Evaluation of Three Manitoba Redroot Pigweed (*Amaranthus retroflexus* L.) Populations Resistant to Acetolactate Synthase-Inhibitors

By

Sampa Sarker

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This thesis is dedicated to my father, Sakhi Charan Das; my mother, Benu Rani Das; and my lovely husband Rubel Chandra Talukder.

Abstract

Redroot pigweed (*Amaranthus retroflexus* L.) is a common broadleaf weed species in Manitoba, Canada. Producers in this region have been reporting reduced response of this weed to commonly used Acetolactate synthase (ALS) inhibiting herbicides. Three suspected resistant populations of redroot pigweed were compared with known susceptible populations in a greenhouse study to determine the level of resistance to the ALS-inhibiting herbicides imazethapyr and thifensulfuron-methyl. A laboratory experiment was conducted to identify the mechanism of herbicide resistance of these three populations. Dose response curves to these herbicides indicated that all three pigweed populations were resistant to imazethapyr (~30 to >170-times), but only one of these populations showed a low level (7-fold) of cross resistance to thifensulfuron-methyl. Acetolactate synthase (ALS) gene sequence analysis detected a Ser653Asn amino acid substitution that was consistent in the two populations ArMB1 & ArMB2 which were highly resistant to imazethapyr. A Ser653Asn/Ile substitution was found in population ArMB3. That population showed high resistance to imazethapyr and low levels of resistance to thifensulfuron-methyl. A malathion treatment study was conducted to determine the presence of non-target site resistance through enhanced metabolism among the three resistant populations. Cytochrome P450 induced metabolism based non-target site resistance (NTSR) did not contribute to resistance to imazethapyr or thifensulfuron-methyl among the three resistant populations. Target site resistance alone was the mechanism conferring resistance to these two ALS inhibitors among the three resistant redroot pigweed populations and that facilitates the development of rapid detection markers to identify the resistant redroot pigweed with these mutations.

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Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

Redroot pigweed (*Amaranthus retroflexus* L.) is an annual C4 dicot weed with worldwide distribution in most field and horticultural row crops in the temperate regions in the world (Costea et al. 2004). It is a native of North America (Sauer 1967). This fast-growing weed can be a strong competitor with crops due to its wide range of environmental adaptability (Hao et al. 2017). It can compete aggressively with crops for water, light and nutrients and affects the quality and yield of major crops severely (Zargar et al. 2020). Photosynthetically active radiation from many crops could be blocked by this weed if it remains unmanaged (Knezevic et al. 1999). It potentially reduces yield of soybean (*Glycine max* L. Merr.), corn (*Zea mays* L.), cotton (*Gossypium barbadense* L.), sugarbeet (*Beta vulgaris* L.), wheat (*Triticum aestivum* L.), sorghum (*Sorghum bicolor* L.), alfalfa (*Medicago sativa* L.) and many vegetable crops (Weaver and MacWilliams 1980, Mykhalska and Schwartau 2022, Heap 2023). In the 2016 weed survey in Manitoba, redroot pigweed was the 5th most abundant broadleaf weed species (Leeson et al. 2017).

The enzyme acetolactate synthase (ALS) also often referred to as acetohydroxy acid synthase (AHAS) (Powles & Yu 2010) is present in plants and microorganisms. ALS is required to catalyze the first step of branched chain amino acid synthesis (leucine, isoleucine and valine) which are essential for the plant's growth and development (Zhou et al. 2007). Herbicides are used to inhibit this enzyme in susceptible weed species. To control redroot pigweed in different agricultural crop fields, ALS-inhibitors are used widely (Anonymous 2021b).

The ALS-inhibitors are popular group of herbicides and have been used on various crops because of their high effectiveness at low doses (Chen et al. 2015). Moreover, these herbicides have broad-spectrum season-long weed control with low toxicity to mammals (Tranel and Wright 2002, Saari

et al. 1994). There are five different families of ALS inhibitors (sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinyl-benzoates (PTB), and sulfonylamino- carbonyl-triazolinones (SCT)); however, in western Canada only four of these are used commercially (Saari et al. 1994, Santel et al. 1999). Unfortunately, because of widespread repetitive use of the ALS inhibitors, weeds have evolved resistance to these herbicides quite frequently (Mallory-Smith et al. 1990, Tranel and Wright 2002). Besides the emergence of resistance to acetolactate synthase (ALS) inhibitors, redroot pigweed also has evolved resistance to protoporphyrinogen oxidase (PPO) inhibitors, photosystem II inhibitors, hydroxyphenyl pyruvate dioxygenase (HPPD) inhibitors and enolpyruvyl shikimate phosphate (EPSP) synthase inhibitors (Heap 2023).

