations up to the present day.

There has been much controversy about *C. sativa* over the past 80 years, after THC was listed as a schedule 1 drug in the U.S.A. (i.e. as a high-risk drug with no medicinal value) and was/is also banned in many other countries (https://www.dea.gov/drug-scheduling). In recent years, however, *Cannabis* is starting to be remembered and recognized for its many applications. Its popularity today is still associated with its textile production, but its rise in popularity is particularly associated with its biochemical properties, as discussed later in the chapter. *Cannabis* has withstood the test of time much like most commonly grown agricultural crops. It's irrefutable that this plant has had and still has a rich cultural connection with humans around the world. The next section will briefly review *Cannabis'* diversity.

1.2 Types of *Cannabis* and their Distribution

Cannabis sativa L. is part of the Cannabaceae family, which also includes hops and hackberries. *Cannabis sativa* is an annual, dioecious species (producing male and female plants) that flowers in response to a shortening photoperiod (it is a short day plant). *Cannabis sativa* has a widespread distribution across the globe, owing to its agricultural uses for fiber products, nutritional products, and medicinal/pharmaceutical compounds, as described above. Agricultural selection has led to the development of two types of *C. sativa,* one commonly referred to as hemp which is grown primarily for fiber, seed and more recently for cannabidiol (CBD), and the other commonly referred to as marijuana, grown primarily for its psychoactive compound Δ^9-tetrahydrocannabinol (THC) (Gaoni & Mechoulam 1964). Morphologically, these two types of *C. sativa* are hard to distinguish; for this, phytochemical analysis is needed to quantify cannabinoid content. Hemp is high in CBD but very low in THC (less than 0.3% of dry weight), while marijuana has high levels of THC but very low levels of CBD (Small and Cronquist 1976; Siniscalco 2001; Taura et al., 2007).

Cannabis' long history in human cultivation makes it hard to definitively pinpoint its place of origin (Soorni et al., 2017). Two centers of diversity have been reported, in Hindustan (India) and Siberia (Chandra et al., 2017). It is thought that *Cannabis'* expansion was linked to the migration of the Aryan and Scythian tribes from Central Asia via ancient Persia to Europe (Chandra et al., 2017). *Cannabis sativa*'s spread across the old world is illustrated in Fig 1.1 (Chandra et al., 2017), showing a North vs. South separation for non-intoxicating (hemp) and intoxicating (marijuana) varieties.

Apart from the cannabinoid-based biochemical/legal distinction between hemp and marijuana, there are three subspecies distinguished within *Cannabis sativa*: *C. sativa* var. *sativa, C. sativa* var. *indica* and *Cannabis sativa* var. *ruderalis*. This can further complicate the unraveling of the history and divergence of *Cannabis.* These subspecies can be distinguished by their morphology

(Fig. 1.2).

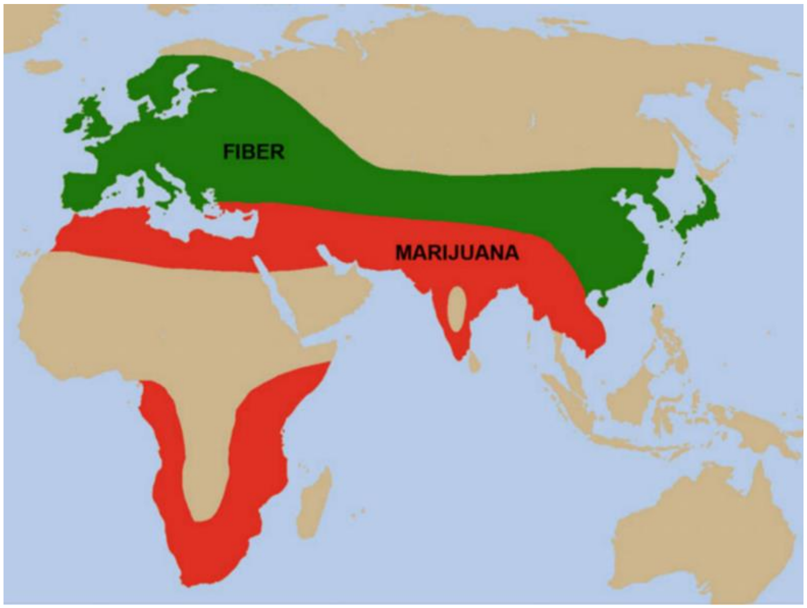

Figure 1.1 Illustration showing the divergence of *Cannabis*, distinguishing psychoactive varieties or marijuana (red) and high-fiber varieties (hemp, green). From: ElSohly et al., 2017.

C.sativa is tall, with a fibrous stalk and narrow leaflets, while *C.indica* is shorter, has a woodier stalk and broader leaflets; *C. ruderalis* is even shorter, has narrow leaflets and will go through early flowering without dependence on short day induction: “autoflowering”. The subspecies

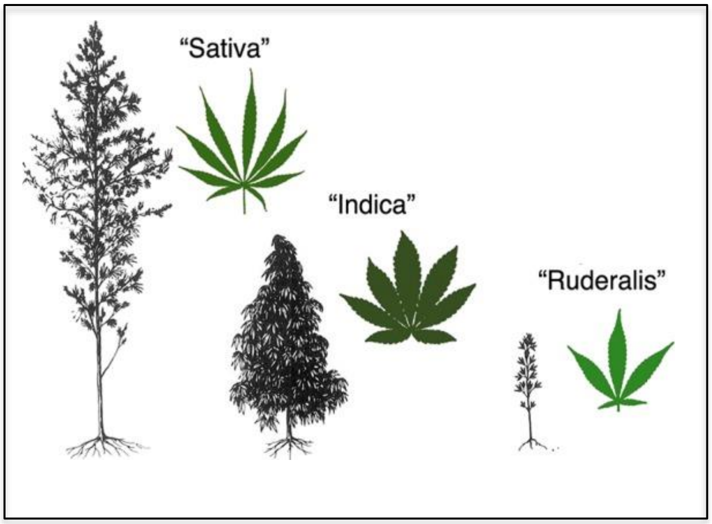

Figure 1.2. Morphological differences between the three subspecies of *Cannabis sativa* L., *sativa, indica, ruderalis*. From: McPartland, 2018.

readily hybridize, resulting in a range of morphological and phytochemical hybrids (McPartland 2018).

1.3 Medicinal properties of *Cannabis*

The medicinal properties of *C. sativa* are highly diverse and are gaining attention in areas of medical research. *Cannabis sativa* compounds have been shown to alleviate and treat a variety of diseases and afflictions, from relieving intraocular pressure from glaucoma to suppressing seizures in epileptic patients. The main class of compounds at the basis of its medical applications are the cannabinoids. Over 90 cannabinoids have been identified in *C. sativa*, the best characterized of which are THC and CBD. Cannabinoids are a diverse class of phenolic terpenoid compounds that serve the plant via protection against UV light at high altitude and as an herbivore deterrent (McPartland 2018). Interestingly, cannabinoids have the ability to interact with mammalian cannabinoid receptors, specifically the G coupling proteins CB1 and CB2 receptors in humans. The presence of CB1 and CB2 allows for the medicinal and psychoactive effects to take place in the patient/user. The cannabinoid receptors CB1 and CB2 have a diverse role in interacting with mammalian endogenous cannabinoid ligands, specifically *N*-arachidonoylethanolamine (anandamide/AEA) (Devane et al., 1992; De Petrocellis & Di Marzo 2009; Serrano 2011; Battista et al., 2012). AEA is a crucial endogenous cannabinoid that has functions in the peripheral and central nervous system. AEA functions in important steps of early stage embryo development, feeding regulation, neural generation, and has been shown to cause inhibition of breast cancer cell proliferation (De Petrocellis et al., 1998; Fride 2008; Sticht et al., 2018). Below is a brief review of the medicinal aspects of THC and CBD, relevant because of the level of interest and amount of research conducted on these specific cannabinoids.

Δ^9-tetrahydrocannabinol (THC), the main compound associated with psychoactive marijuana varieties of *Cannabis*, also has important medical effects: it is prescribed for alleviating a variety of medical conditions such as anorexia, glaucoma, inflammation, movement disorders, nausea caused by chemotherapy, pain, spasticity, and a variety of mental illnesses (Grotenhermen & Russo 2002). Synthetic pharmaceutical variants of THC such as Dronabinol have been produced and can also be prescribed to alleviate these conditions. A notable study by Clifford (1983) found THC to significantly reduce spasticity and ataxia associated with multiple sclerosis (MS). Ninety minutes after oral administration of 5 mg THC, the patients' ability to write and draw was significantly improved. While THC cannot cure MS, it can greatly increase the overall quality of life, allowing for basic tasks to be performed more easily. THC can also improve the quality of life of patients living with chronic pain, offering an alternative for the highly addictive opiates that are frequently prescribed for mild to extreme pain. This is of significance considering the many crippling conditions that arise from opiate dependency; furthermore, THC does not cause respiratory depression, which is the cause of a lot of opiate-related deaths (Grotenhermen & Russo 2002). Another condition THC has been shown to alleviate is the reduction of intraocular pressure in the eye from glaucoma; in some instances it has been shown to decrease pressure up to 50% in individuals, for up to 4-6 hours (Grotenhermen & Russo 2002). These are just some examples of the efficacy of THC as a medicine; for a more extensive review on therapeutic effects of THC, see the book "*Cannabis and Cannabinoids: Pharmacology, Toxicology, and Therapeutic Potential"* by Franjo Grotenhermen and Ethan Russo.

Cannabidiol (CBD) is another cannabinoid that is of high interest for its medicinal therapeutic potential. Cannabidiol is non-psychoactive cannabinoid that has a variety of medicinal properties such anti-epileptic, sedative, neuroprotective, anti-dystonic, anti-inflammatory, anti-anxiety, anti-psychotic properties (Grotenhermen & Russo 2002). As of 2018, CBD has been approved for human clinical trials in the treatment of epilepsy (https://pharmacy.olemiss.edu/marijuana/history/). CBD was shown to reduce overall seizure incidents by 50% or more in a childhood epilepsy study (Rosenberg et al., 2017). Cannibidiol is thought to decrease epilepsy based on its ability to restore hippocampal interneuron functions, acting as a neuro-protectant, and has also been shown to increase adult neurogenesis in mice (Crippa et al., 2018; Khan et al., 2018). The fact that CBD is non-psychoactive makes it a more suitable compound for studies or patients/individuals where psychoactive effects are not desirable.

In conclusion, medicinal application of cannabinoids from *Cannabis* can alleviate debilitating symptoms, particularly from neurological ailments. It does not cure these diseases but can improve the overall quality of life. While the results so far are promising, there is more to be discovered. Research on the medicinal value of cannabinoids has been hampered in the past 80 years by the categorization of *Cannabis* products as a schedule 1 drug. Hopefully in the years to come, with new legislation in the U.S.A. and other countries, further research can be conducted to better understand the workings and applications of *Cannabis* products, including human-based clinical trials.

1.4 Other, emergent uses of Cannabis

In modern times, new uses for *Cannabis* fibers are being developed, apart from the classic uses of *Cannabis* fiber for a variety of textiles such as ropes, hawsers, boat canvas, paper and clothing (Bouloc et al., 2013). For instance, hemp fibers may serve as a plastic reinforcement, reducing the overall need for plastic polymers and increasing structural durability. *Cannabis* can also supply cheap, carbon negative, eco-friendly building materials, from hemp wool for insulation to “hempcrete” for structural building material. Specifically, the concrete known as hempcrete is receiving attention as a light-weight, malleable, noise absorbing, low thermal conductivity alternative to conventional concrete. Furthermore, *C. sativa* of the hemp type is currently being grown in North America (U.S.A, Canada) for its edible seed products and is a common product in health sections of grocery stores, in the form of dehulled seeds (“hemp hearts”), protein powders, and pressed oils. Compared to other plant-based seed oils, hemp seed oil has a particularly rich and balanced composition of essential fatty acids (EFA). The hemp oil pressed from the seeds contain the EFA linoleic acid (~20%, LA, 18:3, omega-6), alpha-linoleic acid (~50%, ALA, 18:2, omega-3), stearidonic acid (~5%, SDA, 18:4, omega-3), and gamma-linoleic acid (~12.5%, GLA, 18:3, omega-6); the remainder is made up of saturated fatty acids. Essential fatty acids also have reported health benefits for consumers, i.e. by controlling inflammation, pain, neural functions, blood pressure, and blood coagulation. Like other medicinal aspects of *C. sativa*, more research is warranted to fully understand the potential of this species’ lipids (Bouloc et al., 2013).

