THESIS

PHYTOREMEDIATION WITH HEMP (*CANNABIS SATIVA L.*): A LOOK AT HEMP'S POTENTIAL FOR ENVIRONMENTAL CLEANUP AND ECONOMIC RECOVERY

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ABSTRACT

PHYTOREMEDIATION WITH HEMP (*CANNABIS SATIVA L.*): A LOOK AT HEMP'S POTENTIAL FOR ENVIRONMENTAL CLEANUP AND ECONOMIC RECOVERY

The aim of this thesis study was to test hemp's (*Cannabis sativa* L.) potential for phytoremediation (environmental clean-up). I tested hemp for tolerance and accumulation of four inorganic pollutants, to evaluate its remediating performance. Hemp has many properties that would make it a likely candidate for phytoremediation however, due to recent regulations, research of this versatile plant has been limited.

Phytoremediation is a process of cleaning polluted sites using plants. In this clean-up method, plants may stabilize the pollutant in situ, or take-up the pollutant into the plant tissue. In the latter, there are a few different fates for the pollutant that include degradation, metabolization, sequestration, and/or volatilization. Phytoremediation is a clean process that reestablishes an onsite ecosystem and is a competitive alternative to more conventional scrape-and-remove methods.

Hemp is a hardy, fast growing species that produces high biomass. Hemp has deep roots that can be used to reach pollutants deep in the ground. These properties make hemp a potential choice for phytoremediation. Contaminated sites create harsh growing conditions that require hardy plant properties in order for a species to survive. An added benefit to using hemp for remediation is the many economic uses of hemp biomass. Each part of the hemp plant can be used to make goods such as clothing, building material, cosmetics, lotions, animal bedding,

fragrances, and medicinal products that have therapeutic qualities. In addition, hemp seeds are nutritious and can be added to the diet.

In chapter one of this thesis, phytoremediation is reviewed to explain the remediation process. This review includes explaining the different phytotechnologies that are employed by plants which depend on the plant used and the type of pollutant encountered. Chapter one also reviews hemp, its history, biology, and the properties that make it a viable choice for phytoremediation.

Chapter two of this thesis is an experimental chapter presenting data for testing hemp seedlings with four different oxyanions: arsenate (As), molybdate (Mo), vanadate (V), and tungstate (W). The parameters considered were biomass, chlorophyll content, chlorophyll fluorescence, pollutant accumulation levels, and pollutant fate. *Brassica juncea* (Indian mustard) was used as a reference phytoremediation species.

The findings of this thesis study present promising results for hemp as a potential remediator. Arsenic was found to accumulate in the root at levels up to 2700 mg kg^{-1} DW. Tungsten also accumulated in the root at levels up to 3100 mg kg^{-1} DW. In both tests, hemp performed well, judged from photosynthetic measurements and relative chlorophyll content, but reduced biomass started at treatments with 3 and 24 mg As L^{-1} in the shoot and root respectively, and 40 and 80 mg W L^{-1} in the shoot and root, respectively. Molybdenum accumulated in the shoot at levels up to 4900 mg kg^{-1} DW and in the root at levels up to 2600 mg kg^{-1} DW. Biomass reduction of Mo started at treatment with 40 mg Mo L^{-1} for both shoot and root, while photosynthetic measurements and relative chlorophyll content remained unchanged. Lastly, V accumulated in the root at levels up to 2100 mg V kg^{-1} DW. Interestingly, hormesis (stimulated growth) was observed in hemp supplied with V: biomass increased at all tested levels.

From this study, it was concluded that hemp may have potential for phytoremediation in cleaning contaminated sites with the four elements tested. Hemp performed competitively with the popular phytoremediation species, Indian mustard (*Brassica juncea* L.) in all levels tested for Mo, V, and W. Hemp's economic recovery with clean post-harvest biomass may offset phytoremediation costs giving this species a unique advantage over other popular phytoremediation choices.

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CHAPTER 1- PHYTOREMEDIATION, CONTAMINATION, AND HEMP (*CANNABIS SATIVA* L.)

1.1 Introduction

There is a great need for environmental remediation in the world (Salt et al., 1998; Ahmed et al., 2016). Toxic spills are becoming more common and there are many abandoned sites that need to be cleaned up (Kukreja and Goutam, 2012). Phytoremediation is an environmentally sound process that removes contaminants from polluted sites using plants (Salt et al., 1998; Crini et al., 2020; Dietz and Schnoor, 2001). This process removes pollutants noninvasively by establishing plants in the soil to remove the pollutants, rather than by removing the soil, with the pollutants, and stripping the biological as well as physical environment (Salt et al., 1998). Conventional methods may include surface digging and removal, incineration, soil washing, and land farming (Rungwa et al., 2013). These methods all remove or harm the biotic aspects of the site leaving a barren wasteland behind (Kukreja and Goutam, 2012). Using plants to remove different pollutants is necessary in rebuilding the immediate environment and ecology around a spill site as soon as possible (Ahmad et al., 2016; Kristanti et al., 2021). Phytoremediation begins reestablishing the ecosystem by stabilizing the soil and stimulating microbial activity (Kristanti et al., 2021). This helps reestablish the ecosystem around the site and encourages native flora and fauna to return and repopulate the area.

One species of plant that is just beginning to be studied for phytoremediation is hemp (*Cannabis sativa* L.) (Kukreja and Goutam, 2012). Hemp may have potential to phytoremediate pollutants, but this will depend on the elements present and levels of toxicity in the contaminated area (Pilon-Smits, 2005). There has been a lack of funding in hemp research in the past due to the

illegal status of this controversial plant (Dietz and Schnoor, 2001). Hemp has been classified as a schedule one drug for over a century and as a result there has been little research done with hemp (Mead, 2019). This includes restrictions on growing, harvesting, and possession, along with scientific research. Hemp and the drug-producing marijuana, both *Cannabis sativa*, have been restricted because it was assumed that they both can be used recreationally (Mead, 2019). Hemp, however, does not give euphoric results when smoked or ingested, like can be achieved with marijuana (Crini et al., 2020; Baker et al., 2003). This misunderstanding has caused hemp, low in the drug compound THC (Δ^9-tetrahydrocannabinol) and marijuana, high in THC, to fall under the same regulations (Crini et al., 2020). Access to hemp for research is important in gaining an understanding of hemp's ability and performance economically as well as biologically. The hemp plant offers many economical uses ranging from food, personal care products, and medicinal uses to clothing, paints, building material, and biofuels (Crini et al., 2020). The economic products of hemp could offset research funding and likely phytoremediation costs as well (Salt et al, 1998; Dietz and Schnoor, 2001). Hemp is now legal in many countries around the world, or experiencing reduced restrictions, allowing for study and research on this very useful plant.

1.2 Phytoremediation

Phytoremediation, or plant-based remediation, is the process of removing pollutants from contaminated sites using plants (Kristanti et al., 2021). This is done by moving pollutants into plant tissue, holding them in the soil, or metabolizing the pollutants after breaking them down (Dietz and Schnoor, 2001). Phytoremediation works best when the whole ecosystem is considered. This includes looking at the current condition of the site, secondary effects like runoff, future productive uses for the site, and possible changes to the current toxic levels from

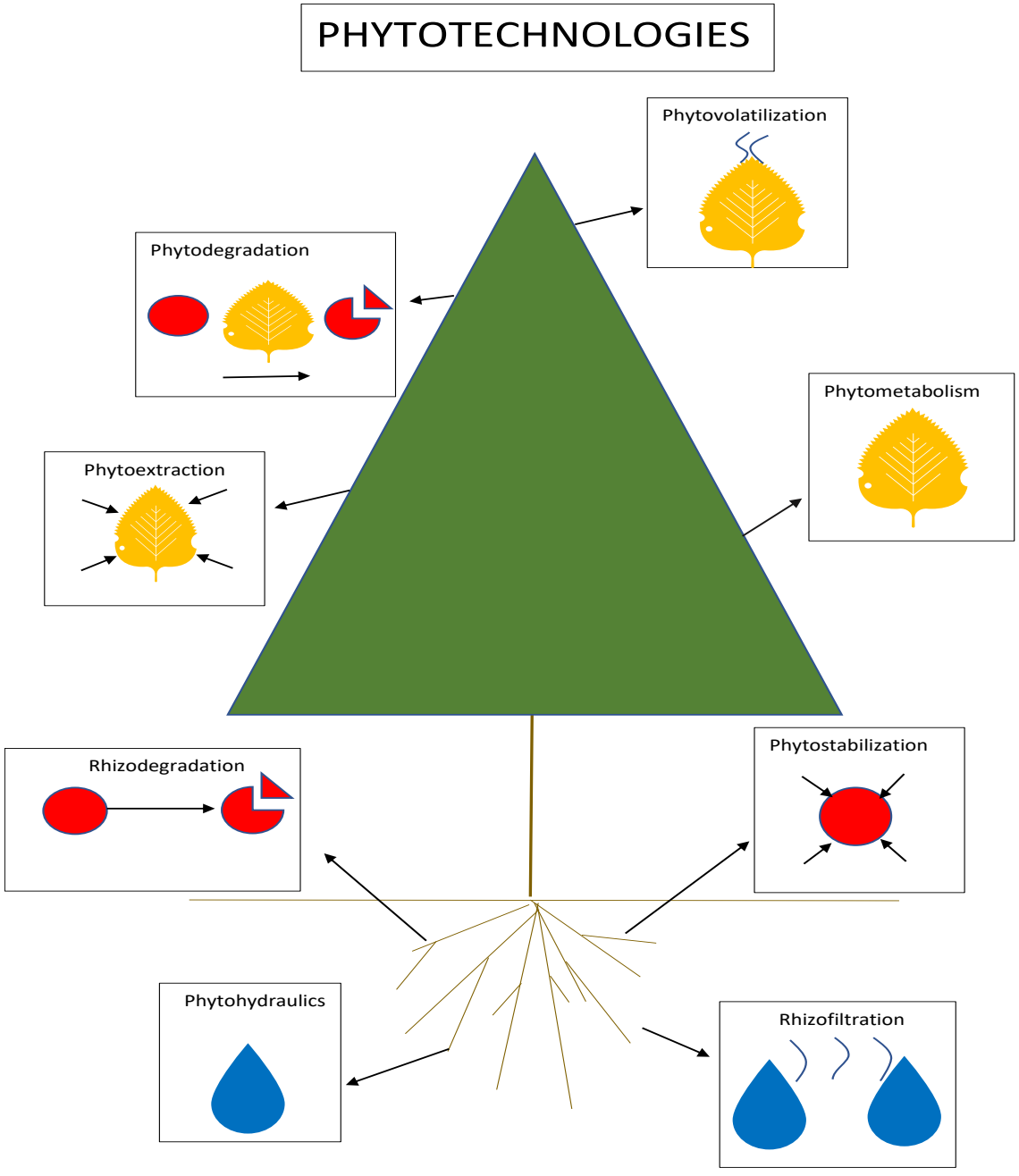

Figure 1.1 Different phytotechnologies used by plants. The utilized method is plant: pollutant specific. Red circles are pollutants. Wavy lines indicate release into the atmosphere. Arrows indicate direction of pollutants. Modified from Kennen and Kirkwood, 2015.

interactions of new species introduced as phytoremediators (Kristanti et al., 2021; Salt et al., 1998). Scientific research under controlled conditions is a common approach to assess these risk factors and determine the best phytoremediation strategy.