Target site modification (target site resistance = TSR) or enhanced metabolism (non-target site resistance = NTSR) are the two main mechanisms for resistance to ALS inhibitors in weeds (Preston and Mallory-Smith 2001). Target site modification is the most frequently reported mechanism, with point mutations causing specific nucleotide substitutions within a specific coding region resulting in a different amino acid substitution near the active site of enzyme (Powles and Preston 2006). The changes to the enzyme reduce the affinity of herbicide to the enzyme by blocking the active site and ultimately confers resistance to the weed species (Powles and Preston 2006). NTSR is another important mechanism which reduces herbicide uptake/translocation and/or increases herbicide metabolism, often via cytochrome P450 enzymes (Yu and Powles 2014). This effectively reduces the herbicide dose that reaches the target site causing resistance. Based on these resistance mechanisms weeds may develop cross resistance to herbicides of the same mechanism of action or resistance to multiple herbicides with different mechanisms of action (Yu & Powles 2014).

The level of resistance to different ALS inhibitors depends on what type of mutations are in the ALS gene of plants (Tranel and Wright 2002). For example, a point mutation causing a Pro197 substitution generally confers resistance to sulfonylureas and triazolopyrimidines, and a Ala122 substitution is responsible for conferring resistance to imidazolinones (Heap 2023). Over the past two decades, 29 amino acid substitutions at 8 positions (Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, Gly654) on the ALS gene have been recorded conferring resistance to ALS inhibitors (Heap 2023). So far, 171 weed species resistant to ALS herbicides have been identified, globally (Heap 2023).

Resistant biotypes of redroot pigweed have been documented in the USA, Canada, France, Germany, Brazil, China, Hungary, Switzerland, Spain, Serbia, Italy, Israel, Greece, Poland, and the Czech Republic (Heap 2023). In Canada, ALS resistant redroot pigweed has been reported in Ontario, Manitoba, Saskatchewan and Quebec (Heap 2023). One population of resistant redroot pigweed to ALS inhibitor (florasulam) was first documented in Manitoba in 2002 (Beckie et al. 2008). Herbicide resistant redroot pigweed, however, is not yet threatening in Western Canada even though some incidences of resistant redroot pigweed have been documented.

Hypotheses:

1) Manitoba redroot pigweed populations suspected to be resistant to the ALS inhibitors imazethapyr and thifensulfuron-methyl will have different dose response curve parameters than susceptible control populations.

2) Resistance in Manitoba redroot pigweed populations is caused by target site resistance (target site mutations) and is not caused by non-target site resistance.

Chapter 2: Literature Review

2.1 Origin & distribution of redroot pigweed (*Amaranthus retroflexus* L.)

Redroot pigweed is a native to the eastern and central U.S., and adjacent regions of southeastern Canada and northeastern Mexico (Sauer 1967). From the evidence of macrofossil seeds which is about 250 years old from Quebec, it is estimated that redroot pigweed was introduced to Canada from southern regions of North America by colonists between the 17th and 18th centuries (Costea et al. 2004).

Redroot pigweed is an annual weed with widespread distribution (Sheibany et al. 2009). It is considered one of the most common agricultural weed species in the areas where it originated (Sauer 1967). Through anthropochory, it now is naturalized in the temperate regions of Asia, Africa, Europe, and Australia (Holm et al. 1979). It is found in most field and horticultural row crops around the world due to its wide range of environmental adaptability (Chen et al. 2015). In Canada, redroot pigweed is found in all provinces and territories excluding the Yukon, Nunavut, and Labrador (Costea et al. 2004).

2.2 Biology of redroot pigweed

Redroot pigweed is a member of *Amaranthaceae* family. It is a common annual dicotyledonous plant and rapidly growing weed species found in many agricultural fields around the world (Horak and Loughin 2000, Alebrahim et al. 2012). It is one of the monoecious weed species among the *Amaranthus* genus which also has many dioecious members (Carvalho et al. 2015). Other closely related *Amaranthus* species of agricultural concern are powell amaranth (*Amaranthus powellii* S.Wats.), spiny amaranth (*Amaranthus spinosus* L.), waterhemp (*Amaranthus rudis* (Moq.) Sauer), tumble pigweed (*Amaranthus albus* L.), smooth pigweed (*Amaranthus hybridus* L.), prostrate

pigweed (*Amaranthus blitoides* S. Wats.), and Palmer amaranth (*Amaranthus palmeri* S. Watson.) (Webster 2006, Sellers et al. 2003, Schonbeck 2012).