1.5 Biofortification

There is an epidemic effecting humanity worldwide, termed the “Hidden Hunger”, which is referring to the billions of people across the globe that are deficient in essential micronutrients. These micronutrient deficiencies are most prevalent in developing areas of the world in populations where human diets are less diverse and plant crops grown have low content of bioavailable essential elements such as Iodine (I), Iron (Fe), Zinc (Zn), Calcium (Ca), and Selenium (Se) (Singh et al., 2016). These essential micronutrients have roles that range from being components of thyroid hormones, essential electron donors and acceptors, essential functional components for thousands of proteins, involvement in skeletal structure, and cofactors for antioxidant enzymes (Lyons et al., 2004). Among the interventions that have been proposed to help alleviate this worldwide epidemic of micronutrient malnutrition, one is a long-term strategy termed biofortification (Zhu et al., 2007).

Biofortification is a (phyto)technology that is based around the development of plant food crops that have enhanced abilities to accumulate bioavailable content of essential micronutrients in edible parts (often seeds/grains). Biofortified crops could lower the incidence of micronutrient malnutrition, and also could maintain nutritional status long-term (Singh et al., 2016). When comparing this potential strategy to other conventional methods for addressing micronutrient deficiencies (vitamin/mineral supplementation), biofortification is the cheapest, most accessible to remote and rural areas, and the only cost-efficient long-term approach to address this hidden hunger (Singh et al., 2016). Development of these enhanced micronutrient crops can also be used to create enhanced feed products to produce fortified livestock (Reddy et al., 2003; Zerbini & Thomas 2003). Crop biofortification can address not only micronutrient malnutrition in consumers across the globe, but can also benefit crop productivity by increasing plant vigor and growth and enhancing resistance to biotic and abiotic stresses (Bouis 2003). Hemp is interesting from the perspective of biofortification, because of its edible seeds that are already well-known for their health benefits to consumers. Micronutrient biofortification of these seeds would further enhance their nutritional value.

1.6 Phytoremediation

Another emergent plant-based (phyto)technology for which hemp is starting to receive attention is phytoremediation. This cheap alternative to mechanical or chemical remediation of contaminated sites has been well-established in the past two decades. Below is a brief review of the different phytotechnologies used in phytoremediation, and arguments why hemp is an interesting species to explore for this purpose.

Since the industrial and agricultural revolutions, various industrial processes, as well as mining, military and farming operations have produced enormous waste streams. These, in combination with improper waste disposal, have led to large areas of polluted land and water with dangerous levels of organic and inorganic pollutants (Peuke et al., 2005). Pollution such as arsenic, cadmium, lead and mercury, as well as organic contaminants poses serious health issues to humans and animals within ecosystems, where contaminants can be biomagnified and accumulated throughout the food chain (Shayler et al., 2009; Miransari 2011; Bhargava et al., 2012; He et al., 2014; Cristaldi et al., 2017). It has been estimated that 3.5 million sites are contaminated in the European Union alone, among which 500,000 are highly contaminated and in desperate need of environmental remediation. In total about 16% of the EU, or 52 million hectares are contaminated

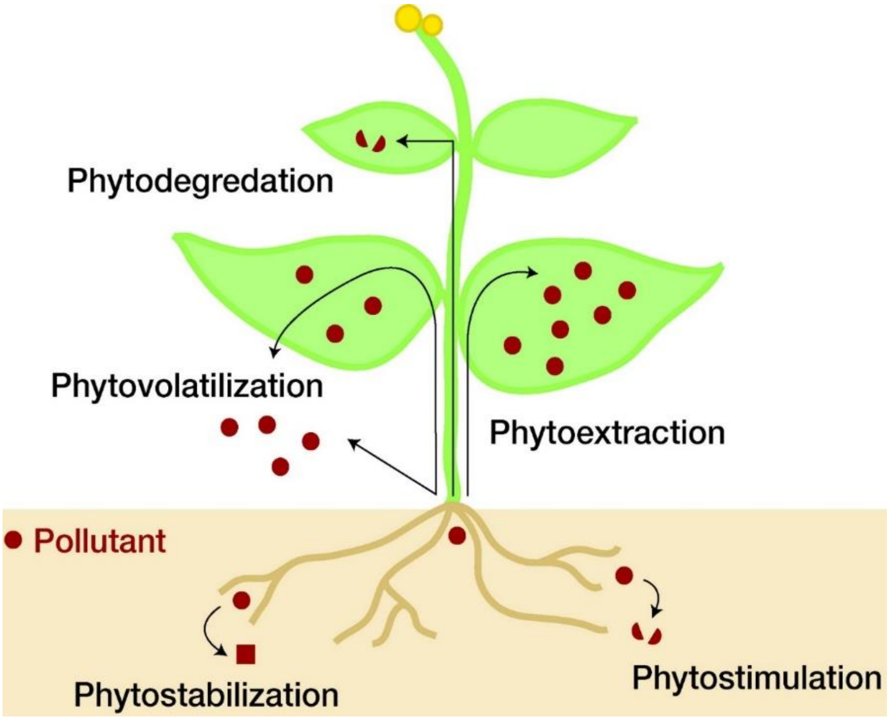

Figure 1.3 Possible fates of pollutants during phytoremediation: the pollutant (represented by red circles) can be stabilized or degraded in the rhizosphere, sequestered or degraded inside the plant tissue, or volatilized.

(Peuke et al., 2005; Mahar et al., 2016). The rapid expansion of contaminated area is expected to continue as the world population grows and there is an accelerated increase of development and urbanization. It is estimated that two-thirds of the world population will be living in large cities by 2050, forming large sources for soil, water, and air contamination (Pilon-Smits 2005). It is imperative that remediation technologies be put in place that are cost-effective and efficient for cleanup of these contaminated areas across the globe. A possible technology that has emerged in the past two decades and is being favored as a cheap effective alternative to mechanical or chemical remediation of contaminated sites, is plant-based remediation, or phytoremediation. Phytoremediation is an environmental cleanup process that involves using specially selected plants and their associated microbes to extract, degrade, volatilize, or stabilize inorganic and organic contaminants in soil, water, and air (Pilon-Smits 2005; Paz-Alberto 2013). The best practices for phytoremediation, and expected success is dependent upon several factors, including class of pollutant, its concentration and bioavailability, the physical/chemical and site climatic conditions, and the species selected (Cristaldi et al., 2017; Sreela & Jayanthi 2017). Among the various phytotechnologies, phytodegradation, phytostabilization and phytoextraction are the most used (for a review see 2005) (Fig. 1.3). Phytodegradation involves breakdown of contaminants inside the plant or in the root zone, and is suitable for organic, bioavailable contaminants including the solvent trichloroethylene (TCE), the pesticide atrazine and the explosive trinitrotoluene (TNT). Specific metabolic pathways and transporters can allow for plants to either completely mineralize these organic compounds or partially degrade or modify them, followed by conjugation and sequestration in plant tissues (Fig. 1.4). Degradation in the root zone (rhizosphere) may involve plant-exuded enzymes or degradation by plant-associated microbes (Peuke et al., 2005; Pilon-Smits 2005). Phytostabilization involves immobilization of contaminants without accumulation, and is suitable for contaminants that are not bioavailable or mobile. Phytoextraction involves accumulation of contaminants in harvestable plant parts, and is very suitable for bioavailable elements that can be accumulated in plant shoots. By cultivating selected plant species on contaminated sites and harvesting the aboveground biomass, the pollutants are removed from the site. It is also possible to recover metals from harvested plant material after ashing (phytomining),

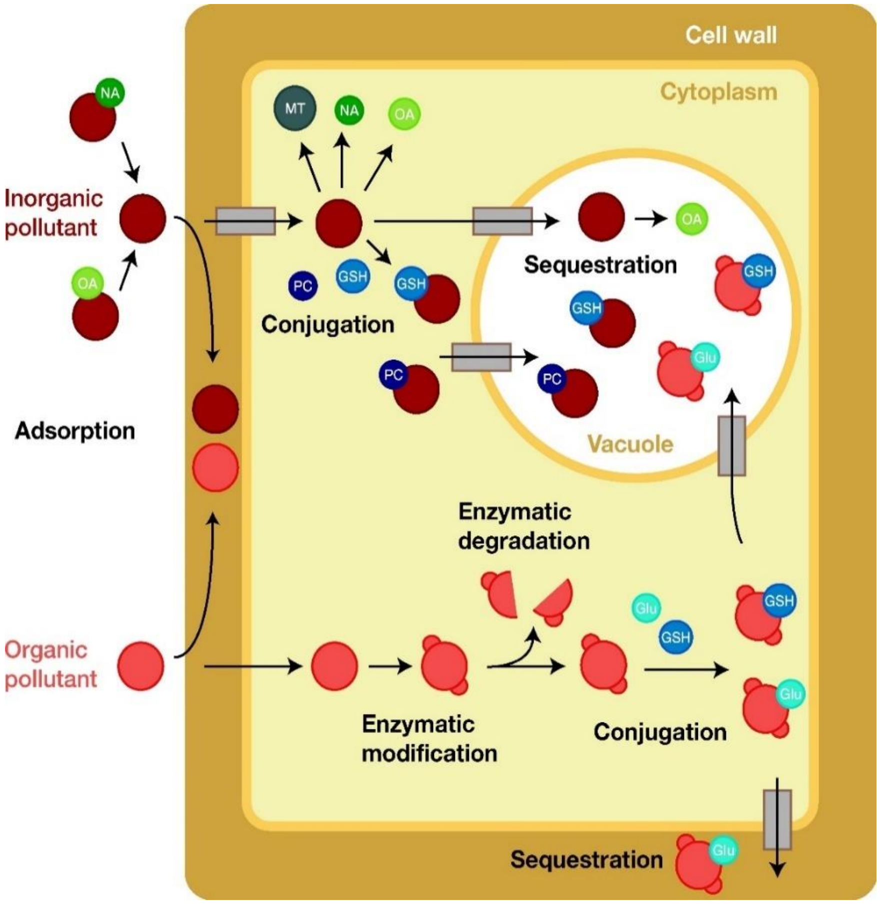

Figure 1.4 Tolerance mechanisms for inorganic and organic pollutants in plant cells. Detoxification generally involves conjugation followed by active sequestration in the vacuole and apoplast, where the pollutant can do the least harm. Chelators shown are GSH: glutathione, Glu: glucose, MT: metallothioneins, NA: nicotianamine, OA: organic acids, PC: phytochelatins. Active transporters are shown as boxes with arrows. From Pilon-Smits, 2005.

which can be economically feasible in some cases, such as for nickel (Cerdeira-Perez et al., 2019)

Phytoremediation has a variety of advantages compared to other methods of soil and water remediation. Overall, phytoremediation is a relatively inexpensive process. Phytoremediation also has the potential to yield plant products with economic value that can offset the phytoremediation costs. In the example of phytomining, it could be possible to remediate a contaminated site while mining valuable metals that pay for the environmental cleanup (Cerdeira-Perez et al., 2019). Potential limitations of phytoremediation are that it can be slower, particularly if phytoextraction is used, which sometimes requires decades to restore a site. Phytoremediation may also be limited by root depth and by the tolerance of the plant to the pollutant in question or the on-site conditions. Depending on the contaminant and phytotechnology used, the plant material may have to be harvested, may be toxic and need special disposal, adding to the cost. Despite its limitations, phytoremediation has established itself as one of the accepted remediation technologies in the realm of environmental remediation, either by itself or in combination with mechanical or chemical remediation methods, e.g. to tackle different areas of a particular site, or to use sequentially.

To optimize phytoremediation, research is important in order to understand processes by which plants absorb, translocate, accumulate, and degrade and detoxify pollutants, in order to identify and develop plant species or varieties that are particularly suitable for remediation of different pollutants. General characteristics of plants that are good for phytoremediation are fast growth, high biomass production both above and below ground, broad geographical applicability, hardiness under extreme conditions, and economic value. Additional, more pollutant-specific characteristics are good capacity to absorb, detoxify or degrade the pollutant(s) in question. *Cannabis sativa* has all the general characteristics of a good phytoremediator species. Whether it also has good pollutant-specific characteristics remains to be investigated, and is one of the topics of this research. The pollutant of interest, in this case, is selenium (Se). This element is interesting to focus on because it occurs naturally in a widespread area of North America where hemp is grown and is considered a pollutant in many areas where human activities concentrate the Se, but also because it is a micronutrient. Plants with elevated levels of Se, e.g. after their use for phytoremediation, thus are interesting from the perspective of biofortification and human/animal health. Below, the various interesting aspects of Se biology are further reviewed.