1.3 Phytotechnologies

There are many phytotechnologies involved in phytoremediation that process pollutants in different ways (Figure 1) (Dietz and Schnoor, 2001). Phytovolatilization is a process where the plant slowly transpires the pollutant into the atmosphere as a gas, once the pollutant has degraded in the plant (Kukreja and Goutam, 2012; Limmer and Burken, 2016). This happens slowly enough to have little impact on the atmosphere. Phytodegradation is the process of degrading pollutants into smaller non- toxic metabolites (Kukreja and Goutam, 2012). Phytometabolism is a process where the pollutant is metabolized into plant biomass (Dietz and Schnoor, 2001). Phytoextraction is a process where the plant moves the pollutant into its tissue for storage, this may be followed by phytodegradation (Kukreja and Goutam, 2012). Rhizodegradation is a process where roots and microbes near the roots, degrade the pollutants in the rhizosphere (Kukreja and Goutam, 2012). This process turns the pollutants into less harmful elements that can be used by the microbes or released into the atmosphere (Dietz and Schnoor, 2001; Limmer and Burken, 2016). Phytohydraulics is a process where plants pull the contaminated water into the plant through deep roots (Martino et al., 2019). This brings the pollutant into the plant tissue which could then be degraded or volatilized.

Table 1.1 Summary and overview of phytotechnologies. Modified from Kennen and Kirkwood, 2015.

Phytotechnology	**Plant location**	**Pollutant outcome**	**Type of pollutant**	**Disposal**
Phytovolatilization	Leaves	Pollutant removed from soil and released slowly as a gas	Nutrients, Metals Chlorinated solvents, Petroleums	None, released to atmosphere
Phytodegradation	Leaves	Pollutant is broken down into metabolites	Chlorinated solvents, Petroleums, Pesticides, Explosives	None, non-toxic elements
Phytometabolism	Leaves	Pollutant turned into plant biomass	Nutrients, Metals	None, non-toxic molecules
Phytoextraction	Leaves	Pollutant taken up and stored in leaf	Metals, Salts	Burned, Landfill, Smelted
Rhizodegradation	Microbes working in Root exudates	Pollutant is broken down by Microbes	Chlorinated solvents, Petroleums, Pesticides, Explosives	None, may release to atmosphere
Phytohydraulics	Roots	Pollutant pulled into roots with water uptake	Nutrients, Metals, Salts, Chlorinated solvents, Petroleums, Pesticides, Explosives	Burned, Landfill, Smelted
Phytostabilization	Roots	Pollutant is held in place by binding or physical cover	Nutrients, Metals, Salts, Radionuclides, Persistent Organic Pollutants (POPs)	None, remains in place and covered
Rhizofiltration	Roots	Pollutants are filtered out of the water	Nutrients, Metals	Burned, Landfill, Smelted

Phytostabilization is the process of stabilizing pollutants in the soil within the rhizosphere, holding them in place via thicker roots (Kukreja and Goutam, 2012). These pollutants are bound by phytochemicals making them less bioavailable. This prevents them from leaching down into groundwater, stabilizing them in the soil. Rhizofiltration is a process of cleaning contaminated water by filtering the water through roots (Kristanti et al., 2021). The pollutants are then stored in the roots and can be removed from the water for harvest. This process works well in constructed wetlands where the plants grow on the water surface allowing their roots to hang down, filtering the pollutants as the water passes below (Kristanti et al., 2021). There is usually some overlap of these technologies in how plants interact with contaminants where more than one technology occurs. Table 1 shows the most prominent technologies used by plants.

1.4 Pollution sites

Phytoremediation can clean up many different sites where pollutants have damaged the environment (Song et al., 2019). These include mining operations and chemical spills from industrial sites where the pollutants may leach into groundwater and eventually enter local drinking water sources (Kristanti et al., 2021). Phytoremediation can be used on abandoned urban brownfields and agricultural fields with over-applied fertilizers that run-off into streams and collect downstream, creating dead zones (Song et al., 2019). Contaminated sites also result from industry waste, military activities, transportation, agricultural practices, and storage breakdowns. Pollutants from these sources cause harm to humans, as well as the animals and plants in the local environment (Kristanti et al., 2010; Song et al., 2019). Contaminated sites are found in urban and rural locations, as well as streams, ponds, and lakes. Many of these sites are used recreationally, where pollutants create health concerns (Kristanti et al., 2021). Depending on the strategy used, phytoremediation may take longer than conventional methods, sometimes

more than 10 years, and plants must be specifically matched to the pollutant(s) and location (Kukreja and Goutam, 2012; Salt et al., 1998; Song et al., 2019).

1.5 Pollutant types

Inorganic pollutants include nutrients, metals, salts, and radionuclides (Newman and Reynolds, 2004). Organic pollutants include chlorinated solvents, petroleums, pesticides, explosives, and persistent organic pollutants (POPs) (Kristanti et al., 2021). Inorganic pollutants cannot be broken down in plants since they are made of simple elements. These toxins however can be changed into non-toxic forms, volatilized, or stabilized through phytoremediation (Pilon-Smits, 2005; Salt et al., 1998). Nutrients are considered pollutants when they reach high levels, because they leach into waterways causing algal blooms from their high concentrations (Bauddh et al., 2020). This results in dead zones downstream, especially when there are high levels of nitrogen and phosphorous (Bauddh et al., 2020). Metals and metalloids are inorganic toxins that come from mining and industrial sites, agriculture, feed- lots, and landfills. These metals become a problem when they are in high concentrations in the soil or water. In addition to phytoremediation, metals can be phytomined. This is a process of extracting valuable metals from the soil using plants. In this situation, the metals are removed from the plants after harvest through smelting. This method recovers the metals that then can be sold for various purposes (Sheoran et al., 2013). Metals are more bioavailable for uptake by plants when the pH is adjusted (Pilon-Smits, 2005; Salt et al., 1998). This can be accomplished with soil amendments or organic matter added to the soil. Sometimes specific chelators are needed to aid in extraction of metals (Salt et al., 1998). The addition of EDDS chelators increased removal amounts of Pb, Zn, and Cd in hemp (Kos and Lestan, 2004). As accumulation was enhanced with these chelators and Cd and Cu accumulation was enhanced in the root of hemp (Petrova et al., 2012). One study with

hemp found that when it was grown on dredged river sediment it greatly reduced many of the heavy metal concentrations, even though most (95%) of the plants died (Loser et al., 2002). Another type of pollutant, radionuclides, can cause damage to the environment when they leach from nuclear reactors, or buried waste, or build up from military activities (Newman and Reynolds, 2004). These can also move into the ground contaminating groundwater (Kristanti et al., 2021). Organic compounds are another pollutant type that cause damage to the environment (Newman and Reynolds, 2004). Organic compounds can be degraded in plants, making them less toxic. These can be volatilized, metabolized, or stabilized in the soil with phytoremediation. Once broken down, these metabolites can be used for plant growth as they are no longer toxic.

1.6 Plant properties

Good plant properties for phytoremediation include hardy plants that can grow in toxic environments. Many sites require a hardy plant to withstand the harsh conditions created by the pollution on-site. Plants that mature quickly, sometimes within one growing season, have an important advantage in phytoremediation. Mature plants have higher root surface area that translates into more plant: pollutant interactions (Dietz and Schnoor, 2001). Another good characteristic is high biomass production by the plant. Higher biomass provides more tissue for pollutant accumulation (Dietz and Schnoor, 2001). Having deep roots is another good plant property, enabling interactions with pollutants further away from the surface. Deep roots reach greater depths into the soil where some pollutants may be located, like in groundwater (Kristanti et al., 2021). Hemp may be a competitive choice for phytormediation with other common phytoremediating species (Table 2) because hemp has many of the growing properties necessary to make a successful remediator.

Table 1.2 Common phytoremediator species. Modified from Kennen and Kirkwood, 2015.

Pollutant	**Species**
Inorganics Nutrients – N, P	*Typha* (Cattail) *Juncus effusus* (Rush) *Lemna minor* (Duckweed)
Metals/ Metalloids	*Pteris vittata* (Chinese Brake Fern) – As *Brassica juncea* (Indian Mustard) – Cu, Cd, Ni *Stanleya pinnata* (Prince's Plume) - Se
Salts	*Brassica* spp. *Helianthus* spp. *Pinus* spp.
Radionuclides	*Helianthus annuus* (Sunflower) *Solanum tuberosum* (Potato) *Triticum aestivum* (Wheat)
Organics Chlorinated Solvents	*Pinus palustris* (Longleaf pine) *Populus* spp. *Serenoa repens* (Saw Palmetto)
Petroleums	*Avena sativa* (Oat) *Fabaceae* (Legumes) *Salix alba* (White Willow)
Explosives	*Helianthus annuus* (Sunflower) *Myriophyllum aquatica* (Parrotfeather) *Triticum aestivum* (Wheat)
Pesticides	*Lemna minor* (Duckweed) *Ceratophyllum demersum* (Coontail) *Pisum sativum* (Peas)
Persistent Organic Pollutants (POPs)	*Morus rubra* (Red Mulberry) *Rumex crispus* (Curly Dock) *Vicia cracca* (Cow Vetch)

1.7 Hemp history

There is evidence of *Cannabis* use 5000 - 10,000 years ago (Small, 2015). *Cannabis* originated in the area north of Iran and spread to China and Russia. The range expanded and shrunk with the movement of the ice ages (Small, 2015). *Cannabis* was used by our ancestors for many every-day needs. Hunter-gatherers began using *Cannabis* for clothing, food, and medicine. *Cannabis'* range spread as these early people roamed from place to place (Clarke and Watson, 2007; Crini et al., 2020). Eventually *Cannabis* use spread along the silk routes both by land and by sea from China, north to Europe and south to Africa (Small, 2015). The northern land route locations grew *Cannabis* for fiber, while the southern sea route destinations concentrated on growing *Cannabis* for drug use (Small, 2015). *Cannabis* spread further west into North and South America from Europe, Africa, and the Middle East. Northern temperate locations continued to favor *Cannabis* varieties for fiber and seed, while the southern tropical locations selected for medicinal and drug varieties (Small, 2015). Discoveries show *Cannabis* uses for rope, nets, bow strings, clothing, medicine, and food. *Cannabis* continued to be used by civilizations until the late 1800's when new uses were added that included ship rigging, paper, and canvas (Crini et al., 2020). *Cannabis* was a common crop used by colonial Americans who were required to grow it by the British government for uses that supported living in the new American colonies (Small, 2015).

1.8 Hemp biology

Cannabis is one of two genera in the family Cannabaceae; the other being *Humulus* (hops); though other genera are recently thought to be in this family now (Small, 2015; McPartland, 2018) These two genera separated around 21 million years ago, making this an old family (Small, 2015). There are three varieties or two subspecies, depending on the source, in the

Cannabis sativa species: *C. sativa* var. *sativa* (hemp), *C. sativa* var. *indica*, and *C. sativa* var. *ruderalis*, or *C. sativa* subsp. *sativa* and *C. sativa* subsp. *indica* (Small, 2015; McPartland, 2018). *C. sativa* var. *sativa* grows taller, and the leaf has longer more narrow leaflets than *C. sativa* var. *indica*. *C. sativa* var. *ruderalis* is a smaller plant than the other two species growing less than two feet high (Small, 2015). Hemp and marijuana, the common name for recreational *Cannabis*, are legally distinguished by having different levels of the cannabinoid drug Δ^9-tetrahydrocannabinol (THC) (Crini et al., 2020; Baker et al., 2003). Hemp has less than 0.3% THC, resulting in no psychoactive effects when ingested. Marijuana has significantly higher levels of THC (up to 20%), which gives marijuana its mind-altering effects (Crini et al., 2020; Baker et al., 2003). Hemp is an annual, herbaceous plant that grows to maturity in 4 months (Small, 2015). It has deep roots reaching down 3-6 feet. Hemp grows in a variety of habitats, including near rivers and water sources (Clarke and Watson, 2007). Hemp is hardy and produces high biomass. It can be grown in two different growing patterns based on how it is planted (Small, 2015). Hemp can be planted close together where it will produce taller stems and higher fiber biomass. In some cases, hemp can grow up to five meters (16 ft.) tall (Clarke and Watson, 2007). Hemp can also be planted further apart where it will have more branching that will provide more flowers and seeds. This will favor more trichomes that can be found on the leaves, flowers, and buds (Small, 2015). Hemp is naturally pest resistant, so pesticides and fungicides are not usually required (Small and Marcus, 2002). Hemp's low pesticide needs are due to its ability to volatilize the aromatic terpenoids and cannabinoid compounds produced from the trichomes, which ward off pests (Ahmed et al., 2019; Citterio et al., 2003; Linger et al., 2002).