With respect to the morphology of redroot pigweed plants, the taproot system of this weed is shallow and may be pink or red in color at the base, and the plant has simple or branched green to slightly red erect stems (Weaver and McWilliams1980). The shoot can be from 0.1-2m in height. Leaves are sparsely hairy, alternate, ovate to rhombic-ovate with long stalk, dull green color on the upper surface and prominent white veins appear on the lower surface of the leaves (Weaver and McWilliams1980). Flowers are numerous, complete, tepals are retuse or obtuse, short, green in color, forming a rigid terminal spike or panicle (5-20 cm long) and crowded into dense blunt spikes in axils of the leaves. Redroot pigweed reproduces by seed. The seed (1-1.2 mm in diameter) is shiny dark brown to black, broadly elliptic and flattened (Weaver and McWilliams1980). The small seed structure facilitates the dispersal of the weed through animals, water, and wind (Costea et al. 2004). A single redroot pigweed plant can produce over 500,000 seeds (Sauer 1967). It has long seed viability. For example, seeds of redroot pigweed were found to remain viable for up to 40 years when buried in soil at a depth of 45 cm (Darlington and Steinbauer 1961). The long seed longevity leads to this species building a persistent soil seed bank (Karimmojeni et al. 2014). In terms of genetics, the chromosome number of redroot pigweed is reported to be 2n = 34 (Murray 1940, Grant 1959, Mulligan 1961).

Redroot pigweed is a C4 weed, and at high temperatures and light intensity the photosynthetic rate is high (Weaver and McWilliams 1980, Kigel 1994). The high photosynthetic rate, high net carbon exchange capacity, more efficient use of use of CO_2, high transpiration efficiency and reduced photorespiration of C4 species facilitate to increase the leaf area which ultimately enable the plant to grow rapidly (Oliver and Schreiber 1974, Costea et al. 2004). Temperature plays a vital role in

these properties of this weed species. The maximum photosynthetic rate of redroot pigweed is about 60 mg $CO_2 \cdot dm^{-2} \cdot h^{-1}$ at 1.0 x 10^5 lx (Mahendra et al. 1974). For net photosynthesis the optimum temperature in this weed species is 32.9 °C (Oryokot et al. 1997). To increase carbon exchange and transpiration rate the optimum temperature and photosynthetic photon flux density (PPFD) rates for this species were 20 to 35 °C, and 0 to 2000 μmol m^{-2} s^{-1}, respectively (Nielsen and Anderson 1994). The leaf appearance rate of this weed species also increased with temperature from 10 to 35 °C (Weaver and McWilliams 1980, McLachlan et al. 1993).

2.3 Weedy nature of redroot pigweed

Redroot pigweed is considered one of the worst weeds in the world (Costea et al. 2004). It is found in a wide range of crop fields in most provinces in Canada (Holm et al. 1991, 1997). The prolonged germination periods, prolific seed production capacity, and wide range of adaptation of this weed in varied environmental conditions help the weed to be competitive with crops (Hao et al. 2017). The high rate of photosynthesis facilitates fast growth of redroot pigweed which makes this weed aggressive and competitive in many cropping areas (Khan et al. 2022).

2.3.1 Yield loss

Redroot pigweed competes with crops for nutrients, water, light and space causing crop yield loss. The yield of soybean can be reduced by 38.0% at redroot pigweed densities of 10.5 plants m^{-2}, when the row spacing was 76 cm (Bensch et al. 2003). When the density of redroot pigweed is high (over 30 plants m^{-2}), yield losses of 90% or more can occur (Costea et al. 2004). Yield of corn was reduced by 5% with a density of 0.5 redroot pigweed m^{-2} (Knezevic et al.1994). Yield of snap bean (*Phaseolus vulgaris* L.) was decreased by 42–58% because of redroot pigweed plants at densities of 8 plants m^{-1} (Aguyoh and Masiunas 2003). Stebbing et al. (2000) found that

sugarbeet yield was decreased by 31% when densities of redroot pigweed were 1.5 plants m^{-2}. Season-long infestation of redroot pigweed at a density of one plant m^{-1} of row reduced yields of marketable potato (*Solanum tuberosum* L.) by 22 to 33% (VanGessel and Renner 1990). The amount of loss depends on redroot pigweed density and duration of interference during crop growth and development.