1.7 Selenium

Selenium is chemically similar to sulfur (S) and can be transported and metabolized via the same mechanisms (Schiavon & Pilon-Smits 2017a). Selenium is essential for mammals and many other animals, where it is incorporated into selenoproteins (25 in humans) in the form of the seleno-aminoacid selenocysteine (SeCys). Selenoproteins often have redox functions and are essential for a number of physiological processes such as thyroid function, immune system response, fertility, and antioxidant function. (Willson 1992; Struss et al., 2017). Important seleno-enzymes with antioxidative functions are glutathione peroxidase and thioredoxin reductase (Rayman 2012). These enzymes scavenge reactive oxygen species (ROS) such as hydrogen peroxide, superoxide and hydroxyl radicals. Removal of these ROS is essential for reducing cellular damage which, if allowed to go uncontrolled, can result in diseases such as cancer (Rayman 2012). Other selenoproteins are the thyroid hormones known as deiodinases, involved in controlling the activation and deactivation of thyroid hormones. Thus, Se deficiency can increase chances of hypo- and hyperthyroidism (Rayman 2012). Unfortunately, Se deficiency is impacting over 1 billion people worldwide in areas where soils are low in available Se and thus there is a lack of adequate dietary Se (Lyons 2003). Due to this Se deficiency, people are at a higher risk for cancers, infections, thyroid issues and fertility problems.

While Se is an important micronutrient, it can also become toxic at high concentrations. Selenium is considered a pollutant in many areas of the world that experience high concentrations of this element, because of naturally occurring Se in (seleniferous) soils and anthropogenic activities like the burning of high-Se containing fossil fuels (Schiavon & Pilon-Smits 2017b). Selenium is present in some industrial and agricultural wastewaters and mine tailings. Fortunately, plants readily accumulate bioavailable forms of Se and can thus be used to both clean up excess Se where it exists (phytoremediation), while producing Se-fortified edible products that may be useful for alleviating the Se deficiency in low-Se areas (Schiavon & Pilon-Smits 2017b).

In plants, Se is not an essential micronutrient, but because of its similarity to S, Se is non-specifically absorbed and assimilated into organic forms via S transporters and pathways (Terry et al., 2000). Specifically, plants take up Se most commonly in the bioavailable inorganic forms selenate (SeO_4^{2-}) or selenite (SeO_3^{2}), and can assimilate these into organic Se compounds, e.g. selenocysteine (SeCys) and selenomethionine (SeMet). Selenate is the most common form in oxic environments, such as most soils. Selenite is a less commonly occurring form and found in areas associated with anaerobic environments, e.g. wetlands. Selenate is absorbed by roots and translocated to the shoot via sulfate transporters (Lass and Ulrich-Eberius, 1984; Hawkesford et al., 1993). After uptake, selenate is assimilated via the sulfate assimilation pathway, which begins with ATP sulfurylase, the enzyme responsible for coupling selenate (and sulfate) to ATP, forming either adenosine 5′-phosphoselenate (APSe) or adenosine 5′ phosphosulfate (APS) (Leustek 1994; Pilon-Smits et al., 2009; et al., 2015) (Fig. 1.5). APSe can be converted to selenite by APS reductase (APR). Selenite can be further reduced to selenide (Se^{2-}), either by sulfite reductase (SiR) or via non-enzymatic conversion to GSSeSG, forming GSSeH, which is then converted to selenide via glutathione reductase (GR) (Hsieh & Ganther 1975) (Pilon-Smits 2012; White 2015). Selenide can be then be incorporated into SeCys, via the cysteine synthase enzyme complex (CS) (White 2015; Pilon-Smits 2012). Selenocysteine can further be converted to either methyl-SeCys (MeSeCys), SeMet, or volatile dimethyldiselenide (DMDse), or be broken down into elemental Se and alanine, preventing SeCys incorporation into proteins (Pilon-Smits et al., 2009). Investigation and manipulation of S transporters and S assimilation pathway enzymes has benefited phytoremediation and biofortification (Schiavon and Pilon-Smits, 2017b).

1.8 Scope of this thesis work

The goal of this Master thesis project was to explore the potential of hemp for phytoremediation and biofortification of Se. The reason hemp was explored for its phytoremediation potential was because it has many of the characteristics that make plants good candidates for phytoremediation such as fast growth, high biomass production, hardiness, deep penetrating roots, and a variety of valuable economic products. Selenium was chosen for this study because it can be a pollutant in both industrial and agricultural wastewater, and occurs naturally at high levels in some areas where hemp is cultivated, including Colorado, U.S.A. One of the commercial products from these areas is edible seed, which warranted investigation into the fate of Se in these hemp plants. Because Se is not only toxic, but also an important human/animal micronutrient, better knowledge of the capacity of hemp to absorb and metabolize Se is relevant for consumers, and potentially for biofortification applications. This is of significance considering that one billion people worldwide are estimated to be Se deficient. The technologies of phytoremediation and biofortication may even be combined: hemp that has accumulated bioavailable Se in its edible seeds after phytoremediation could be used as Se supplement for populations with Se deficiency.

As described in Chapter 2, the studies included two approaches: a field analysis of Se levels in industrial varieties of hemp grown on naturally seleniferous CO soils, and controlled greenhouse experiments. In the latter, varying concentrations of selenate were administered to hemp, both at the seedling and mature plant level. Biomass was determined as a measure of Se tolerance, and tissue levels of Se and other elements were measured in the different plant organs. Furthermore, the Se effects on photosynthesis and valuable phytocompounds (CBD, terpenoids) was investigated, and the chemical speciation of the Se in the seeds was analyzed.

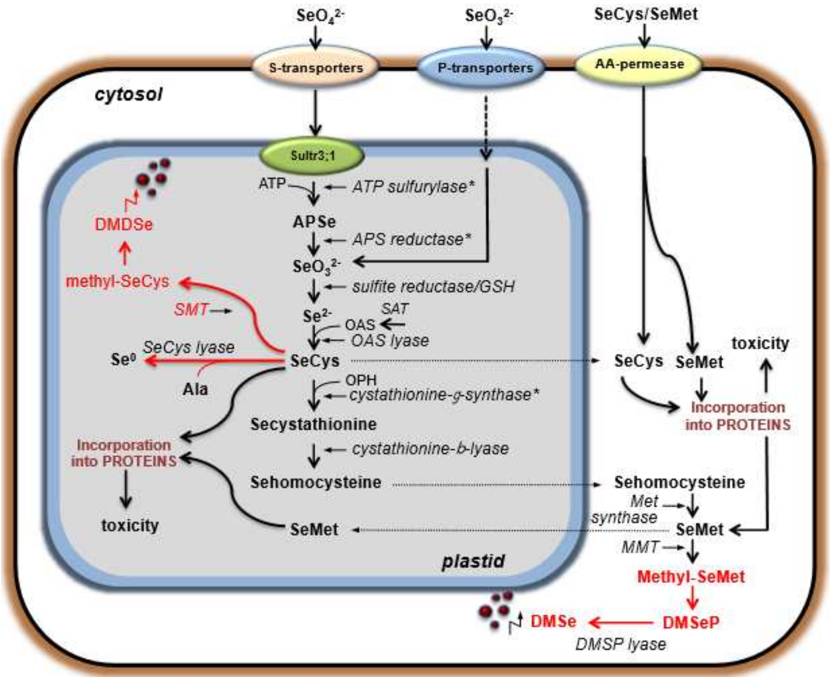

Figure 1.5. Schematic model of Se assimilation and metabolism in plant mesophyll cells. Red text and arrows indicate Se hyperaccumulator processes. Asterisks indicate enzymes overexpressed via genetic engineering. Sultr: sulfate/selenate cotransporters; APSe: adenosine phosphoselenate; GSH: glutathione; SAT: serine acetyltransferase; OAS: O-acetylserine; (Se)Cys: (seleno)cysteine; OPH: O-phosphohomoserine; (Se)Met: (seleno)methionine; MMT: methylmethionine methyltransferase; DMSeP: dimethylselenoproprionate; DM(D)Se: dimethyl(di)selenide (volatile); SMT: selenocysteine methyltransferase. From Schiavon & Pilon-Smits, 2017b.

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CHAPTER 2: EXPLORING THE POTENTIAL OF HEMP (*CANNABIS SATIVA* L.) FOR SELENIUM BIOFORTIFICATION AND PHYTOREMEDIATION

2.1 Introduction

Cannabis sativa (hemp) has been described as "the plant of 50,000 uses". It is one of the oldest agricultural crops, dating back to ancient Asian cultures, already found 5,000 B.C. in the Yellow River basin in China (Booth, 2005). *Cannabis sativa* was mainly utilized for its fiber, to make rope, clothing and sails, but also has a long tradition in Chinese medicine, owing to its cannabinoid and terpenoid compounds. Some varieties of *C. sativa* (known as marijuana) contain a psychoactive compound (Δ-9-tetra-hydrocannabinol (THC). Hemp varieties have below-psychoactive levels of this compound, but do contain numerous (~90) other cannabinoids and (~100) terpenoids, most notably the medicinal compound cannabidiol (CBD) (ElSohly & Slade 2005; Brenneisen 2007; Radwan et al., 2009; Fischedick et al., 2010). Because of marijuana, *C. sativa* cultivation has been banned throughout most of the 20th century in the U.S.A. However, in the last two decades, *C. sativa* is increasingly being recognized for its many uses and benefits, leading to its gradual legalization. In 2012, Colorado and Washington were the first states to legalize recreational use of marijuana and indoor cultivation. In 2014, low-THC hemp was approved for large-scale outdoor cultivation throughout the U.S.A., which was clarified and re-affirmed in 2018 (www.agriculture.senate.gov/2018-farm-bill). This legalization has led to a prolific increase in hemp cultivation for CBD, terpenoids and seed products, and its associated hemp industries: in 2016 there were more than 20,000 hemp-related companies (www.statista.com/statistics/596641/us-cannabis-businesses-number/), earning a collective 6.2 billion USD; this revenue is projected to reach 20.2 billion USD in 2021 (www.forbes.com/sites/debraborchardt/2017/01/03/marijuana-sales-totaled-6-7-billion-in-

2016/#10c1c2eb75e3). The legalization of *C. sativa* has also opened up the opportunity for research into the biology of this interesting ancient crop.

The increased interest in hemp has led to many emerging hemp products. In addition to fiber, seed products, and a variety of medicinal compounds, "hempcrete" made from hemp biomass is gaining popularity as a lightweight building material (Pecenko et al., 2014). Hemp has other emerging phytotechnological applications, including environmental cleanup, a technology known as phytoremediation. Hemp is a hardy, fast growing, deep-rooted, high-biomass crop, all desirable properties for phytoremediation. Hemp has already shown promise in phytoremediation of various metals, radionuclides and polyaromatic hydrocarbons (PAHs) (Parisa et al., 2012; Ahmad et al., 2016; Iqbal et al., 2018).

In some cases, a pollutant is also an animal and plant micronutrient, e.g. zinc (Zn), iron (Fe), manganese (Mn), cobalt (Co), chromium (Cr) and the topic of this study, selenium (Se). Thus, plants that accumulate these elements in phytoremediation may have elevated levels of these elements in their edible parts, an example of biofortification (Guerinot & Salt 2001; Broadley et al., 2006). Crop biofortification is receiving a lot of attention recently, in view of its global importance, considering that billions of people worldwide have micronutrient deficiency (https://www.who.int/nutrition/topics/WHO_FAO_ICN2_videos_hiddenhunger/en/). Crops may be biofortified with micronutrients via fertilization, or cultivated on soils particularly rich in the element in question. Hemp biofortification is an interesting area of study; hemp seeds are already known for their high nutritional value, including essential fatty acids (e.g. omega-3 and -6) associated with cardiovascular health. Biofortification of hemp seed with micronutrients may further enhance their nutritional benefits.