1.9 Economic uses of hemp

1.9.1 Hemp stems

Hemp has many uses that derive from different parts of the plant (Figure 2). Like many ancient crops, each part of the hemp plant can be used to make different products (Small, 2015). There are even more products made when hemp parts are combined. This is true for stem and seed, wood and fiber, and terpene and cannabinoid combinations. Hemp products are renewable, recyclable, and biodegradable, enabling economic off-sets to the cost of phytoremediation (Crini et al., 2020). Hemp stems have 2 parts that are used economically (Small, 2015). The inner harder hurd or wood, and the outer flexible bast or fiber. The hurd is made up of rigid lignin from the xylem tissue and the bast is made up of flexible cellulose from the phloem tissue. The wood is used for making hempcrete (a material similar to concrete), paper, fiber board, and animal bedding (Crini et al., 2020). The fiber is used for making clothing, rope, and insulation. Wood and fiber materials are strong, even when wet. They are lightweight, breathable, insulating, have antimicrobial properties, and block UV light (Crini et al., 2020). Fermented hemp stems are used in making bioethanol. This is a clean biofuel that has the advantage of replacing some fossil fuels in existing systems and can be used in transportation needs (Crini et al., 2020; Kumar et al., 2017).

1.9.2 Hemp seeds

Hemp seeds are edible and used in making both cooking and biofuel oils.

One of these biofuels, biodiesel, is a renewable and clean energy source (Kumar et al., 2017; Crini et al., 2020). Hemp seeds are pressed, ground, or hulled in making different products (Crini et al., 2020). Hemp oil is pressed from the seeds and can be eaten or made into personal care products and creams. Beer, milk, flour, animal feed, and paints are all made from hemp seeds. The seeds are nutritious and offer an alternative nutritional source to the diet (Crini et al., 2020).

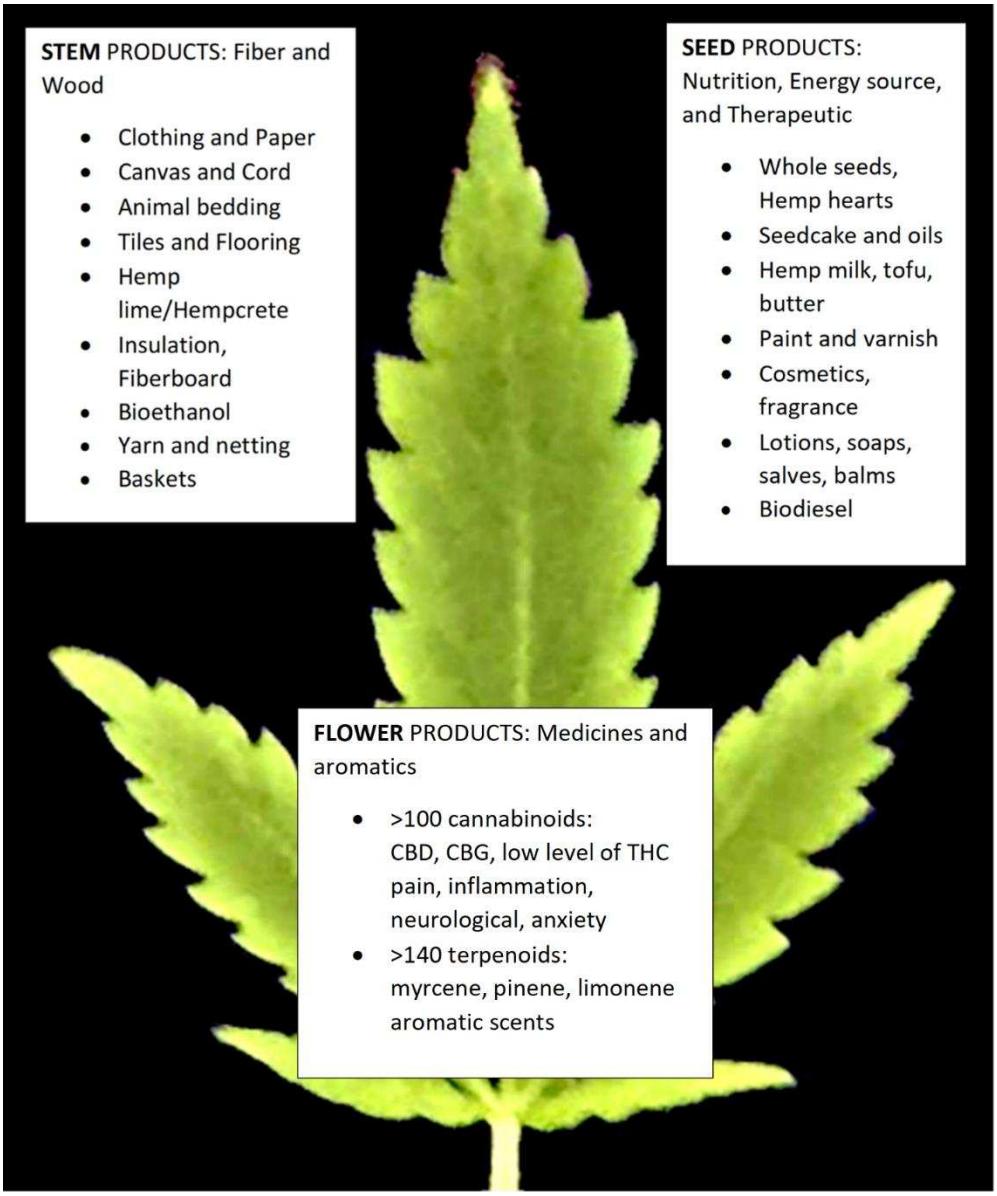

Figure 1.2 Economic products from hemp.

Elements like Se, Co, Zn, Mn, and Fe can build up in the edible parts of hemp like the seeds and the flowers and will fortify a diet (Stonehouse et al., 2019). Hemp seeds are known as superfoods because of their health benefits. The seeds can be eaten whole, boiled, roasted, or ground into other foods. They contain 30% protein, 30% carbohydrates, and 30% oil (Crini et al., 2020). They have a range of minerals like P, K, Mg, S, Ca, Cu, Fe, and Zn and vitamins like Vitamin A,

C, E and many of the B vitamins. Hemp oil is polyunsaturated and contains omega 3, 6, and 9 essential fatty acids for cell membrane health (Crini et al., 2020). These make hemp seeds a viable source of protein and oil which is easy to digest as it is beneficial for sensitive stomachs and reduces inflammation. Hemp seeds (with shell) and hemp hearts (without shell) are both used, depending on the needs. Hemp hearts have much less fiber and are easier to use in some recipes.

1.9.3 Hemp flowers

Hemp roots, leaves, buds, and flowers have glandular trichomes that are full of cannabinoids and terpenoids (Livingston et al., 2020; Andre et al., 2016). The highest density of these trichomes is located on the female flowers (Crini et al., 2020). There are over 100 cannabinoids and more than 140 terpenoids in hemp (Small, 2015). Hemp terpenoids include pinene (pine), borneol (mint), limonene (lemon), and myrcene (citrus and cloves). Myrcene is the compound responsible for hemp's (and hops') familiar smell (Andre et al., 2016). Cannabinoids derive from Acetyl Co-A in respiration where they are synthesized in the Mevolonate pathway (Andre et al., 2016). Geranyl diphosphate (GPP) and Olivetolic acid (OA) combine to form Cannabigerolic acid (CBGA) via CBG synthase. With the loss of the COOH group, CBG becomes the precursor to Cannabidiol acid (CBDA), Δ^9-tetrahydrocannabinolic acid (THCA), and Cannabichromonic acid (CBCA) resulting in CBD, THC, and CBC compounds after they also lose their COOH groups (Salentijn et al., 2015). THC and CBD compounds have been researched more than the others and are known for their mind altering and medicinal properties. Hemp and marijuana produce these two compounds in a ratio where hemp has high CBD:THC ratio (Crini et al., 2020). This higher CBD ratio offers therapeutic benefits without a mind altering high. Marijuana produces the opposite ratio with higher THC and lower CBD. Higher levels of THC will

produce a recreational high with use. THC gives intoxicating results when ingested, smoked, or placed under the tongue as a tincture (Small, 2015). Both terpenoids and cannabinoids can be purified or used as crude extracts. These compounds may be combined giving added benefits of therapeutic synergism known as the entourage effect, but further research is needed to understand these benefits (Russo, 2011).

1.10 Hemp's performance

Hemp has shown phytoremediation potential with many different pollutants. (Stonehouse et al., 2019; Hoseini et al., 2012; Ahmad et al., 2016; Iqbal et al., 2018). This means that hemp may be able to accumulate pollutants in one area while repurposing another part of the plant. Hemp growing on soil contaminated with cadmium, nickel, and chromium, showed no reduction in biomass (Citterio et al., 2003). Copper has been found to accumulate in the upper leaf epidermis and trichomes, leaving the bast available for future wood uses (Arru et al., 2004). Hemp studies with nickel (Ni), lead (Pb), and cadmium (Cd) found the highest toxin accumulations in the leaves (Linger et al., 2002,). There were no effects on the quality of the fiber so it could potentially be used in industry. Hemp selenium (Se) studies found that hemp seeds are a good source of dietary Se, at the recommended serving size of 30 g. Additionally, there was a healthy Se concentration in hemp beer, providing 25% of daily required selenium per bottle (Stonehouse et al., 2020).

1.11 Conclusions and scope of this thesis

The review above summarizes the properties of hemp and its potential for phytoremediation. As presented, hemp has many of the qualities necessary for successful remediation: it attains high biomass, is fast growing, has deep roots, has no need for pesticides and is hardy in a wide variety of climates and conditions. These properties make hemp a good candidate to study for

phytoremediation because they are expected to maximize plant growth and performance while growing in disturbed, polluted areas. Hemp's properties would favor its use in phytoextraction, phytodegradation, phytometabolism, phytovolatilization, phytohydraulics, and rhizodegradation. Due to the legal holdings on hemp, funds have been limited for research on hemp, including phytoremediation research. In the limited studies so far, hemp has shown promise as a promising phytoremediation species, on par with other popular phytoremediation species. Phytoremediation success depends on the specific interaction between the plant species and the pollutant, which supports a need for continued research on hemp's phytoremediating abilities for different pollutants.