2.3.2 Other harvest challenges

In addition to yield reductions, harvest efficiency also was affected by redroot pigweed interference with crops. Besides competitive interference, redroot pigweed with its allelopathic effects was able to affect the seedling growth of soybean, wheat, bean (Costea et al. 2004). Bhowmik and Doll (1982) found when residues of redroot pigweed remained in the field over a winter, soybean yield was decreased by 14 to 20% in the next year. In a greenhouse experiment, addition of 8 g of dried shoot residues of redroot pigweed per kg of soil, resulted in reduction of wheat shoot dry weight by 39% and root dry weight by 26% (Qasem 1995a). In laboratory experiment, plant residues and shoot extracts of redroot pigweed was found effective at reducing germination of wheat, corn, barley (*Hordeum vulgare* L.), soybean, sunflower (*Helianthus annuus* L.) and a wide variety of vegetable crops (Beres and Kazinczi 2000, Qasem 1995b).

Additionally, redroot pigweed was found as an alternate host for many parasites, pathogen and insects that affected a variety of crops including tomato (*Lycopersicon esculentum* Mill.), potato, sugarbeets, peach (*Prunus persica* [L.] Batsch), apple (*Malus domestica* [Suckow] Borkh.) and pepper (*Capsicum annuum* L.) (Weaver and Mcwilliums 1980). In tomato fields, redroot pigweed was found as an alternate host for *Orobanche ramosa* L., a devastating root parasitic weed species commonly known as branched boroomrape (King 1966). In apple and peach orchards, redroot

pigweed has been found as a host for green peach aphid *Myzus persicae* (Sulzer) (Tamaki 1975). *Cucumber mosaic virus* uses redroot pigweed as an alternate host and affected the pepper fields (Lockhart and Fischer 1976). Infection of redroot pigweed by *Rhizoctonia solani* (Kuhn), *Fusarium oxysporum* f. sp. Betae and *Rhizoctonia* spp. was observed in the fields of sugarbeets (Coulombe 1975), tablebeets (MacDonald and Leach 1976) and potato, (King 1966) respectively. Redroot pigweed not only reduces crop yields, but also affects harvest efficiency and dockage which are additional costs to the producer.

2.4 Herbicides for redroot pigweed management

Redroot pigweed is readily managed by a number of selective and non-selective herbicides. The label and rate of most of the herbicides do not differentiate among different pigweed species (Costea et al. 2004). Some popular selective herbicides that are used to control redroot pigweed in Canada are Acetolactate synthase (ALS) inhibitors, (e.g., imazethapyr, thifensulfuron-methyl, imazamox), synthetic auxins (e.g., 2,4-DB, dicamba) and Photosystem II (PSII) inhibitors (e.g., atrazine) which belong to group 2, group 4 and group 5, respectively (Anonymous 2021a). The ALS inhibitors inhibit the normal function of acetolactate synthase (ALS) which is essential for the synthesis of branched chain amino acids (protein). Lack of these amino acids and accumulation of phytotoxic compounds because of the interference of that enzyme by these herbicides causes plants to die (Sherwani et al. 2015). Synthetic auxins interfere with the growth of young plant cells on leaves and stems by inhibiting synthesis of proteins and normal cell division, resulting in malformed growth and callus tissue. Photosystem II (PSII) inhibitor herbicides inhibit the plastoquinone-binding protein in the electron transport chain in photosystem 2 (Sherwani et al. 2015). Among the non-selective herbicides EPSP synthase inhibitor belong to group 9 (glyphosate) and glutamine synthase inhibitor belong to group 10 (glufosinate) also control *amaranthus* species

effectively (Krausz et al. 1996, Jordan et al. 1997, Coetzer et al. 2002). Glyphosate inhibits the synthesis of aromatic amino acids (e.g., tryptophan, tyrosine, and phenylalanine) by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Sherwani et al. 2015). Glufosinate inhibits glutamine synthetase which is the key enzyme for nitrogen assimilation in plants (Lea 1984, Tachibana 1986, Sauer 1987).