In order to optimize the applications of hemp phytoremediation and biofortification, more research is needed regarding what hemp can internally do with the elements (pollutants/micronutrients) of interest: i.e., mechanisms of uptake, translocation, sequestration, metabolism, and tolerance.

One of the elements of interest and the topic of this study is Se. This element is interesting because it is both essential at low concentrations to many life forms (including mammals), but also a toxic pollutant at higher levels. There is a very narrow window between Se deficiency and toxicity, and both are prevalent worldwide, depending on local soil Se concentrations (Schiavon & Pilon-Smits 2017a). Large areas of Western U.S.A., including areas in Colorado with large hemp acreages, has naturally Se-rich (seleniferous) soil, while other areas in the U.S.A. are low in Se (Fig. 1A). Parts of South America, Europe, China, Africa, Australia and New Zealand also have low-Se soils (Jones et al., 2017). Crop products naturally biofortified with Se from high-Se soils offer an attractive source of Se for populations in low-Se areas. The recommended daily allowance (RDA) by the world health organization (WHO) for adults is 55-75 μg Se d^{-1}, while the maximum allowable daily intake is 400 μg Se d^{-1} (Institute of Medicine 2000). Essential functions of Se in mammals are typically redox-related: selenocysteine (SeCys) is incorporated specifically into so-called selenoproteins, which are involved in antioxidant scavenging, immune function, thyroid function, and fertility (Rayman 2012). It has been estimated that one billion people are Se deficient, which may be associated with higher susceptibility to infections and cancer, as well as thyroid and fertility problems (Combs 2005; Lyons, 2003). This human Se deficiency problem is projected to get even worse with global climate change, because of reducing soil Se levels in low-Se regions in Europe and China (Jones et al., 2017). At high concentrations in organisms, Se becomes toxic because it is similar to sulfur (S) and may non-specifically replace S in proteins, interfering with protein function; inorganic forms of Se may also cause oxidative stress (Van Hoewyk 2013).

Selenium in the environment is present mainly as selenate (in oxic conditions like soil), or selenite (in anoxic conditions like wetlands) (White et al., 2015). Plants can absorb these forms by means of sulfate transporters (selenate) or phosphate transporters (selenite) and assimilate them into organic forms like SeCys and Se-methionine (SeMet), using the S assimilation pathway (Terry et al., 2000). There is a large variation between different plant species in their capacity to absorb, metabolize, and tolerate Se (Sors et al., 2005). Based on plant tissue concentrations in natural settings, three categories are distinguished: non- Se accumulators (< 100 mg Se kg^{-1} dry weight), Se accumulators (100 - 1000 mg Se kg^{-1} dry weight) and Se hyperaccumulators (>1,000 mg Se kg^{-1} dry weight), (Schiavon & Pilon-Smits 2017b). The hyperaccumulators are also hypertolerant to Se, because of their capacity to methylate SeCys into methyl-SeCys (MetSeCys), which is non-toxic because it does not get incorporated into proteins (Neuhierl & Bock 1996). For plants, Se is not essential, but it still can stimulate antioxidant capacity by enhancing the production of antioxidant enzymes and metabolites; this offers growth and stress protection benefits through scavenging of reactive oxygen species produced in photosynthesis and respiration, especially under stress (Schiavon & Pilon-Smits 2017b). At high concentrations, Se can also offer plants herbivory protection due to Se-mediated deterrence and toxicity (Schiavon & Pilon-Smits 2017b).

Here, we study hemp’s ability to absorb, translocate, accumulate, metabolize and tolerate Se, both at the seedling stage and the mature plant stage. Two approaches were used. First, a field survey was carried out of Se levels in different organs of hemp grown throughout Colorado on naturally seleniferous soil, as well as commercial edible hemp products derived from these plants, to investigate hemp’s potential for producing Se biofortified products. Second, to study hemp's phytoremediation potential, seedlings and mature hemp plants were cultivated under controlled conditions and supplied with selenate (as sodium selenate), the most common and bioavailable form in seleniferous soils. The accumulation of Se across the different plant organs was investigated, as well as the forms of Se present in the (edible) seeds. Furthermore, the effects of Se on photosynthesis, biomass production, and cannabinoid and terpenoid concentrations were investigated.

2.2 Materials and Methods

2.2.1 Field surveys

Hemp seed, flower and leaf samples (as available) were collected during September and October of 2017 from four agricultural areas in Colorado (Fig. 2.1 A): Colorado State University Agriculture Research Development and Education Center (ARDEC) in Fort Collins, two commercial hemp fields in Eaton and Otis farmed by Colorado Cultivars Co., and the Arkansas Valley Research Center in Rocky Ford. The hemp samples were dried at 50°C for 3 days, and then weighed and acid-digested for elemental analysis as described below.

To assess hemp biomass production per plant and per hectare, seven entire shoots of mature hemp plants were collected around the time of harvest (early October), dried to constant weight, and the weight recorded. Also, the distance between plants in the field was determined, both within and between rows. The plants in the field in question were grown (in Eaton, CO) for seed and CBD and contained both males and females.

2.2.2 Seedling experiments

A low-tetrahydrocannabidiol (THC) variety of hemp (*Cannabis sativa L.*) was used (variety "Workhorse", Colorado Cultivars Co., Eaton, CO). The seeds were germinated in covered petri dishes with a piece of Whatman 1 filter paper wetted with deionized water. After the root tip (radical) emerged from the seed, 30 seedlings were transplanted into 4 x 4 cm pots containing Turface®, a gravel growth medium. Pots were placed in 25 x 50 cm trays and kept separate for each Se treatment. Five different concentrations of Se used were, 0, 40, 80, 160, and 320 µM applied as Na_2SeO_4. The seedlings were watered via the trays with the respective concentration of Se mixed into a one-fifth strength Hoagland's nutrient solution (Hoagland & Arnon 1938) from transplanting to harvest, 25 days later. The plants were grown under 200 µE light intensity with a photoperiod of 12:12 light:dark at 22°C. Chlorophyll fluorescence data were collected the day before harvest.

At harvest, the plants were carefully separated from the growth medium and washed, then dried at 50°C for 3 days. The plants were then separated into roots and shoots and weighed individually. The root and shoot samples were acid-digested for elemental analysis as described below. For the highest (160 and 320 µM) Se concentration treatments, multiple plants were pooled to obtain sufficient biomass for acid digestion.

2.2.3 Mature plant experiments

Seeds of *C. sativa* var. "Workhorse" were germinated in petri dishes between moist Whatman 1 filter paper, using 50 seeds per plate. After three days, when seedlings emerged, 72 germinated seeds were transplanted into an equal number of 2 L pots filled with Turface® (a gravel growth medium), and grown in greenhouse conditions under 1000 watt high pressure sodium lights (200 µE light intensity) at a 16:8 light:dark photoperiod for a total of 15 weeks. The plants were watered as needed and supplied once a week with half-strength Hoagland's nutrient solution (Hoagland & Arnon 1950). After 3 weeks, the Se treatments were started; once-weekly for a total of 12 weeks the plants were supplied with different selenate concentrations water (0, 5, 10, 20, 40, 80 µM Na_2SeO_4, n=12); ½ Hoagland's nutrient solution was also still applied once a week.

Chlorophyll fluorescence parameters were collected at 12 weeks old. Just before harvest, at 15 weeks old, female hemp flower and seed samples were collected for biochemical analyses of secondary plant compounds and selenocompounds. Then roots and shoots were harvested, dried and weighed, and subjected to elemental analysis as described below.

2.2.4 Selenium effects on Chlorophyll fluorescence

Chlorophyll fluorescence parameters (Phi2, PhiNPQ, and relative chlorophyll content) were collected using the Photosynq Multispeq (Kuhlgert et al., 2016) and the manufacturer's standard protocol. Seedling data were collected the day before harvest on the first set of true leaves, from five replicates each from the 0, 40, 80, and 160 µM treatments, and 3 replicates from the 320 µM Se treatment. Mature plant data were collected on the youngest mature leaves of the 0, 10, 40 and 80 µM selenate treatments (n=5).

2.2.5 Selenium effects on secondary plant compounds in mature hemp

Female flower samples (n=3) from the 0, 10, and 40 µM selenate treatments were shipped to Phytatech Inc. (Denver, CO), where they were extracted and analyzed for cannabinoids and terpenoids via gas chromatography (Agilent GC 6890) using the company's standard protocols.

2.2.6 Chemical speciation of Se in hemp seeds

After harvest, the hemp seeds were stored at -80ºC until further analysis when they were prepared for liquid chromatography mass spectrometry (LC-MS) to determine Se speciation of Se in the seeds. Seed samples (n=5) from the 0, 10, 40 µM selenate treatments were ground with mortar and pestle in liquid nitrogen, and then ~400 mg samples were extracted with 2 mL g^{-1} fresh weight of acidic di-ionized water (pH 2.5). After 24h extraction at 4ºC, the supernatant was collected after centrifugation at 2,500 x g for 10 min. The Se speciation in the plant extract was determined using LC-MS as documented by Dumont et al. (2006), comparing the elution profile to a mixture of standard organic Se compounds (SeMet, selenocystine, MetSeCys, γ-glutamyl-MetSeCys, seleno-diglutathione).

2.2.7 Elemental analysis

The concentration of Se and other elements in the various hemp organs was determined using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), as described by Fassel, (1978), after acid-digestion described by Zarcinas et al., (1987). The plant material was dried at 50°C in an oven, and then 100 milligrams of dried sample (root, stem, leaf, flower, and seed) was digested in 1 mL of concentrated nitric acid by heating for 2 h at 60°C and 6 h at 120°C. The digested samples were diluted to 10 mL with deionized water and then analyzed by ICP-OES, using appropriate quality controls and standards. The limit of quantification using this method is 50 $\mu g\ L^{-1}$ in the digest, or 1-2 $mg\ kg^{-1}$ DW in the sample material.

Selenium concentration in beer was determined after 6-fold dilution in 1% nitric acid, according to Rodrigo et al. (2015) using ICP-mass spectrometry (in O_2 DRC mode), and appropriate quality controls and standards. The limit of quantification using this method is 0.1 $\mu g\ L^{-1}$ in the diluted sample.

2.2.8 Statistical analysis

The software JMP® PRO (13.2.1, SAS Institute, Cary, NC) was used for statistical data analysis. A student's *t*-test was used to compare differences between two means. Analysis of variance (one-way ANOVA) followed by a post-hoc Tukey Kramer test was used when comparing multiple means. It was verified that the assumptions underlying these tests (e.g., normal distribution, equal variance) were met.

2.3 RESULTS

2.3.1 Field Survey

Hemp plants grown on naturally seleniferous soils in four locations in Colorado (Fig. 1A) were analyzed for their Se levels in different organs (seed, flower and leaf, as available). The four locations collectively are a good representation of the variation in soil Se concentration in Colorado (Fig. 2.1A). The soil Se levels in the corresponding counties of the four sampling sites, USGS data, (https://mrdata.usgs.gov/geochem/doc/averages/se/usa.html) are 0.29±0.15 (ranging from 0.10-1.11) for Otis, 0.55±0.35 (ranging from 0.11-2.55) for ARDEC, 0.58±0.42 (ranging from 0.10-3.18) for Eaton, and 1.30±0.56 (ranging from 0.46-3.47) for Rocky Ford. Among the different plant organs, the seeds had the highest Se concentrations (Fig. 2.1B), averaging 15-25 mg/kg DW. Flowers and leaves typically had 2-fold lower levels than seeds, around 5-10 mg Se/kg DW. Among the four test sites, the hemp plants at Rocky Ford (the site with the highest reported soil Se concentration) had the highest Se concentrations and Otis (the site with the lowest soil Se concentration) the lowest (Fig. 2.1A).