The objective of the project described in this master's thesis was to further investigate hemp's potential to remediate different elements, specifically arsenic (As), molybdenum (Mo), vanadium (V) and tungsten (W). Chapter 2 of this thesis describes how oxyanion forms of these elements were supplied to hemp seedlings under controlled conditions, followed by measurements of plant tolerance, overall performance (biomass, Chlorophyll fluorescence) and root and shoot accumulation of the toxic element. It is hoped that this research will help elucidate hemp's potential to aid in removing spills and restoring polluted sites. We need to recapture the many uses of hemp that our ancestors knew only too well, as hemp was part of their everyday life and could therefore benefit our modern lives. There are many different known uses for each of the plant's organs as well as many of its produced compounds but using hemp for phytoremediation is less well known or studied. With knowledge from studies like these, in the future hemp may be used to restore contaminated areas or degraded land while producing valuable products that offset the costs.

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CHAPTER 2- THE POTENTIAL OF HEMP (*CANNABIS SATIVA* L.) FOR PHYTOREMEDIATION OF FOUR OXYANIONS: ARSENATE (As), MOLYBDATE (Mo), VANADATE (V), AND TUNGSTATE (W)

2.1 Introduction

Phytoremediation, a process of removing pollutants using plants and their interacting microbes, is an environmentally friendly way of reestablishing an ecosystem in a damaged area (Pilon-Smits, 2005; Dietz and Schnoor, 2001). This process encourages a natural recovery of the ecosystem and is a cost-effective alternative to traditional excavation and soil washing methods (Salt et al., 1998; Manzoni et al., 2011). There are several phytotechnologies used in phytoremediation. In root-associated soil, pollutants can be stabilized or degraded by microbes in processes called rhizostabilization or rhizodegradation (El Aafi et al., 2012; Kukreja and Goutam, 2012). Pollutants in water can be filtered out by roots in rhizofiltration or taken up with water in phytohydraulics (Kristanti et al., 2021; Pilon-Smits, 2005; Martino et al., 2019). Once pollutants are taken up into the plant, a process known as phytoextraction, they may be stored in the vacuole, volatilized through the leaf stomata or stem lenticels in phytovolatilization, degraded to smaller compounds in phytodegradation and metabolized into biomass in phytometabolism (Tack and Meers, 2010; Newman and Reynolds, 2004; Reinhold et al., 2011; Limmer and Burken, 2016). Inorganic pollutants can be stabilized or extracted. In the latter case they are often stored in the vacuole or cell walls, and in rare cases volatilized (Dietz and Schnoor, 2001; Pilon-Smits, 2005; Kukreja and Goutam, 2012). Organic pollutants may be stabilized or degraded in the rhizosphere or extracted and subsequently degraded, volatilized, or metabolized into biomass (Rungwa et al., 2013; Dietz and Schnoor, 2001). Disposal, if needed,

of contaminated biomass may involve burning or burying; if it contains valuable metals these may be recovered through smelting, a process called phytomining (Brooks et al., 1998; Sheoran et al., 2013).

The best plant species for phytoremediation show high tolerance to the pollutant and depending on the phytotechnology have high accumulation or good degradation rate (Abdelsalam et al., 2019; Kukreja and Goutam, 2012). These plants should also be hardy, fast growing, produce high biomass, and have a deep and elaborate root system; furthermore, if phytoremediation species have economic value, this may help offset the remediation costs (Pilon-Smits, 2005; Dietz and Schnoor, 2001; Kukreja and Goutam, 2012). Species most popular for phytoremediation so far include poplar (*Populus sp*.), Indian mustard (*Brassica juncea*), cattail (*Typha sp*.) and various deep-rooting prairiegrasses (Burken and Schnoor, 1999; Kukreja and Goutam, 2012; Kumar et al., 2015).

A relatively new species being tested for phytoremediation is hemp (*Cannabis sativa* L.). Hemp is an herbaceous annual crop. It is a hardy, fast growing plant that produces high biomass and deep roots, attractive for phytoremediation. Hemp has a woody stem and may reach a height of five meters in four months (Small, 2015; Kumar et al., 2015). Hemp has been cultivated for more than 10,000 years and has countless economic uses that derive from all parts of the plant (Small, 2015; Kumar et al., 2015). Tall cultivars are grown for the stems, producing bast fiber for textile, rope, and paper; the woody core also has several uses in building materials (Small, 2015; Kumar et al., 2015). Other, less tall and more branched cultivars are grown for seeds (food, oil) or for flowers (cannabinoids, terpenoids) (Small, 2015; Aluko, 2017; Clarke and Watson, 2007). Hemp seeds have high nutritional value and the oils are used for personal care products, varnish and biofuels. Hemp flowers contain cannabinoid and terpenoid compounds that are used for

medicinal and recreational purposes and have antimicrobial properties (Kumar et al., 2015; Small, 2015; Clarke and Watson, 2007). Other products include phytochemicals and compost from the roots, and animal feed or bedding from the leaves. The high economic value of recoverable hemp biomass may offset the cost for phytoremediation, an area traditionally limited in funding (Crini et al., 2020; Mihoc et al., 2012). Other benefits of hemp are that it does not have a need for pesticides, it is considered to improve soil quality, and it has many cultivars that are adapted to grow under very diverse conditions worldwide (Kumar et al., 2015; van der Werf and Turunen, 2008).

Several studies have already been carried out to test hemp's phytoremediation potential. Hemp has been reported to show high tolerance for polycyclic aromatic hydrocarbons (PAHs), significantly reducing their concentration in soil (Linger et al., 2002). Furthermore, hemp accumulated per- and polyfluoroalkyl substances (PFAS) from firefighting foam (Nason et al., 2021). Hemp also accumulated several metals to some extent, including cadmium (Cd), copper (Cu), chromium (Cr), nickel (Ni), lead (Pb), and zinc (Zn) (Campbell et al., 2002; Citterio et al., 2003; Meers et al., 2005; Ahmad et al., 2016).

In a previous study (Stonehouse et al., 2020) we have investigated hemp's capacity for selenium (Se) uptake, metabolism and tolerance, and the implications for phytoremediation and biofortification. Hemp was found to be highly tolerant to selenate and to accumulate Se to high levels in all organs of the plant. Hemp seeds from naturally seleniferous soils were found to contain healthy organic selenocompounds, making these a good source for dietary Se. In the study presented here, we tested hemp's capacity, along with reference species Indian mustard for tolerance and accumulation in seedlings of several other oxyanion pollutants: arsenate, molybdate, vanadate and tungstate. Because most contaminated sites contain more than one

pollutant, it is important to investigate multiple pollutants for uptake and accumulation. All these pollutants are found naturally in the earth's crust (Taylor and Mclennan, 1985; Teng et al., 2011; Senesi et al., 1988), and molybdenum (M) is also used in plants and animals as a trace element (Vyskocil and Viau, 1999). Anthropogenic activity increases the naturally occurring levels of these elements. In many cases, these high levels cause pollution in the local environment resulting from overuse or spills. As and V enter the environment through mining operations, fossil fuel burning and fertilizer applications (Tripathi et al., 2006; Gen et al., 2020). W is used in industrial process such as welding and lighting, and lines ammunition cases used in the military (Senesi et al., 1988; Sadiq et al., 1992). Mo is used in mining where it leaches through the ground, contaminating drinking water (Vyskocil and Viau, 1999). Toxic levels of these elements contaminate the environment due to poor management practices, inadequate storage, and neglect. Previous research with transgenic Indian mustard has shown promising phytoextraction capacities with these metals where overexpression of the enzyme ATP sulfurylase was found to increase tolerance of As and decrease tolerance of Mo and V in seedlings. Another study showed that overexpression in *B. juncea* of a sulfate transporter gene (*Stylosanthes hamata* SHST1) increased root accumulation of V and W and decreased tolerance in Mo and V (Wangeline et al., 2004; Lindblom et al., 2006). This current study aims to leads to a better understanding of hemp's potential for phytoremediation of these elements and form a foundation for future phytoremediation research.

2.2 Methods and Materials

2.2.1 Arsenic experiment

Cannabis sativa L. (hemp) seeds obtained from Colorado Cultivars Company (Eaton, CO), variety "Workhorse", were dark- germinated between Whatman 1 filter paper wetted with

deionized water in taped petri dishes for 5 days. The germinating seeds were then transferred into an inert clay growth medium, Turface®, in 5 x 5 cm pots and cultivated under a 8 light: 16 dark photoperiod at 709 mE photosynthetically active radiation (PAR), 28°C and 32% Relative Humidity. The seedlings were covered initially to maintain moisture. Treatments consisted of 1/5th Hoaglands's nutrient solution (Hoagland and Arnon, 1938) with 1 of 5 different arsenic (As) concentrations (0, 3, 6, 12, and 24 mg L^{-1} Na_2AsO_4) added, throughout the experiment. Each treatment was given to 12 replicate plants. The plants were harvested after 29 days.

2.2.1.1 Photosynthetic measurements

Just prior to harvest, photosynthetic measurements were taken from the hemp plants. Photosynq multispeq (Kuhlgert et al., 2016) a hand-held apparatus that can obtain phenotypic and environmental measurements non-invasively, was used to take measurements from the plants. These measurements included relative chlorophyll content (leaf greenness), Phi2 (% of absorbed light going to photochemistry) , and PhiNPQ (% of absorbed light going to non-photochemistry, heat) taken from the plant leaves. Light intensity (PAR), ambient temperature (°C), and relative humidity (%) were also measured at this time, n=5.

2.2.1.2 Tolerance measurements

After harvest, hemp shoots and roots were separated. The roots were washed twice in tap water and both shoots and roots were dried at 50 °C for 3 days.

Plant tissue was weighed, and dry weights (mg) were recorded, n=5 for 0-12 mg As L^{-1}, n=3 for 24 mg As L^{-1}.

2.2.1.3 Accumulation measurements

For elemental analysis, dried hemp shoot and root samples were weighed and transferred to glass acid digestion tubes (25 mm diameter, 30 cm length) with 100 mg per sample. Each tube

received 1 mL of trace-element grade nitric acid (HNO_3), and the tubes were covered with a glass funnel (40 mm diameter). The samples were digested for 2 hours at 60 °C, and then for 6 hours at 130 °C (Zarcinas et al., 1987). The resulting digests were diluted up to 10 mL with ultrapure water. Samples were analyzed by inductively coupled plasma – optical emission spectrometry (ICP-OES) for elemental composition. Concentrations of As were recorded for each sample, n=5.

2.2.2 Metals Experiment

Cannabis sativa L. (hemp) seeds from variety "Workhorse", were germinated and transferred to Turface® in 5 x 5 cm pots as described above. *Brassica juncea* L. (Indian mustard) seeds, accession no. 173874 from the North Central Regional Plant Introduction Station (Ames, IA) were sown directly into Turface®. *B. juncea* was chosen as a reference species in this experiment because it is often used for phytoremediation of inorganic pollutants. The plants were grown under controlled conditions with a 16 light: 8 dark photoperiod in a growth room that had an ambient temperature of 27 °C and 31% Relative Humidity. Both species were grown under ceiling lights (201 PAR). Plants were covered, but vented, the entire experiment. Treatments consisted of $1/5^{th}$ Hoaglands's nutrient solution (Hoagland and Arnon, 1938) with 1 of 4 different elemental concentrations added, depending on the species. The three different elements tested were molybdenum (Mo), vanadium (V), and tungsten (W). In hemp, the concentrations were (0, 40, 80, and 120 mg L^{-1} Na_2MoO_4), (0, 2, 4, and 8 mg L^{-1} Na_2VO_4), and (0, 40, 80, and 120 mg L^{-1} Na_2WO_4). In each of these concentration treatments, the middle concentration was estimated to give a 50% reduction in growth for the *B. juncea* reference species, based on earlier experiments (Lindblom et al., 2006). Thus, the upper and lower concentrations may help identify limits of hemp in these elements. In *B. juncea*, the

concentrations were (0 and 80 mg L^{-1} Na_2MoO_4), (0 and 4 mg L^{-1} Na_2VO_4), and (0 and 80 mg L^{-1} Na_2WO_4). *B. juncea* was only evaluated at the middle concentrations due to the expected 50% reduction in growth there. This measurement then provided a good reference in the control species for hemp. Treatments of 500 mL were given 1-3 days apart. Each treatment was given to 12 replicate plants. Plants were harvested after 23 days.