2.5 Acetolactate synthase inhibitors

Acetolactate synthase also known as acetohydroxyacid synthase (AHAS) catalyzes early steps in the formation of the three branched chain amino acids leucine, isoleucine and valine (Saari et al. 1994, Devine et al. 1991). For the biosynthesis of leucine and valine 2-acetolactate is derived from two pyruvate molecules, and for isoleucine synthesis 2-acetohydroxybutyrate is synthesized from 2-ketobutyrate and pyruvate (Zhou et al. 2007). These branched chain amino acids are essential for the growth and development of plants (Zhou et al. 2007). Acetolactate synthase inhibitors are used to interfere with the function of this acetolactate synthase enzyme and thus affect plant growth and development. ALS herbicides are readily absorbed by leaves and roots and translocated in the phloem and xylem to meristematic regions in plants (Singh and Shaner 1995, Zhou et al. 2007). Phytotoxic effect on mature tissue takes longer to appear because the mature tissue has a high quantity of amino acids and protein stored in them. The stored proteins also can be used further for the synthesis of amino acids (Shaner and Singh 1993) contributing to the delay of the onset of symptoms in older tissue.

Among the selective herbicides, the ALS-inhibitor herbicides are used widely to control redroot pigweed in different crops. The application of these herbicides has increased steadily since the introduction of the ALS-inhibitors into the marketplace in the early 1980s. By 1991, the market

value of the two dominant families of ALS inhibiting herbicides, the sulfonylureas and imidazolinones, was $1.3 billion dollars globally (Zhou et al. 2007). The popularity of using ALS-inhibitors is high because of their high efficacy at low doses (2 to 75 g ai ha^{-1}), their ability to be used in a number of different crops, season-long, broad-spectrum weed control in by some of the active ingredients in this mode of action, and low mammalian toxicity (Saari et al. 1994, Brown 1990, Tranel and Wright, 2002). These herbicides are used in a number of important crops including pulses e.g., peas (*Pisum sativum* L.), beans, lentils (*Lens culinaris* Medik.); cereals e.g., wheat, barley, oats (*Avena sativa* L.); canola (*Brassica napus* L.), corn and soybeans in western Canada (Anonymous 2021b, Anonymous 2022, Wilson 2012). Five different families of ALS inhibitors have been commercialized, including imidazolinones (IMI), sulfonylureas (SU), pyrimidinyl-benzoates (PTB), triazolopyrimidines (TP), and sulfonylamino- carbonyl-triazolinones (SCT) (Saari et al. 1994, Santel et al. 1999). Among the group 2 herbicides, the sulfonylurea (e.g., thifensulfuron-methyl, metsulfuron-methyl, tribenuron-methyl), imidazolinone (e.g., imazethapyr, imazamox, imazamethabenz,) and triazolopyrimidine (e.g., florasulam) are most widely used herbicides in western Canada (Wilson 2012, Anonymous 2018, Anonymous 2022).

The interaction between the ALS inhibitors and the ALS enzyme is complex. Studies on the crystal and molecular structure of the ALS enzyme in yeast (*Saccharomyces cerevisiae* Meyen *ex* E.C. Hansen) and *Arabidopsis thaliana* (L.) Heynh. have provided a comprehensive understanding of the interaction between the herbicides and ALS (McCourt et al. 2005, 2006). The ALS enzyme is comprised of catalytic subunits and regulatory subunits (Nakka et al. 2016). The herbicide binding site on ALS is different from its active site, but the two sites are in close proximity (Tranel and Wright 2002). The regulatory subunit helps to activate the catalytic site by responding to the

substrate or the products (branched-chain amino acids). The catalytic subunit is inside a channel of the protein, and the herbicide cannot attach to the catalytic site directly (McCourt et al. 2006). ALS inhibitor herbicides attach to a domain that is close to the channel entrance and thereby block the substrate access to the active catalytic site. As a result, the synthesis of leucine, isoleucine, and valine is blocked by the ALS-inhibitor and because of the shortage of the three essential amino acids, protein synthesis is reduced, eventually causing cell death (Zhou et al. 2007). Besides the effects on the synthesis of branched chain amino acids and protein synthesis, ALS inhibition has some other secondary effects thought to contribute to cell death including, but not limited to the formation of 2-aminobutyrate or 2-ketobutyrate, photoassimilate translocation disruption, DNA synthesis inhibition and anaerobic respiration (Zhou et al. 2007).