Three commercial consumption products originating from hemp grown at the Eaton site were also analyzed for Se: hemp hearts (dehulled seeds, provided by Colorado Cultivars), hemp protein powder (prepared from pressed seed, provided by Colorado Cultivars) and commercially purchased hemp beer brewed using these same Eaton hemp hearts (Hemperor, produced by New Belgium Brewing). Hemp hearts and protein powder are both popular plant-based health food products that may be sprinkled over breakfast cereal or mixed into smoothies. The hemp hearts contained 27.6 mg Se/kg DW, and the protein powder 13.9 mg Se/kg DW. Thus, the Se levels in these seed-derived products are similar (protein powder) or slightly greater (hemp hearts) than those found

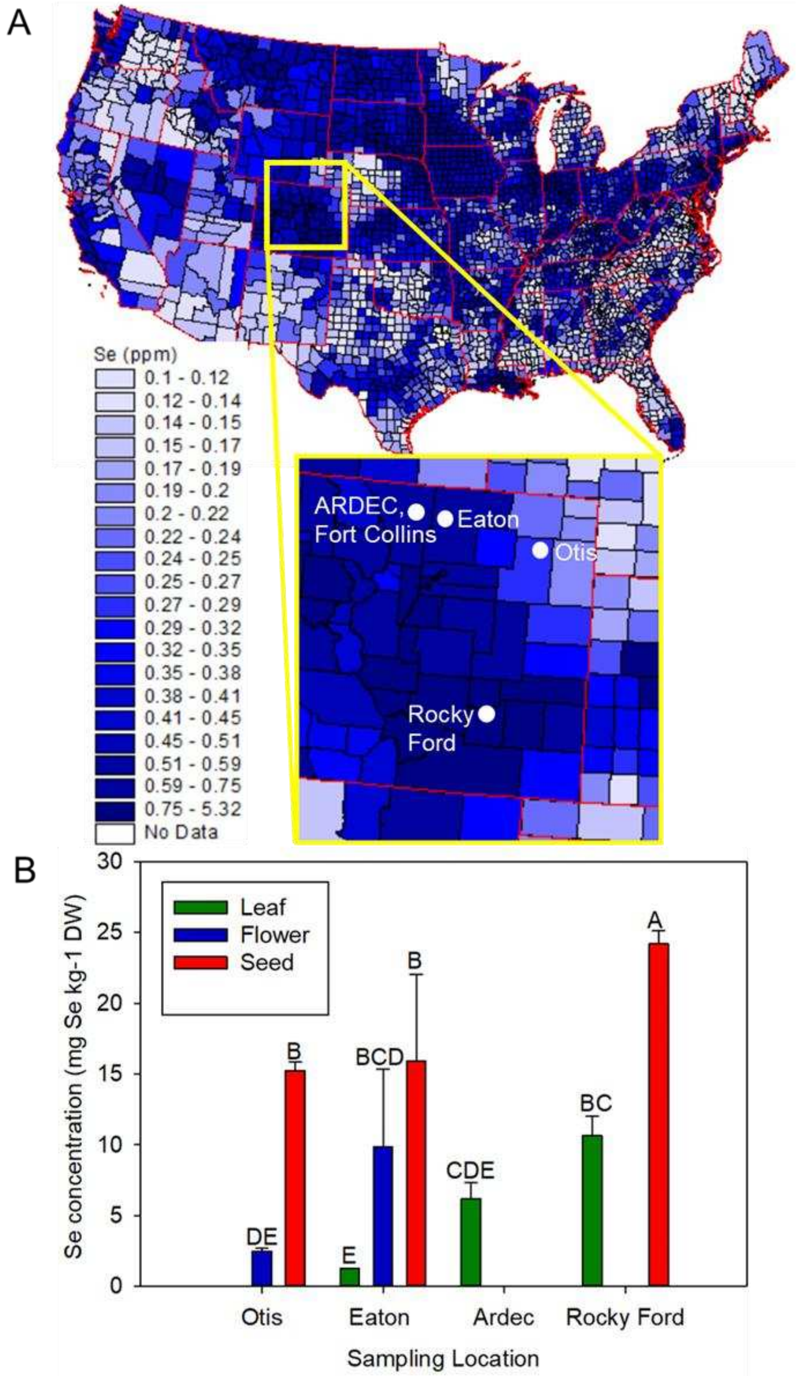

Figure 2.1. A. Soil Se levels in the continental United States (source: US Geological Survey, https://mrdata.usgs.gov/geochem/doc/averages/se/usa.html). The inset shows a close-up of Eastern Colorado and the sampled locations. B. Selenium levels in different hemp organs, sampled from the four agricultural areas indicated in A. Shown are mean and standard error of the mean (n=3-9). Different letters above bars denote significantly different means (ANOVA, Tukey-Kramer, a = 0.05).
Note: not all organs were available from each site (hence the absent bars).

in unprocessed seeds from the same site (Fig. 2.1B). The hemp beer was found to contain 42 µg Se/L, which corresponds with 15 µg Se per 354 mL bottle. Four other beers were tested for comparison, three from Colorado (Citradelic IPA, 40 µg Se/L, Easy Street Wheat, 7 µg Se/L and Blue Moon Belgian style white, 16 µg Se/L) and one from Belgium (Hoegaarden, 9 µg Se/L). None of these were advertised as having hemp as an ingredient. Thus, the Se concentration in the hemp beer was the highest (2.5-fold higher than the average of the other four), but did not stand out dramatically.

To estimate hemp total biomass productivity in Colorado (relevant for phytoremediation), hemp plants being grown in an Eaton field for seed and CBD were collected shortly before harvest, four months after seeding. The plants varied in height from 180-300 cm and the individual hemp shoots weighed 338±52 g DW (n=7). The plants were spaced 25 cm distance apart in their rows, with rows 90 cm apart; therefore the plant density would be 52,480 plants per ha at full density. Based on this density, maximum hemp biomass production may be estimated at 17,712 kg DW per ha (17.7 tons/ha). This is comparable with reported fiber hemp dry-stem yield of 7-15 tons/ha (https://www.ers.usda.gov/webdocs/publications/41740/15859_ages001ei_1_.pdf?v=0). At an average Se concentration of 15 mg/kg DW (Fig. 1B) and a yield of 17.7 tons/ha, the total shoot Se accumulation per hemp crop would be 266 g Se/ha.

2.3.2 Seedling Experiments

Selenium treatment affected hemp seedling growth differently depending on the concentration, stimulating growth at lower levels but becoming toxic at the highest levels. Three week-old seedlings treated from germination with 40 µM selenate had attained ~2-fold higher root and shoot biomass compared to control plants not supplied with Se ($P<0.05$, Fig. 2.2). At 80 µM selenate, biomass was not different from the control, while 160 and 320 µM selenate resulted in reduced root and shoot biomass (Fig. 2.2).

Photosynthetic performance, as judged from Chlorophyll fluorescence analysis, decreased at Se treatments above 80 µM selenate. Photochemical quenching (light energy dissipation via the photosynthetic light reactions) was 3-fold reduced at 320 µM Se ($P<0.05$, Fig. 2.3A). Non-photochemical quenching (light energy dissipation via non-

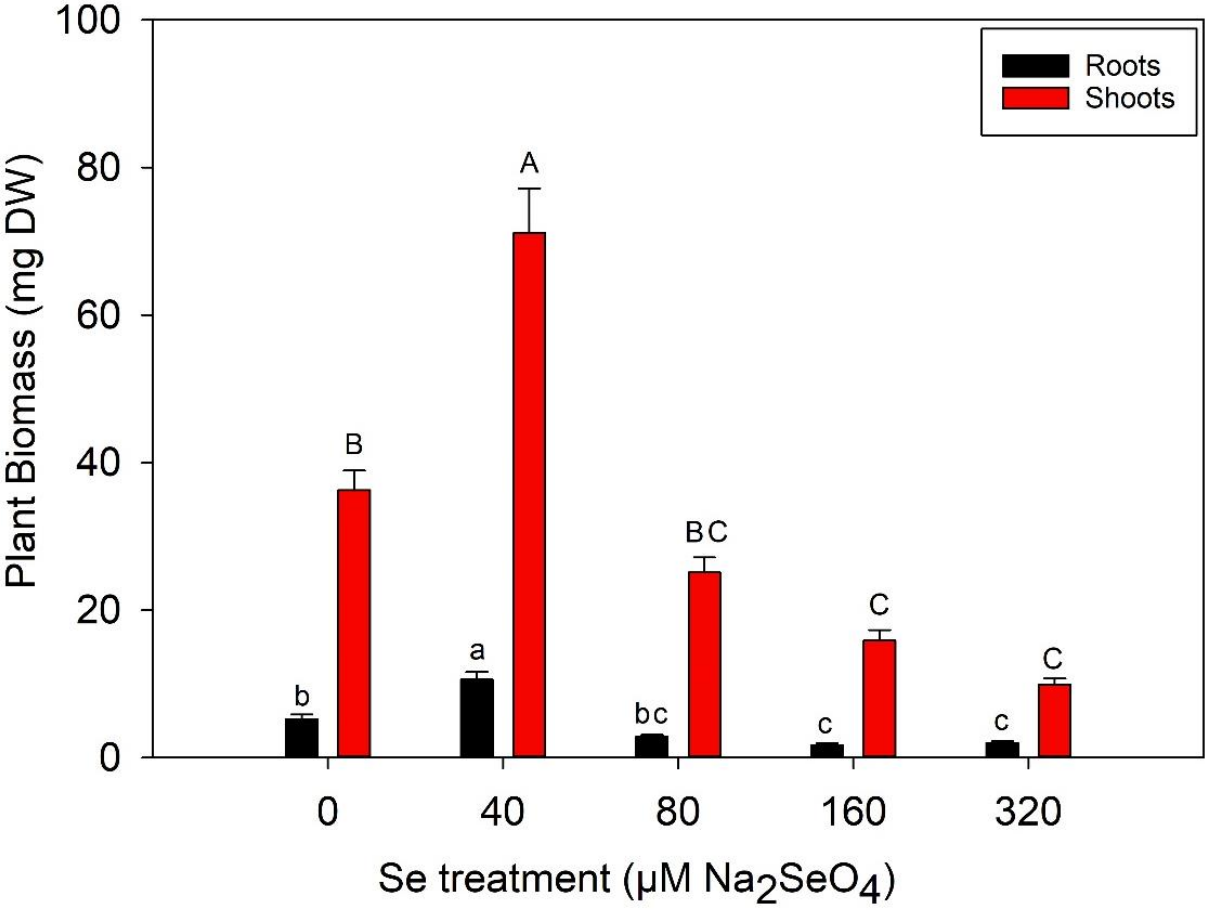

Figure 2.2 Hemp seedling biomass after germination and growth on different concentrations (0-320 µM) of sodium selenate. Different letters above bars denote significantly different means across Se treatments for either shoot (capital letters) or roots (lower case letters); α = 0.05, ANOVA and Tukey Kramer, n=32.

photosynthetic mechanisms, some photoprotective) increased progressively with Se concentration, becoming significantly elevated at 80 µM Se (Fig. 2.3B). Leaf chlorophyll concentration was reduced above 80 µM Se (Fig. 2.3C).

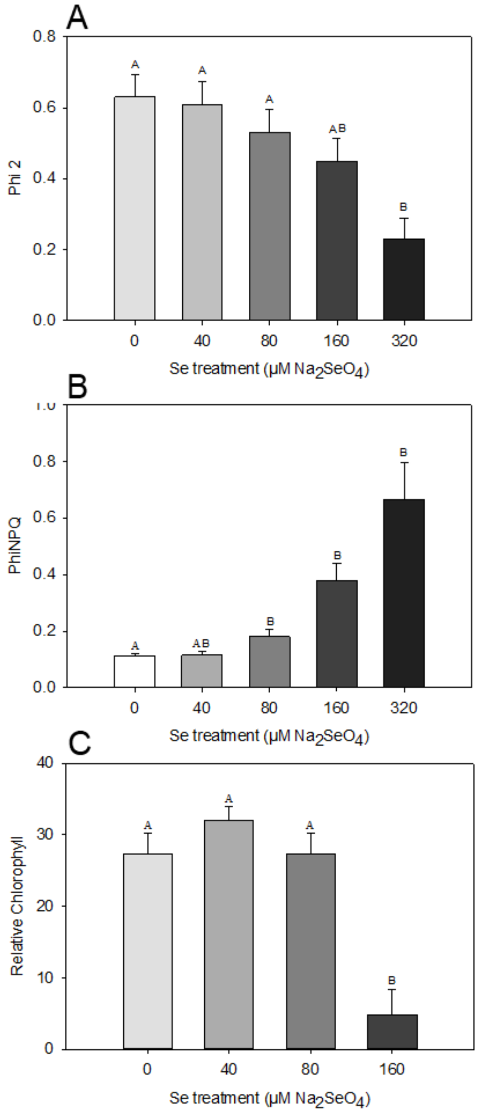

Figure 2.3 Chlorophyll fluorescence measurements from hemp seedlings supplied with different concentrations of sodium selenate. A. Fraction of light energy quenched photochemically due to electron transport through photosystem 2 (Phi2); B. Fraction of light energy quenched non-photochemically due to photoprotective mechanisms (PhiNPQ); C. Relative Chlorophyll content (note: no reliable measurement could be taken from the 320 µM treatment). Letters above bars denote means do not differ significantly across Se treatments. $\alpha = 0.05$, ANOVA and Tukey Kramer, n=7.