2.2.2.1 Photosynthetic measurements

Photosynthetic measurements were taken from hemp and *B. juncea* plants as described above, n=3 for Mo, n=5 for V, W.

2.2.2.2 Tolerance measurements

After harvest, hemp and *B. juncea* shoots and roots were dried and the plant tissue weighed as described above, n=5.

2.2.2.3 Accumulation measurements

For elemental analysis, hemp and *B. juncea* shoot and root dry biomass samples (100 mg, n=5) were weighed and acid-digested as described above, and the concentrations of elements of interest (Mo, V, W) recorded.

2.3 Statistical Analysis

Data were analyzed with Real Statistics Using Excel©. Single factor analysis of variance (ANOVA) with Tukey's post- hoc test was used with multiple means to identify significant differences

($p < 0.05$).

2.4 Results

In aiming to understand how *Cannabis sativa* L. (hemp) tolerates and accumulates toxic elements, hemp plants were grown from seed for four weeks in the presence of different

concentrations of As, Mo, V and W, and photosynthetic performance, dry weight production and shoot and root tissue concentrations were determined.

2.4.1 Arsenic experiment

2.4.1.1 Photosynthetic performance with and without arsenic

Photosynthetic measurements were collected before the plants were harvested, while they were actively photosynthesizing. The relative chlorophyll content showed a decreasing trend with increasing As treatment above 3 mg L^{-1}, leading to a significant decrease at 24 mg L^{-1} (Fig. 2A). The fraction of absorbed light energy channeled toward photochemistry (photochemical quenching, Phi2) showed a slight decreasing trend with increasing As treatment, while the fraction of absorbed light energy used for photoprotection (non-photochemical quenching, PhiNPQ) showed a slight trend to increase with As treatment, although neither parameter changed significantly (Fig 2B and C).

2.4.1.2 Arsenic tolerance

Seedlings of hemp showed significant reduction in shoot biomass at 3 mg L^{-1} arsenic acid and higher concentrations (Fig. 1A). At 3 mg L^{-1} the biomass was 2-fold lower, compared to control conditions. There was no further significant reduction in shoot biomass from 3- 24mg L^{-1}, although biomass continued to reduce down to 4-fold with increasing As concentration (Fig. 1A). For the root, biomass also reduced with increasing As concentration, but was significantly reduced (5-fold) only for the 24 mg L^{-1} treatment. Thus, hemp seedlings could survive arsenate concentrations up to 24 mg L^{-1} but clearly were impacted by As already at the lowest concentration tested, 3 mg L^{-1}, producing no more than half of the control treatment's biomass.

2.4.1.3 Arsenic accumulation

After treatment with arsenic acid, the roots of the hemp seedlings contained much higher As concentrations than the shoot (Fig. 1B). Strikingly, none of the As treatments' As shoot concentrations were even significantly different from the control. In contrast, the As concentration in the root increased dramatically with As supply, up to 2700 mg kg^{-1} DW. These data clearly show that hemp plants exposed to arsenate accumulate As almost entirely in or on the root. Overall, these data indicate that the hemp plants' photosynthesis was coping well up to 3 mg L^{-1} As by adjusting photosynthetically, but experienced increasing toxicity from the As at and upwards of 6 mg L^{-1} As (above root and shoot As concentrations of 2700 and 50 mg kg^{-1} DW, respectively).

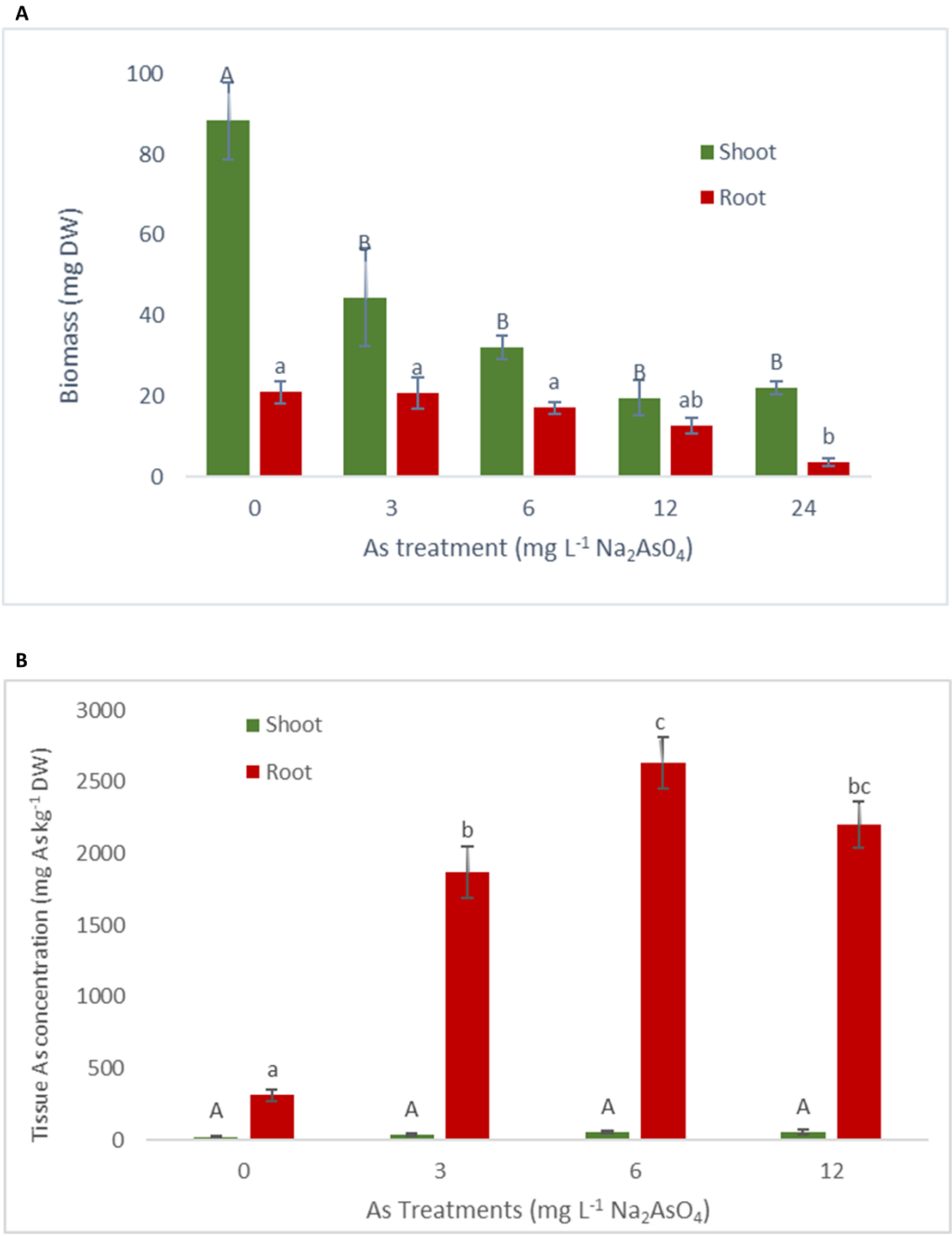

Figure 2.1 Arsenic tolerance and accumulation by *C. sativa* seedlings grown for 29 days on 5 different concentrations (0- 24 mg L^{-1}) of arsenic acid. (A) Biomass of shoot and root. (B) Tissue As accumulation in shoot and root. Shoot accumulations were 22, 35, 54, and 55 mg kg^{-1} respectively for 0-12 mg L^{-1} . No data for 24 mg L^{-1} for lack of sufficient material. Letters above bars denote statistically significant differences for shoot (capital) and root (lowercase). Shown are means and SEM. ANOVA with Tukey-Kramer, α =0.05, n=5 for 0-12 mg L^{-1} and n=3 for 24 mg L^{-1} biomass, n=5 for accumulation.

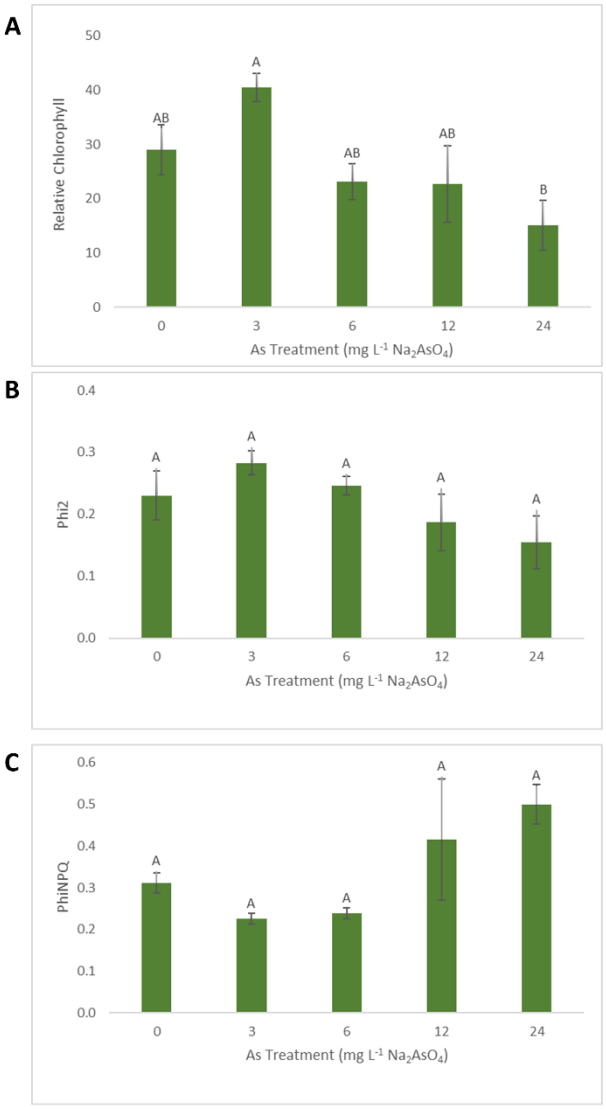

Figure 2.2 Photosynthetic performance of *C. sativa* seedlings grown for 29 days on 5 different concentrations (0-24 mg L^{-1}) of arsenic acid. (A) Leaf relative chlorophyll content. (B) Leaf Phi2 which is the fraction of absorbed light energy photochemically quenched from electrons going through photosystem 2. (C) PhiNPQ which is the fraction of absorbed light energy non-photochemically quenched and released as heat. Letters above bars denote statistically significant differences between As treatments. Shown are means and SEM. ANOVA with Tukey-Kramer, $\alpha = 0.05$, n=5.

2.4.2 Metals Experiment

2.4.2.1 Photosynthetic performance with and without molybdenum

The relative chlorophyll content in hemp was 4-fold lower at the 80 mg Mo L^{-1} treatment compared to control conditions (Fig. 4A). In Indian mustard, this parameter decreased by only 2-fold, but the two species did not differ significantly from each other at either concentration (Fig. 4A). In hemp, there was a slight trend (NS) for Mo treatment to result in an increase in the fraction of light energy channeled toward photochemistry (Phi2); there were no changes in the fraction of light energy used for photoprotection (PhiNPQ). *B. juncea* showed no difference in Phi2 between Mo and control conditions; its PhiNPQ measurement was on average higher for the Mo treated plants, but not significantly so (Fig. 4B and C). Thus, judged from these data both species appeared to have lost chlorophyll significantly, but their photochemistry was not significantly impacted by these Mo treatments.