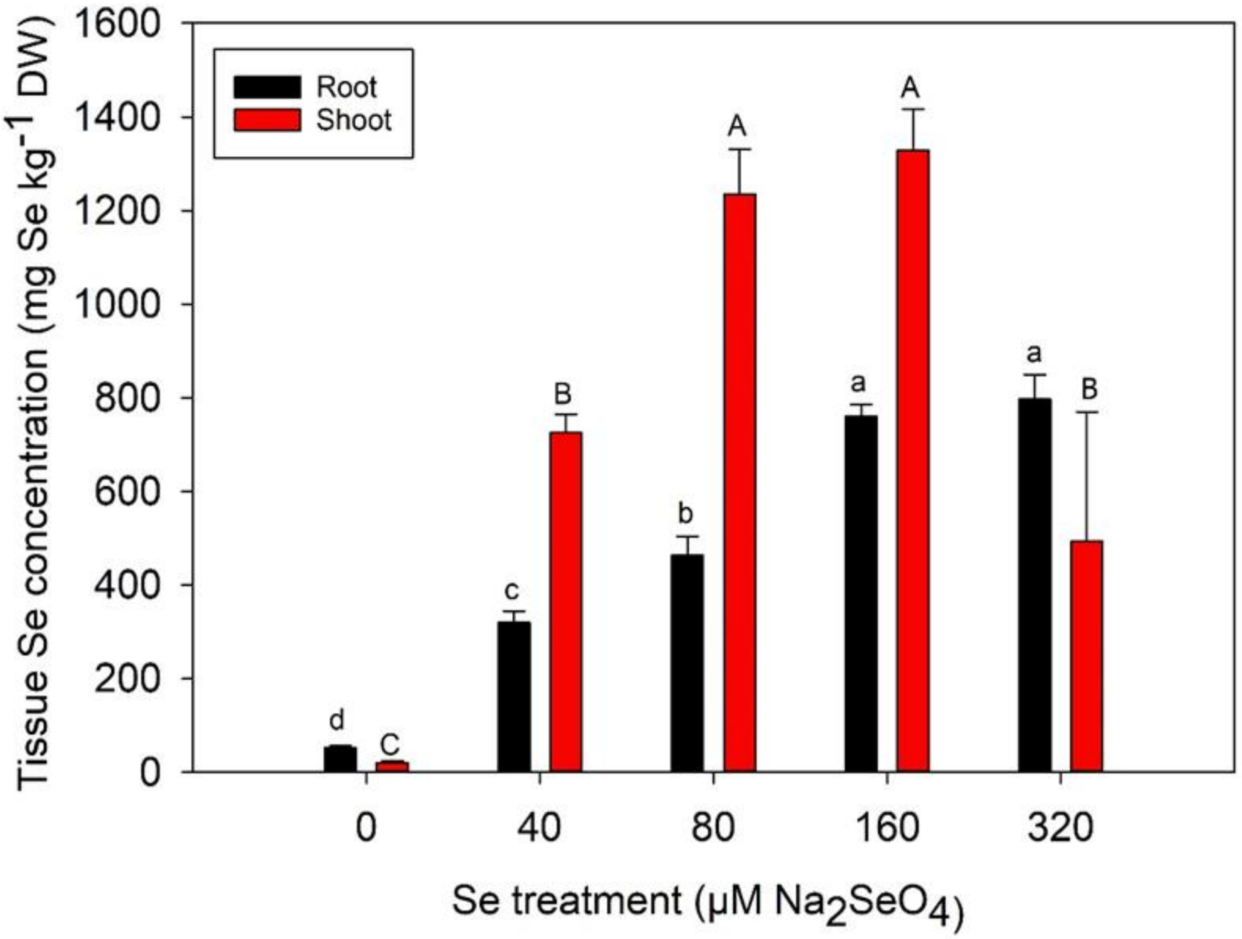

Figure 2.4. Hemp seedling root and shoot Se accumulation after growth on different concentrations (0-320 μM) of sodium selenate. Shown are mean and SEM. Different letters above bars denote significantly different means across Se treatments for either shoot (capital letters) or roots (lower case letters); $\alpha = 0.05$, ANOVA and Tukey Kramer, n=12.

Increasing concentrations of selenate treatment generally resulted in increasing root and shoot Se accumulation, reaching tissue concentrations of 1,300 mg Se kg^{-1} DW in the shoot and 800 mg Se kg^{-1} DW in the root (Fig. 2.4). However, at 320 μM Se, there was significantly less Se accumulation in the shoot compared to that observed for the 80 or 160 μM Se treatments.

2.3.3 Mature Plant Experiments

At the mature plant stage, hemp productivity (biomass) was unaffected by selenate up to 40 µM, but shoot biomass decreased significantly (~2-fold) at 80 µM selenate (Fig. 2.5). Plant height showed a similar response to Se, being unaffected up to 40 µM (results not shown). At 15 weeks of age (in the late stage of flowering, and females in the process of setting seed) the plants were around 150 cm tall.

Photosynthetic performance was not affected up to 80 µM selenate, judged from photochemical quenching, non-photochemical quenching, and chlorophyll content (Fig 2.6A, B, C).

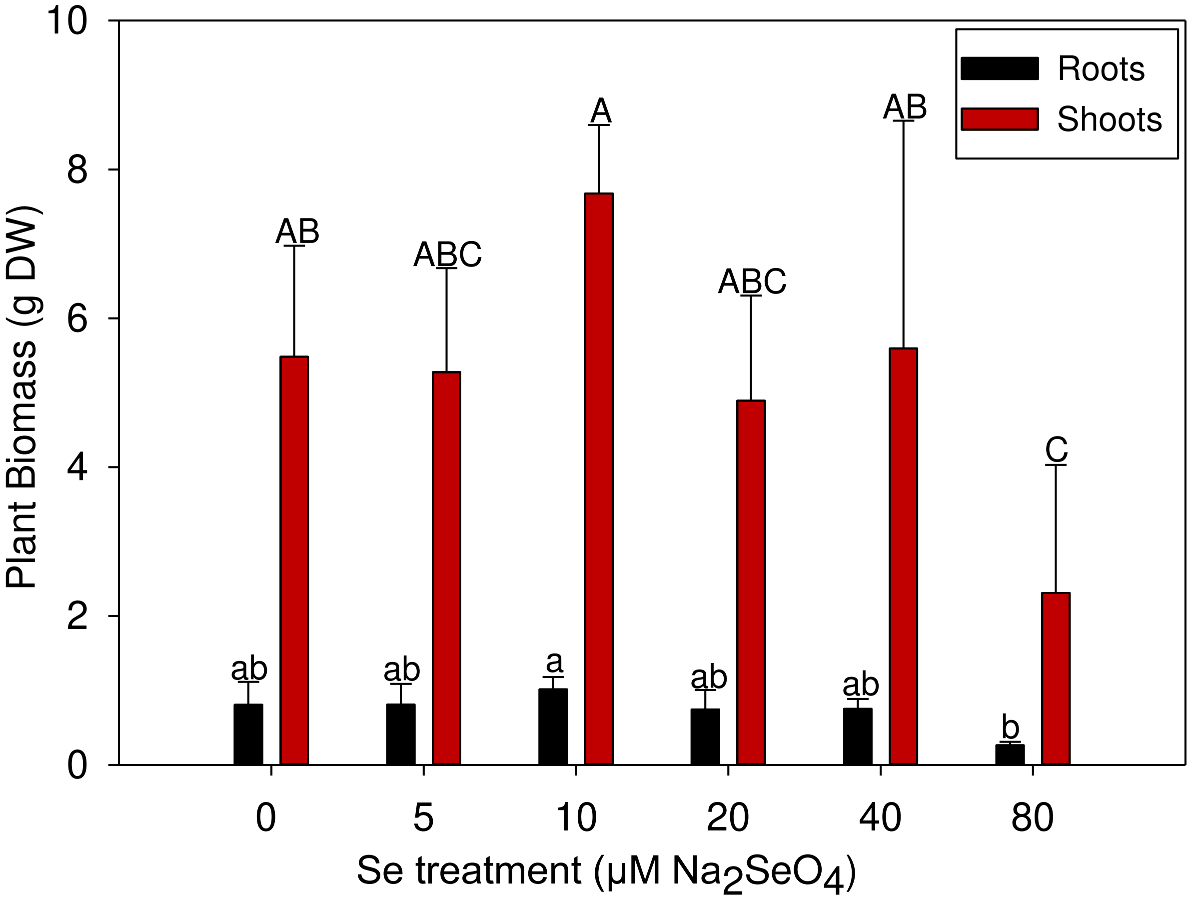

Figure 2.5. Effects of selenium (0-80 µM sodium selenate) on root and shoot biomass production of greenhouse-grown hemp plants. Different letters above bars denote significantly different means across Se treatments for either shoot (capital letters) or roots (lower case letters); α = 0.05, ANOVA and Tukey Kramer, n=12.

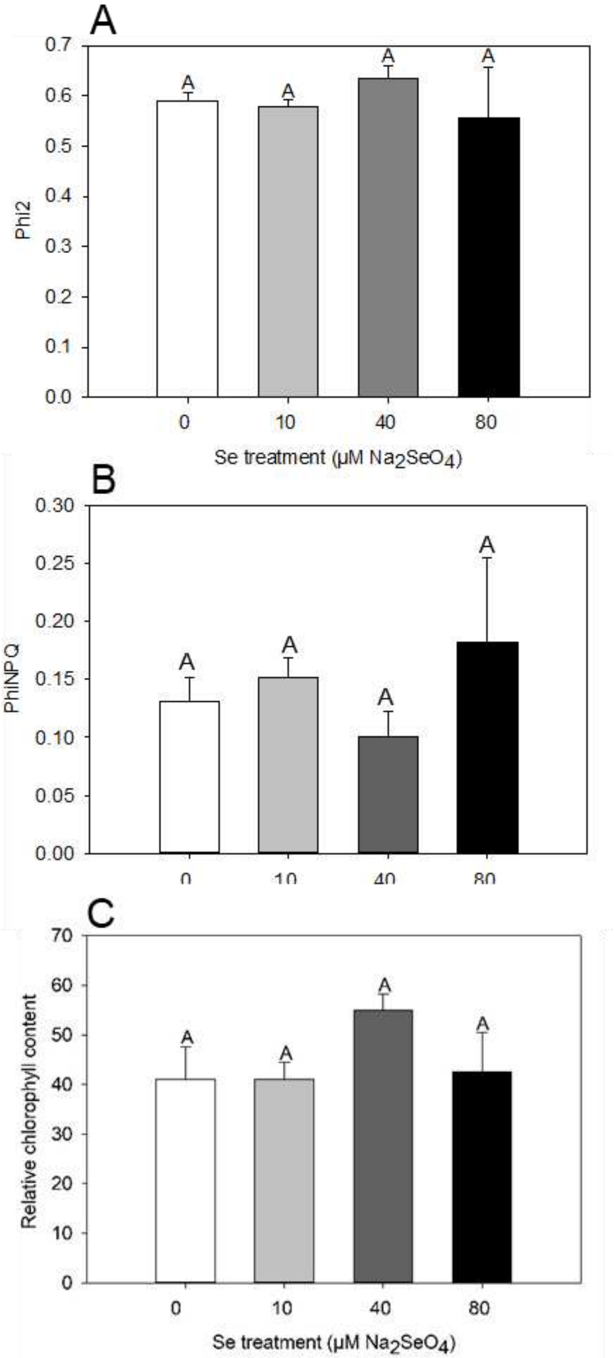

Figure 2.6. Chlorophyll fluorescence measurements from mature hemp plants supplied with different concentrations of sodium selenate. A. Fraction of light energy quenched photochemically due to electron transport through photosystem 2 (Phi2); B. Fraction of light energy quenched non-photochemically due to photoprotective mechanisms (PhiNPQ); C. Relative Chlorophyll content. Letters above bars denote means do not differ significantly across Se treatments. $\alpha = 0.05$, ANOVA and Tukey Kramer, n=5.

Increasing concentrations of selenate treatment resulted in increasing root, stem, leaf, flower and seed Se accumulation, but reached a plateau around the 40 µM Se treatment. The greatest concentrations of Se obtained in leaf tissue was 200 mg Se kg^{-1} DW, and in flower and seeds 150 mg Se kg^{-1} DW; stems and roots had significantly lowerconcentrations of Se, at 50 and 80 mg Se kg^{-1} DW respectively (Fig. 2.7). Male and female plants did not show significant differences in Se accumulation (data not shown).

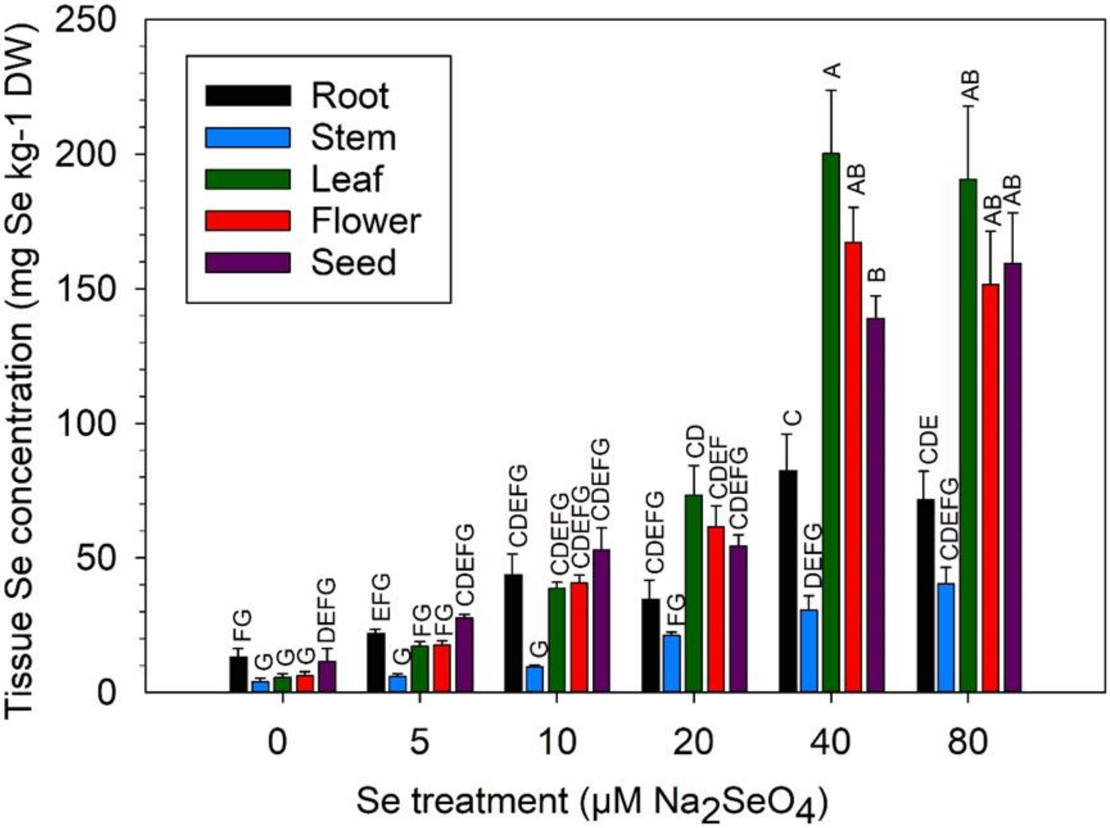

Figure 2.7. Tissue Se concentration in root, stem, leaf, flower and seed material collected from mature hemp plants supplied with different concentrations of sodium selenate. Different letters above bars denote significantly different means across all Se treatments and plant organs ($\alpha = 0.05$, ANOVA and Tukey Kramer, n=12).

Mineral content of seeds was investigated in relation to Se supply, not only to investigate how Se affects plant mineral nutrition, but also because of dietary importance to consumers.

Seeds from selenate-supplied plants had 2-3 fold greater potassium (K) concentrations compared to control seeds (Table 2.1); this effect was found for all Se treatments, but only significant for the 10 µM treatment. For the other elements analyzed, no Se-related effects were observed (Table 2.1).

Table 2.1) Effects of selenate treatment (0-80 µM Na_2SeO_4) on seed nutrient concentrations (mg kg^{-1} DW) in mature hemp plants. Different superscript letters denote means that differ significantly across Se treatments (α = 0.05, ANOVA and Tukey Kramer, n=12).

	0 µM Se	**5 µM Se**	**10 µM Se**	**20 µM Se**	**40 µM Se**	**80 µM Se**
	--------------------------------------- mg kg^{-1} ---------------------------------------					
Ca	1175 ± 477^{a}	968 ± 223^{a}	4312 ± 3386^{a}	872 ± 152^{a}	1133 ± 133^{a}	1036 ± 330^{a}
Cu	40.6 ± 29^{a}	46.5 ± 16.1^{a}	36.7 ± 17.4^{a}	52.1 ± 15.5^{a}	44.6 ± 14.7^{a}	49.5 ± 16.4^{a}
Fe	57.7 ± 47^{a}	53.2 ± 5.9^{a}	65.4 ± 9.9^{a}	51.4 ± 4.2^{a}	55.9 ± 4.4^{a}	47.1 ± 7.1^{a}
K	5444 ± 2149^{b}	10413 ± 586^{ab}	17658 ± 5141^{a}	10412 ± 983^{ab}	15744 ± 2371^{ab}	15782 ± 2706^{ab}
Mn	97.3 ± 5.4 ^{a}	101.9 ± 4.4 ^{a}	214.8 ± 103.7^{a}	86.9 ± 4.2 ^{a}	82.4 ± 4.2 ^{a}	66.8 ± 23.9 ^{a}
Mo	6.5 ± 0.5^{a}	4.5 ± 0.5^{a}	8.8 ± 3.8^{a}	4.6 ± 0.4^{a}	4.9 ± 0.9^{a}	4.5 ± 0.5^{a}
P	5867 ± 530^{ab}	5819 ± 305^{b}	7729 ± 447^{a}	5716 ± 342^{b}	6697 ± 150^{ab}	5481 ± 265^{b}
S	2176 ± 108^{a}	2546 ± 9^{a}	3220 ± 1004^{a}	2038 ± 58^{a}	2223 ± 43^{a}	2177 ± 60^{a}
Zn	36.9 ± 1.7^{a}	37.7 ± 5.2^{a}	47.1 ± 7.9^{a}	37.3 ± 1.3^{a}	37.7 ± 1.4^{a}	34 ± 4.3^{a}

Selenium speciation in seeds was investigated because of the relevance to consumers as a dietary source. Two organic selenocompounds were detected in aqueous extracts, selenomethionine and methyl-selenocysteine (Fig. 2.8). The concentration of these compounds was correlated with selenate supply, but the ratio of selenomethionine and methyl-selenocysteine was 3:1 in the 10 µM selenate treatment and close to 1:1 in the 40 µM selenate treatment. The LCMS analyses in positive and negative modes did not detect any selenate or selenite, nor the other organic Se compounds available as standards, γ-glutamyl-MetSeCys, Se-diglutathione, and Se-cystine.

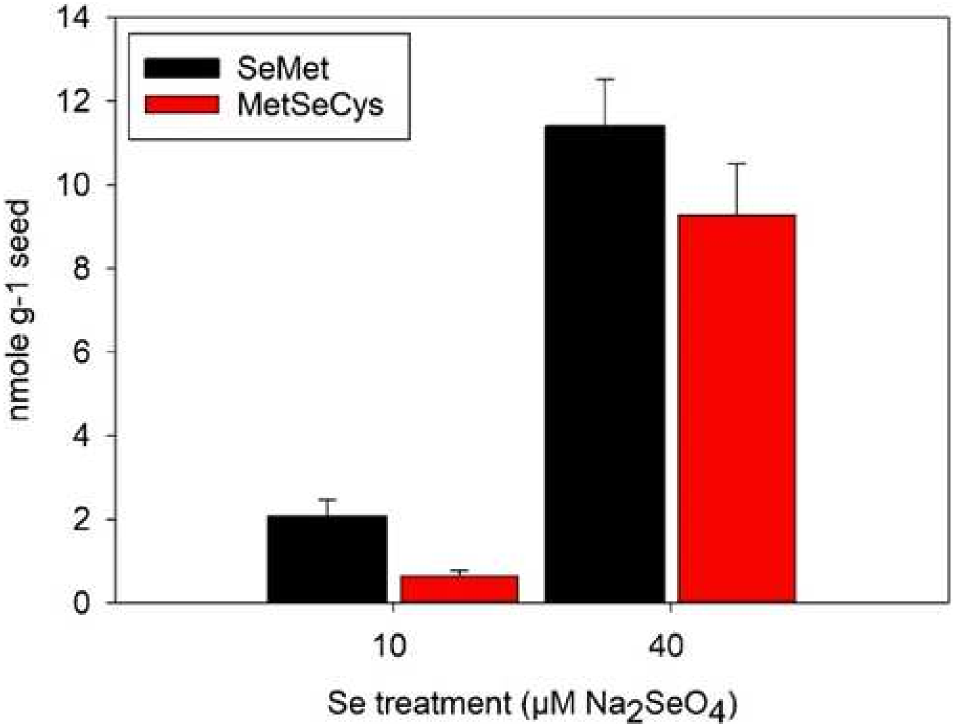

Figure 2.8. Concentration of organic selenocompounds selenomethionine (SeMet) and methyl-selenocysteine (MetSeCys) in seeds collected from mature hemp plants supplied with different concentrations of sodium selenate. Note: there was no detectable γ-GluMetSeCys, selenocystine or seleno-diglutathione.

The effect of Se on the secondary compounds CBD and terpenoids was measured because of their economic value. Selenium did not have any effect on the levels of CBD and total terpenoids when compared to the control treatment (Fig. 2.9A, B). Terpenoid profiles were also not affected by selenate treatments, and consisted mostly of α-Pinene, β-Caryophyllene, and Humulene (Fig. 2.8C).