2.4.2.2 Molybdenum tolerance

Plant productivity decreased as Mo treatment concentrations increased for both plant species (Fig. 3A). Compared to the control treatment, hemp shoot biomass was reduced by 2-fold at 40 mg Mo L^{-1} and 4-fold at 80 mg Mo L^{-1}. The reference species, *B. juncea* (Indian mustard) had a even greater reduction, namely 10-fold at 80 mg Mo L^{-1}. Hemp root biomass also decreased significantly (4-fold) at 40 mg Mo L^{-1}, and slightly more at 80 mg Mo L^{-1}. In comparison, *B. juncea* decreased 44-fold at 80 mg Mo L^{-1}. Plant biomass did not significantly differ between the two plant species, both under control conditions and in the presence of 80 mg Mo L^{-1}. From these data it appears that this hemp cultivar is at least as resistant to Mo as this Indian mustard, and similar in overall productivity.

2.4.2.3 Molybdenum accumulation

Hemp Mo accumulation was high in both shoot and root for both species and did not differ significantly between the species (Fig. 3B). In hemp, the shoot Mo concentration increased with Mo treatment up to 4900 mg Mo kg^{-1} DW for the 80 mg Mo L^{-1} treatment; no further increase was observed at the higher Mo concentration treatment. In roots, the Mo levels were around 2600 mg Mo kg^{-1} DW for each of the three Mo treatments. Indian mustard had equal Mo levels in shoot and root, approaching 4400 mg Mo kg^{-1} DW for the 80 mg Mo L^{-1} treatment in the shoot and 4700 mg Mo kg^{-1} DW for the 80 mg Mo L^{-1} treatment in the root. Thus, it appears that this hemp cultivar is comparable to Indian mustard in its capacity to accumulate Mo from molybdate and to translocate Mo to their shoot and root. Both species can concentrate extremely high Mo levels in both shoots and roots, and thus appear to be quite tolerant to Mo.

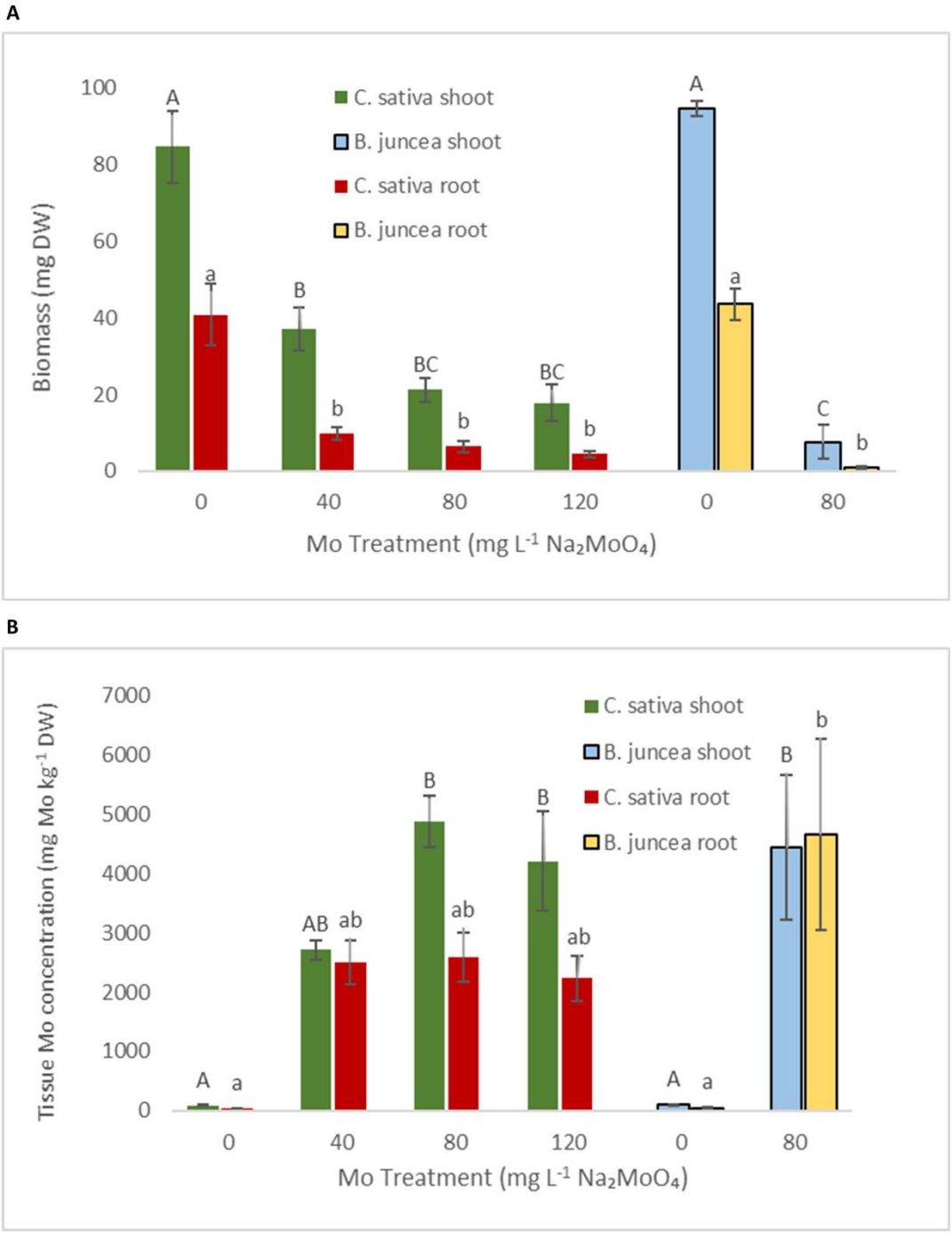

Figure 2.3 Molybdenum tolerance and accumulation by *C. sativa* and *B. juncea* seedlings grown for 23 days on different concentrations (0-120 mg L^{-1}) of sodium molybdate. (A) Biomass of shoot and root. (B) Tissue accumulation of shoot and root. *C. sativa* control shoot and root accumulations were 83 and 38 mg kg^{-1} respectively. *B. juncea* control shoot and root accumulations were 98 and 46 mg kg^{-1}. Letters above bars denote statistically significant differences for shoot (capital) and root (lowercase). Shown are means and SEM. ANOVA with Tukey-Kramer, α =0.05, n=5.

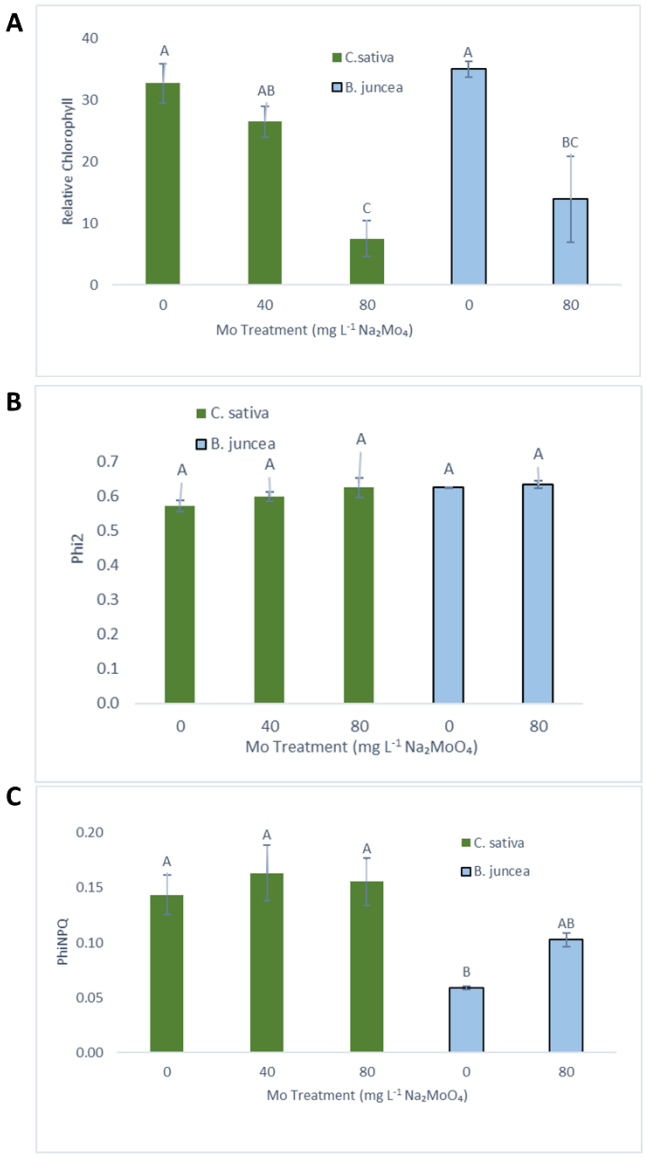

Figure 2.4 Photosynthetic performance of *C. sativa* and *B. juncea* seedlings grown for 23 days on different concentrations (0-120 mg L^{-1}) of sodium molybdate. (A) Leaf relative chlorophyll content. (B) Leaf Phi2, which is the fraction of absorbed light energy photochemically quenched (C) Leaf PhiNPQ, which is the fraction of absorbed light energy non-photochemically quenched. Letters above bars denote statistically significant differences for Mo concentrations. Shown are means and SEM. ANOVA with Tukey-Kramer, α =0.05, n=3. Note: No data could be obtained for 120 mg L^{-1}.

2.4.2.4 Photosynthetic performance with and without vanadium

Photosynthetic measurements with and without V were not significantly different for any of the parameters tested for hemp (Fig 6A-C). *B. juncea* showed one significant difference: a decrease in non-photochemically quenched light energy with V. Overall, there is no indication from these data that photosynthesis was much affected by these V treatments.

2.4.2.5 Vanadium tolerance

Hemp shoot and root biomass increased with increasing vanadate concentration treatment and was significantly higher than the control treatment at 8 mg V L^{-1} for shoot (2-fold) and root (5-fold) (Fig. 5A). For Indian mustard the results were similar to those for hemp (Fig. 5A). Thus, it appears that at the concentrations tested, vanadate was not toxic to the two species, but this non-essential element actually had a positive physiological effect (hormesis).

2.4.2.6 Vanadium accumulation

Hemp shoot V concentration showed no significant differences between the treatments, but there was a general trend for it to increase with increasing V supply (Fig. 5B). Hemp root accumulation, however, increased significantly with V supply, reaching approximately 2100 mg V kg^{-1} DW (Fig. 5B). Indian mustard shoot and root V concentrations were not significantly different from those in hemp (Fig. 5B), higher for 4 mg L^{-1}. Thus, both hemp and Indian mustard accumulated V predominantly in or on the root.