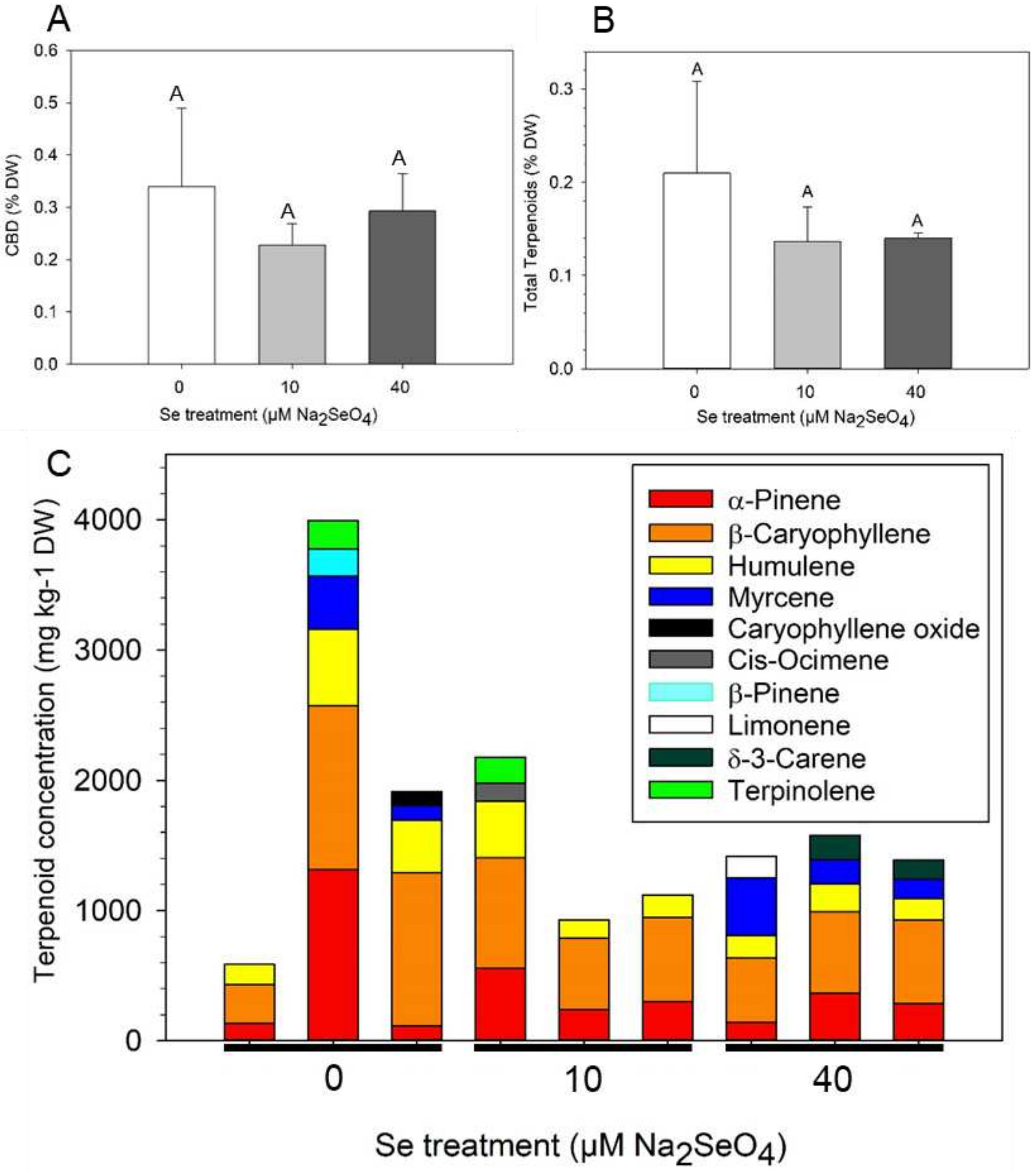

Figure 2.9. Effects of selenate treatment on (female) floral levels of secondary plant compounds in greenhouse-grown plants. A. cannabidiol (CBD); B. total terpenoids; C. individual terpenoids. Letters above bars denote means that do not differ significantly across Se treatments (α = 0.05, ANOVA and Tukey Kramer, n=3).

2.4 DISCUSSION

In this study, hemp's ability was tested to absorb, translocate, accumulate, metabolize and tolerate Se, both at the seedling level and the mature plant level. The first approach was a field survey of hemp grown on four naturally seleniferous Colorado soils; the second approach was a controlled greenhouse experiment where plants were supplied with selenate, the main form of bioavailable Se in soil. The field survey was conducted to investigate hemp's potential for Se biofortification. Hemp seeds contained Se levels ranging from 15-25 mg kg^{-1} dry weight depending on the location; the variation in plant Se content corresponded with USGS-reported variation in soil Se levels. The Se content was similar for intact and dehulled seeds (hemp hearts), and so the Se appeared to be concentrated in the embryo and endosperm, and not in the seed coat. Based on the Se levels found in these Colorado-grown hemp seeds, approximately 4 g of these seeds can provide the recommended daily Se intake (55-75 μg Se). A recommended serving size of 30 g, i.e. three table spoons (according to USDA nutrient database and commercial packages of hemp hearts) of these Colorado-grown seeds would provide 450-750 μg Se, which corresponds with the maximum allowable daily Se intake (https://ndb.nal.usda.gov/ndb/). Thus, it can be concluded that these Colorado-grown hemp seeds are a good source of dietary Se; as a cautionary measure, the official recommended serving size of 30 g should not be exceeded. Beer produced using these hemp hearts contained 42 μg Se/L. Thus, one bottle would provide 15 μg Se, 20-25% of an adult's daily Se requirement, which may also be considered a valuable source of dietary Se. The Se concentration in the hemp beer was higher than in four reference beers tested that were not brewed with hemp, which averaged 18 μg Se/L, but these showed substantial variation in Se, likely from the use of other ingredients (grains, hops, water) that may vary in Se content depending on their origin. Overall, the U.S.

beer Se concentrations were comparable to those reported by Rodrigo et al. (2015), which averaged 12 μg Se/L and ranged from 5 – 18 μg Se/L.

The field survey showed that Se was not only present in seeds, but in all plant organs tested. Flowers and leaves had Se levels around 5-10 mg kg^{-1} dry weight. These findings are relevant because this material may also be utilized for various purposes, and the Se may affect these uses. The flowers are primarily used for extraction of cannabinoids and terpenoids (using non-polar solvents or CO_2), which is not expected to extract the (polar) selenocompounds. The remaining shoot material may be used as animal feed, among other applications. Any of this dried, Se-containing shoot biomass may be considered Se-biofortified, and would be a potential source of dietary Se for livestock or as fertilizer (https://catalog.extension.oregonstate.edu/sites/catalog/files/project/pdf/em9094.pdf) for low-Se areas. Hemp biomass could be added as a feed supplement to provide a suitable concentration (0.1-0.3 mg kg^{-1} dry weight) to satisfy daily livestock requirements. The hemp biomass may also be used as green manure for crop fertilization (Bañuelos et al., 2015, 2016). A fertilization rate of 5-10 g Se per 4,000 m^{-2} (one acre) would correspond to 0.25 kg m^{-2} of hemp biomass containing 5-10 mg Se kg^{-1} dry weight.

For Se biofortification, not only is the Se concentration important, but also the Se form. When selenate was supplied to hemp under controlled conditions (chosen because selenate is the predominant bioavailable form of Se in soils), hemp seeds accumulated several organic forms of Se: the seleno-amino acids SeMet and MetSeCys. These were readily water-extractable without any protease treatment, so likely were present in seeds in their free form, rather than incorporated in proteins. No selenate or selenite could be detected in the extracts, nor the other organic Se compounds γ-glutamyl-MetSeCys, Se-diglutathione, or Se-cystine. Therefore, free SeMet and

MetSeCys may be the main selenocompounds present in hemp seeds. These forms of Se are considered the most effective for biofortification (Navarro-Alarcon and Cabrera-Vique, 2008; Rayman, 2012). Selenomethionine has also been found in seeds of several other species including wheat and Brazil nut, but as part of proteins and thus not in its free form (Zhu et al., 2009; Vonderheide et al., 2002). Methyl-SeCys is found in many Se hyperaccumulator species (Neuhierl & Bock 1996), as well as in broccoli and garlic (Schiavon & Pilon-Smits 2017a). Thus, hemp seeds from seleniferous areas like Colorado have added value owing to their healthy selenocompounds, in addition to the other hemp nutraceuticals, including the essential fatty acids, linoleic acid (Omega-6), alphalinolenic acid (Omega-3) and gamma linoleic acid ("Super" Omega-6) (Erasmus 1989). Whether other hemp organs (flowers, leaves, stems) also accumulate organic forms of Se remains to be investigated. Another important nutrient, K, was found to be enhanced 2-3 fold in Se-supplied plants; other elemental content was not affected by Se. It is not clear what may be the underlying mechanism for the Se-related increase in seed K levels. Perhaps the uptake of K^+ is a response of the plants to the accumulation of Se oxyanions, to balance its overall electric charge; it has been reported before for corn (Pazurkiewicz-Kocot et al., 2003). For consumers, it is valuable to know that Se-fortified hemp seeds may also be K-fortified.

In the controlled selenate-supply experiments, the hemp readily accumulated Se in all its organs tested, up to 1,400 mg kg^{-1} dry weight for seedlings and 200 mg kg^{-1} dry weight for mature plants. There are several explanations for the observation that Se concentrations were higher in seedlings than in mature hemp plants. First, the seedlings were given Se continuously rather than once a week. Second, the seedlings were fed one-fifth Hoagland's nutrient solution while the mature plants received half-strength Hoagland's solution, so there was less competition of selenate uptake by sulfate for the seedlings. Third, the seedlings were supplied with Se directly after germination, when the casparian strip of the root endodermis (barrier for translocation from the root to the shoot) may not yet have been fully developed (Karahara et al., 2004).

In the mature hemp plants, the highest Se concentrations were found in seeds, flowers, and leaves, as compared to stems and roots. Thus, Se was readily remobilized within the plant; this has also been reported for other plant species supplied with selenate (de Souza et al., 1998). Comparison of greenhouse and field hemp data show some differences in this respect. In the field, Se concentration was higher in seeds than in flowers and leaves, while in the greenhouse these levels were comparable, especially at higher levels of Se supply (above 5 μM). This difference may be explained by the difference in Se exposure: in the field the Se supply was likely lower and slower, giving the plant more time to metabolize and remobilize Se to the seed. The plant may preferentially sequester the Se in its seeds, at the expense of other organs, and thus a more limited, slow Se supply (in the field) may lead to relatively more Se ending up in the seed.

Hemp appears to be quite tolerant to Se, based on biomass production, which was not affected up to 40 and 80 μM for mature plants and seedlings, respectively. Selenium actually had a positive effect on seedling growth at 40 μM, a concentration that is quite toxic to many other species (Pilon-Smits et al., 1999; Zhang et al., 2006). A similar response was observed for the mature plants where the 10 μM treatment was on average 45% larger than the control treatment (NS). It is known that Se is a beneficial element for plants, but usually these effects are found at lower tissue levels (<10 ppm). Beneficial effects reported for Se include higher antioxidant levels, which scavenge reactive oxygen species. The observed positive growth response of hemp to Se may mean that the levels of Se found in seleniferous soils may positively affect plant performance. On Se-polluted sites, the Se levels can reach higher levels, but will rarely reach levels that would be toxic to hemp, estimated from these studies.

Plant photosynthetic performance, (as judged by Chlorophyll fluorescence) was not significantly affected by Se up to 80 μM and 160 μM for mature plants and seedlings, respectively. The other metabolic processes measured, production of CBD and terpenoids, were also not significantly affected by Se up to 40 μM. Within each treatment, there was a large variation in terpenoid concentration and profile, which may be due to genetic variability. *Cannabis sativa* is known for its large genetic variation, likely resulting from uncontrolled crossing and mixing due to a lack of controlled breeding programs (Sorni et al., 2017; McPartland 2018). All replicate plants contained humulene, α-pinene and β-caryophyllene, compounds well-known to occur in this species. Thus, Se up to 40 μM does not appear to negatively affect the overall functioning of hemp in terms of productivity and metabolic activities. The finding that CBD and terpenoid levels remained unaffected by Se is important, because of the economic value of these compounds. Cultivation of hemp on soils elevated in Se therefore, likely will not affect the yield of these metabolites.

The finding that hemp is highly tolerant to Se and can accumulate Se to substantial levels in its shoot make hemp a promising candidate for Se phytoremediation, particularly for phytoextraction where pollutants are accumulated and the biomass harvested. On naturally seleniferous Colorado soils at full planting density, the total shoot Se accumulation per hemp crop was estimated in this study to be 266 g Se/ha, and at higher Se supply levels these values may be an order of magnitude higher.

It can be envisioned, for instance, that hemp crops could be irrigated with Se-containing agricultural drainage water or oil refinery waste water, or fertilized with high-Se biosolids from sewage treatment facilities. Additional phytoremediation qualities of hemp are its hardiness, fast growth and high productivity. So, how would hemp compare with other species, in terms of Se phytoremediation? While it is hard to compare species if they are not grown under identical conditions, it is interesting to discuss hemp's potential with that of *Brassica juncea* (Indian mustard), the best Se phytoremediation species known so far (Banuelos & Meek 1990). Based on published data, *B. juncea* may reach higher tissue Se concentrations, but hemp may be more tolerant to Se and may produce more biomass (Pilon-Smits et al., 1999; Prins et al., 2011). Of course, productivity will be influenced by climate, soil, cultivar, and agronomic practices used. Hemp will likely perform better than *B. juncea* under arid and other marginal conditions.

It may be concluded from these field and greenhouse studies that hemp is a promising candidate for Se biofortification and phytoremediation. On naturally seleniferous soils, hemp produces various Se-fortified products (seeds, flowers, leaves) that may be utilized as a source of dietary Se for humans or livestock. This will be particularly applicable in areas where Se deficiency is a problem. Selenium in seeds is present in the forms of SeMet and MetSeCys, which are ideal for Se supplementation and have been reported to have anticarcinogenic properties. When supplied with high levels of selenate, hemp showed remarkable Se tolerance, judged from biomass and photosynthetic parameters, and its levels of valuable cannabinoids and terpenoids were not affected by Se. Thus, hemp may be used for Se phytoremediation while still producing CBD, terpenoids, and Se-biofortified products.

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