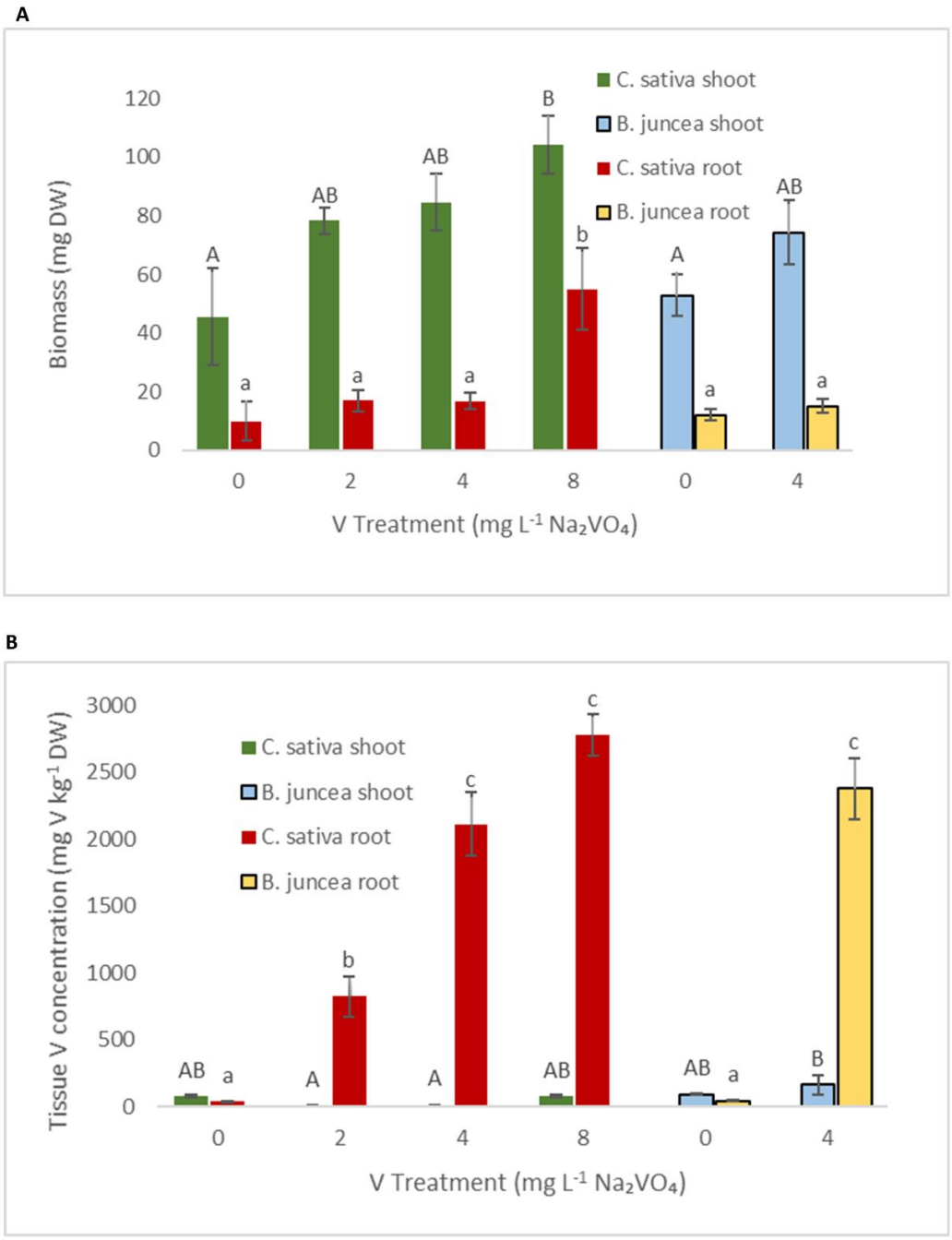

Figure 2.5 Vanadium tolerance and accumulation for *C. sativa* and *B. juncea* seedlings grown for 23 days on different concentrations (0-8 mg L^{-1}) of sodium vanadate. (A) Shoot and root biomass. (B) Tissue accumulation of shoot and root. *C. sativa* shoot accumulations were 83, 11, 16, and 83 mg kg^{-1} for 0-8 mg L^{-1} respectively. *C. sativa* root accumulation was 38 mg kg^{-1} for the control. *B. juncea* shoot accumulations were 95 and 164 mg kg^{-1} for 0 and 4 mg L^{-1}. *B. juncea* root accumulation was 46 mg kg^{-1} for the control. Letters above bars denote statistically significant differences for shoot (capital) and root (lowercase). Shown are means and SEM. ANOVA with Tukey-Kramer, α =0.05, n=5.

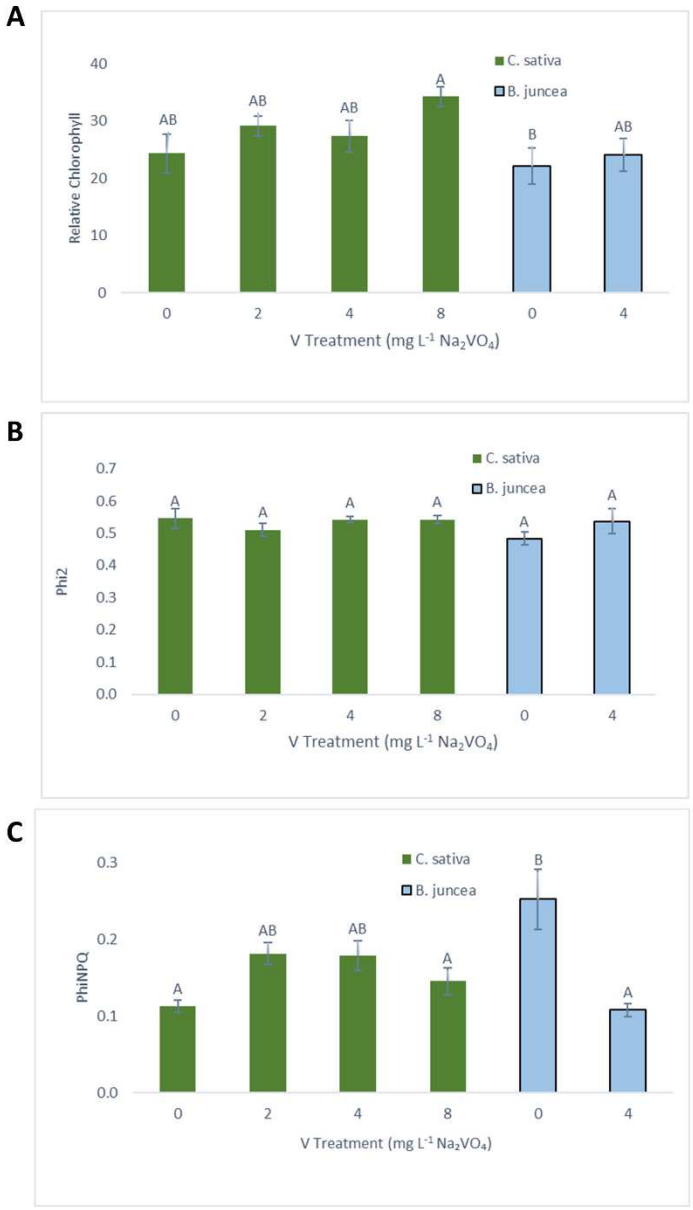

Figure 2.6 Photosynthetic performance of *C. sativa* and *B. juncea* seedlings grown for 23 days on different concentrations (0-8 mg L^{-1}) of sodium vanadate. (A) Leaf relative chlorophyll content. (B) Leaf Phi2, i.e. the fraction of absorbed light energy photochemically quenched. (C) Leaf PhiNPQ, i.e. the fraction of absorbed light energy non-photochemically quenched. Letters above bars denote statistically significant differences for V concentrations. Shown are means and SEM. ANOVA with Tukey-Kramer, $\alpha = 0.05$, n=5.

2.4.2.7 Photosynthetic performance with and without tungsten

Photosynthetic performance measurements showed that relative chlorophyll content remained almost unchanged up to 80 mg W L^{-1} for both plant species but decreased sharply at 120 mg W L^{-1} in hemp (Fig. 8A). Photochemical quenching and non-photochemical quenching did not change significantly for either species at any of the concentrations (Fig. 8B, C). It is interesting to point out, however, that the trend was opposite for the two species: in hemp, the fraction of energy corresponding with photochemical quenching went down with increasing W treatment and that corresponding with non-photochemical quenching went up; in Indian mustard these trends were opposite. Overall, these photosynthetic data indicate there was little effect of W up to 80 mg L^{-1} on photosynthesis in hemp and Indian mustard, corresponding with shoot and root concentrations of 200-300 mg W kg^{-1} DW and 3100 mg W kg^{-1} DW, respectively, suggesting that both species were quite W tolerant. However, further increasing W concentration to 120 mg L^{-1} clearly led to toxicity in the only species tested i.e., hemp.

2.4.2.8 Tungsten tolerance

The shoot biomass of hemp decreased significantly by W treatments: by 2-fold at 40 mg W L^{-1} and 16-fold at 120 mg W L^{-1}; root biomass for hemp also decreased significantly, by 2-fold at 80 mg W L^{-1} (Fig. 7A). There were no significant differences between the two plant species in root and shoot biomass with or without W (Fig. 7A). Indian mustard shoot biomass was only slightly affected by 80 mg W L^{-1} (NS), but root biomass was significantly lower (2-fold). From these data it appears that this hemp cultivar is similar or slightly less resistant to W than Indian mustard. Furthermore, tungsten appears to be less toxic than Mo and As, leading to a 2-fold reduction in shoot biomass when treated with 80-100 mg W L^{-1}.

2.4.2.9 Tungsten accumulation

Shoot W accumulation was at maximum around 200 mg W kg^{-1} DW in hemp and 300 mg W kg^{-1} DW in Indian mustard (Fig. 7B). Root accumulation was much higher in both species, around 3000 mg W kg^{-1} DW for the 80 mg W L^{-1} treatment (Fig. 7B). Further increasing the W concentration in the growth medium to 120 mg W L^{-1} did not lead to a further increase in tissue concentration. There were no significant differences between the plant species in root or shoot W concentration. Overall, these data show that hemp and Indian mustard plants exposed to tungstate accumulate W predominantly in or on the root, and that hemp is comparable in its W accumulation properties to Indian mustard.

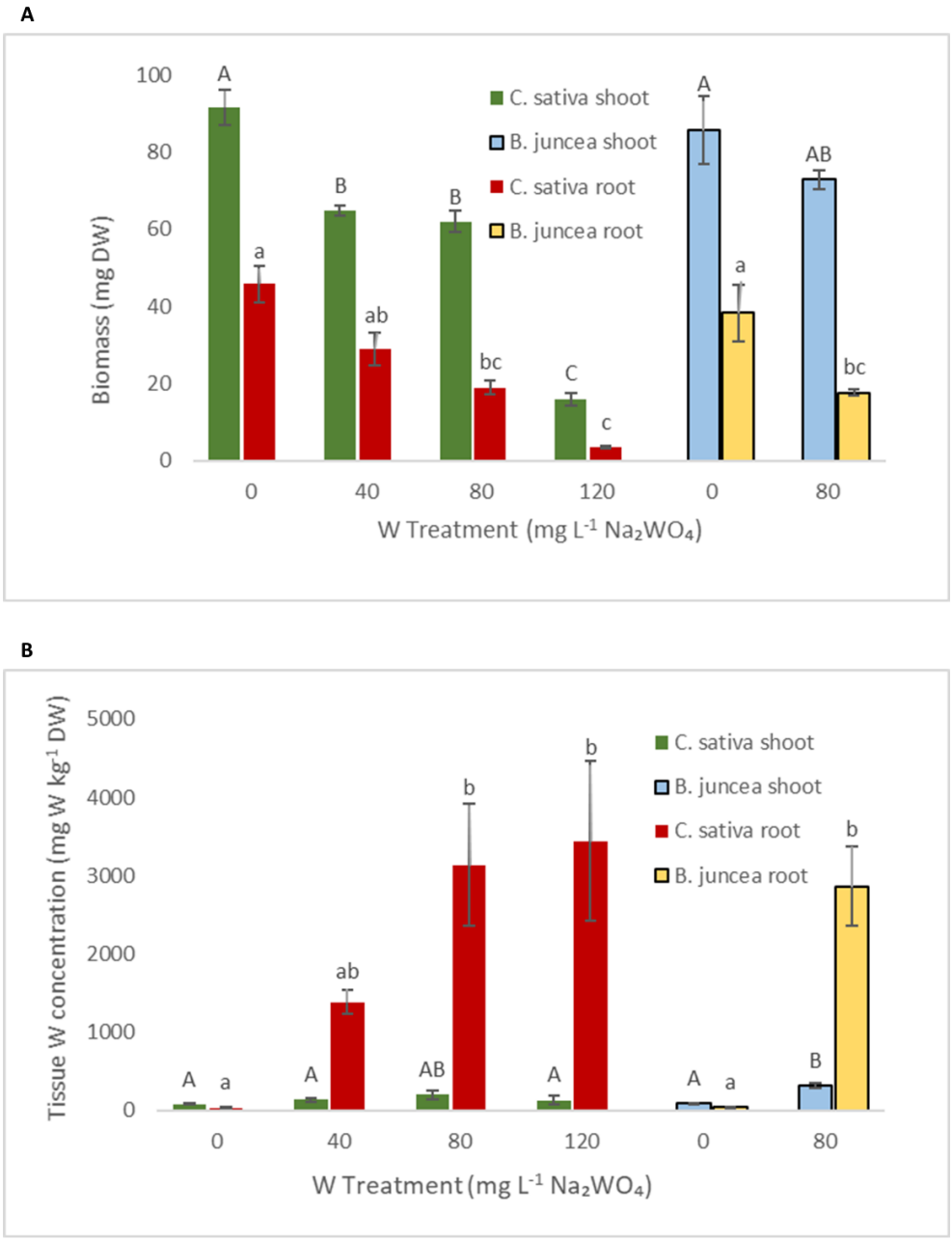

Figure 2.7 Tungsten tolerance and accumulation for *C. sativa* and *B. juncea* seedlings grown for 23 days on different concentrations (0-120 mg L^{-1}) of sodium tungstate. (A) Shoot and root biomass. *C. sativa* root biomass for 120 mg L^{-1} was 4 mg kg^{-1}. (B) Shoot and root accumulation. *C. sativa* shoot concentration were 83, 137, 204, and 135 mg kg^{-1} for 0-120 mg L^{-1} respectively. *C. sativa* root concentration was 38 mg kg^{-1} for the control. *B. juncea* shoot concentration were 95 and 316 mg kg^{-1} for 0 and 80 mg L^{-1}. *B. juncea* root concentration was 46 mg kg^{-1} for the control. Letters above bars denote statistically significant differences for shoot (capital) and root (lowercase). Shown are means and SEM. ANOVA with Tukey-Kramer, α =0.05, n=5.

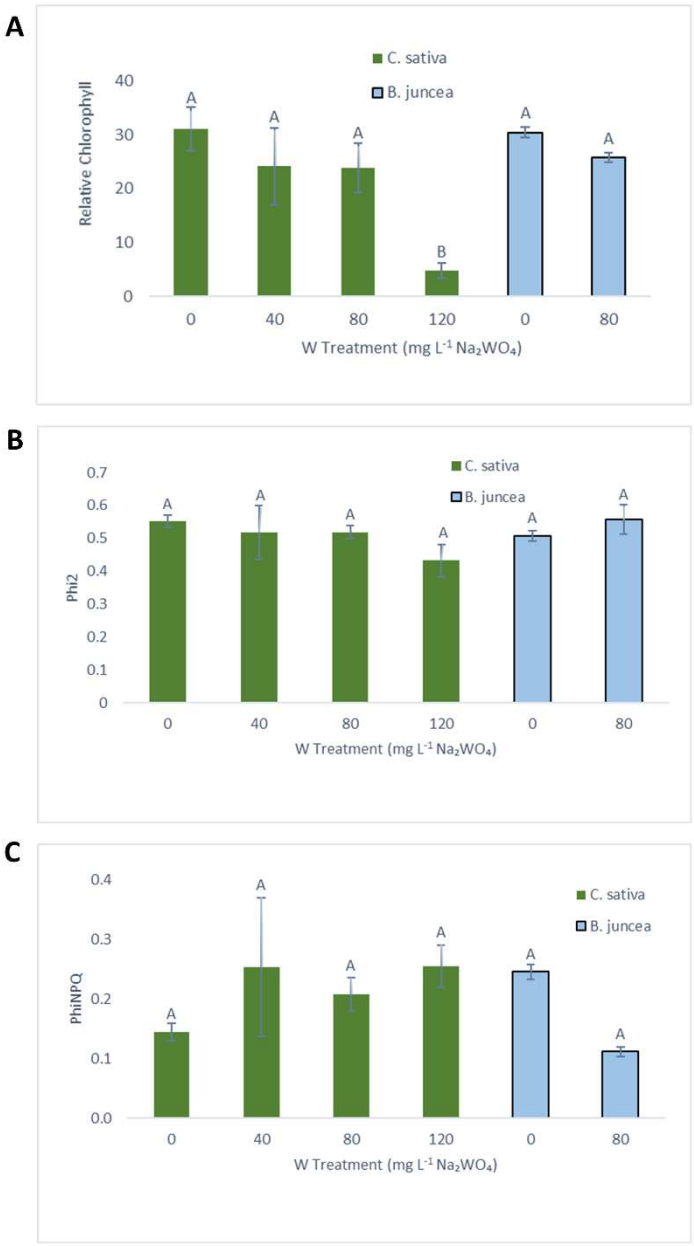

Figure 2.8 Photosynthetic performance of *C. sativa* and *B. juncea* seedlings grow for 23 days on different concentrations (0-120 mg L^{-1}) of sodium tungstate. (A) Leaf relative chlorophyll content. (B) Leaf Phi2, the fraction of absorbed light energy photochemically quenched. (C) Leaf PhiNPQ, the fraction of absorbed light energy non-photochemically quenched. Letters above bars denote statistically significant differences for W concentrations. Shown are means and SEM. ANOVA with Tukey-Kramer, α =0.05, n=5.

2.5 Discussion

In this study, hemp seedlings were tested with four different oxyanions of As, Mo, V and W, to evaluate hemp's ability to tolerate, take-up, and accumulate these toxic elements as well as to determine their fate in the plant. It was hypothesized that hemp would perform in a way comparable to Indian mustard (*Brassica juncea*) and be able to accumulate the elements in its tissues. The findings indicate that hemp shows promise for phytoremediation of these elements, although the economic value after harvest remains to be determined using mature plants. Tolerance, accumulation, and fate of the elements tested were comparable to the popular phytoremediation species Indian mustard. There were no significant differences between hemp and Indian mustard in any of the experiments for tolerance or accumulation. These findings are significant because they suggest that hemp is a competitive phytoremediating species for these metals compared to Indian mustard. Having a viable alternative species to grow in polluted sites is important in phytoremediation where there may not be many capable or successful species to choose from. Hemp could be a strong choice in the remediation process because its hardy and fast-growing characteristics will withstand the harsh environments usually found at contaminated sites. Using hemp could be a cost-effective choice, because the economic payoff of using mature hemp biomass in industry and for biofuels could off-set set up and maintenance costs of the phytoremediation process.

The findings indicate that As, V, and W were mostly accumulated in the root tissue while Mo accumulation occurred in both the shoot and the root. This could be because molybdenum is an essential trace element in plants and has been shown to translocate to the shoot (Hale et al., 2001; Hawkesford, 2003). This was found to be especially true when anthocyanins were present suggesting an association between Mo and anthocyanins (Hale et al., 2001). In the Hale et al.,

2001 study, anthocyanins were found to bind to Mo resulting in a blue color change. Mo, found mostly in leafy vegetables and legumes, is found in drinking water worldwide at levels up to 0.2 mg L^{-1} (Vyskocyl and Viau, 1999). Mining activity has caused increased levels as high as 0.4 mg L^{-1} in drinking water in Colorado where this amount of contamination is near the higher end of estimated safe levels for humans of 0.15 – 0.5 mg L^{-1} (Vyskocyl and Viau, 1999). The hemp seedlings in this experiment show promise for Mo phytoremediation as they were able to remove up to 4900 mg Mo kg^{-L} DW when treated with 80 mg L^{-1}. The levels obtained here are all toxic of course, eliminating possible post-harvest economic use of the shoot biomass.

The arsenic shoot accumulation at the lowest treatment of 3 mg L^{-1} was 35 mg kg^{-1} DW, which is the highest acceptable accumulation level for shoot biomass used for economic purposes. The safe maximum As level (when ingested by children) is 40 mg As kg^{-1} DW in the tissue (Dudka and Miller, 1999). This means that hemp shoot biomass could only be used economically when grown in As levels below 3 mg L^{-1} Na_2AsO_4. A complication in this arsenic experiment occurred with the control plant roots, which measured almost 300 mg As kg^{-1} DW. A level this high indicates that these plants were inadvertently treated at least once. These results can be remedied by retesting with stronger barriers between the control and treated plants.

In the vanadium experiment, plant biomass increased with higher treatment concentrations of V, for both hemp and Indian mustard. The increase in biomass suggests treatment levels (0-8 mg L^{-1} Na_2VO_4) were not toxic to the plants tested, but rather stimulated their growth. This phenomenon is known as hormesis: it occurs when a toxic element stimulates growth rather than inhibiting it. In ground water, V contamination levels from mining activities have been measured from 0.07 – 0.2 mg V L^{-1} (Teng et al., 2011). The levels treated here were higher than reported contaminated mining levels. All supplied V levels were tolerated and accumulated by hemp, suggesting hemp

has potential to tolerate and accumulate V at contaminated sites. For comparison, it was found earlier that tobacco plant growth was inhibited by V treatments greater than 2 mg L^{-1} (Wu et al., 2021). Further studies with increased V levels are needed to find the V toxicity threshold level for hemp.

Tungsten is found naturally at 0.2 – 2.4 mg kg^{-L} in soils; plant toxicity levels are not well-characterized, due to limited research (Adamakis et al., 2012; Strigul et al., 2005; Senesi et al., 1988). Toxicity may vary among plant species, but also depending on the form of W, not only for plants but also animals. It is believed that W toxicity causes leukemia and other forms of cancer, increasing the importance of removing W from polluted sites (Witten et al., 2012). In this hemp study, W toxicity occurred in hemp when treated with 120 mg W L^{-1}. This level of W is just beyond toxic levels found from anthropogenic sources where 100 mg L^{-1} was considered toxic (Adamakis et al., 2012). This suggests that hemp may be able to take up W at some contaminated sites, although those W levels may be near hemp's uptake limit.

Of course, in choosing to only test hemp seedlings, this study offers limited understanding of what mature hemp performance could be. The As, Mo, V and W phytoremediation performance of hemp at mature plant level may be looked at in further studies. In past studies, Se tolerance and accumulation data from hemp seedlings corresponded well with those of the mature plant, which is encouraging (Stonehouse et al., 2020). Another consideration to study further in this experiment is the use of the clay inert growth medium, Turface®. Due to ion exchanges, there is a possibility that treatments may have built up throughout the experiment resulting in higher-than-normal concentrations in the data. Additional studies to this research should also include field studies where the environmental conditions can be evaluated, as they play an important role in real world contaminated sites. Follow-on mature plant studies in the field may reveal new

insights that differ from this seedling experiment. Testing plants growing outside the greenhouse could reveal different result where competition may play a role due to outside interactions (Danh et al., 2014).

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