2.6 Herbicide resistance

Plants show their responses to herbicides in different ways. The responses are susceptibility, tolerance or resistance. Susceptibility of plants is defined as the state of being harmed by herbicide treatment. On the other hand, tolerance and resistance are the ability of plants to withstand herbicide treatment (Holt and Lebaron 1990). Specifically, tolerance means the inherent capacity of a species to survive and reproduce after herbicide treatment at a normal use rate. Tolerance to specific modes of action such as broadleaf tolerance to ACCase inhibiting herbicides is ubiquitous within a species. This is considered as the naturally occurring mechanisms that enable plants to survive exposure to a herbicide with a certain mode of action (Holt and Lebaron 1990).

According to Herbicide Resistance Action Committee (HRAC) herbicide resistance is defined as the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type (Poston et al. 2000). Herbicide resistance in weeds is considered

an evolutionary process (Jasieniuk et al. 1996). In response to repeated application of same herbicides or different active ingredients from the same mode of action, the genetic makeup of a weed population can be changed resulting in the production of more resistant individuals through the increase of the resistant allele frequency (Jasieniuk et al. 1996).

Weeds resistance to herbicides can be divided into three groups: single resistance, cross resistance, and multiple resistance. Single resistance can be defined when weeds are resistant to only one active ingredient in one herbicide mode of action (Nakka 2016). Cross resistance is resistance is when a weed biotype is resistant to two or more active ingredients with the same mode of action. This resistance may arise without the weed population ever being exposed to one of the herbicides. Multiple resistance occurs when weeds develop resistance to two or more active ingredients with different modes of action (Nakka 2016).

2.7 Factors affecting evolution of herbicide resistance

The evolution of herbicide resistance is common. Some of the important factors that lead to the evolution and spread of herbicide resistance in weeds include: herbicide selection pressure, biological and genetic characteristics of weeds, herbicide characteristics and agronomic practices (Powles and Yu 2010).

Herbicides impose a significant selection pressure on weeds if the same herbicide or herbicides from same mode of action are used repeatedly on the same weed population. On a population level, it is normal to have some individuals that are resistant to herbicides whether the herbicides are used or not (Jasieniuk et al. 1996, Gasquez 1997). Each time a herbicide is applied, the susceptible individuals die while the resistant individuals survive and potentially reproduce, increasing the frequency of the resistance allele in the population. Resistant individuals can spread rapidly

through regeneration and eventually become the dominant genotype in the population (Nakka 2016). Selection pressure is considered greater when herbicides kill a larger number of the sensitive biotype (Anonymous 2021c).

The biological characteristics of weed species can also influence the evolution and spread of herbicide resistance. High fecundity of weeds, high germination percentage, wide range of emergence, wide seed dispersal, and a high frequency of resistant individuals are some examples of biological characteristics of weeds that can facilitate the development of herbicide resistance (Nakka 2016). Some *Amaranthus spp* produce large numbers of seeds and if a resistant individual from a mixture of susceptible and resistant biotypes can survive and reproduce, the resistant portion in the population will increase.

Evolution of herbicide resistance depends on the genetic factors of weeds. Frequency of resistant alleles, presence of dominant resistant alleles and fitness cost of resistant genes influence the development of resistant weeds (Jasieniuk et al. 1996, Délye et al. 2013). For instance, high frequency of the resistant alleles in the weed population at the initial stages helps to develop resistant weeds rapidly (Nakka 2016).

Herbicide characteristics such as structural properties of the active ingredient, mode/site of action of herbicide, residual activity of the herbicide also can contribute to the development of herbicide resistance (Nakka 2016). Herbicides that bind to one highly specific site of action are usually more prone to develop resistant weeds as a single alteration in only the gene coding for the herbicide target may be enough to affect the binding potential of a herbicide to the active site of action. When a herbicide has multiple sites of action, it is less likely that weeds develop resistance rapidly to such a herbicide (Gunsolus 2023).

Agronomic practices can also contribute to the development of herbicide resistance (Nakka 2016). For example, application of herbicide at a lower rate than the recommended dose can contribute to the development of herbicide resistance in weeds (Nakka 2016). Complete dependency on herbicides for weed control and monocropping culture are also some examples of agronomic practices which can greatly enhance the occurrence of herbicide resistant weeds (Nakka 2016).

2.8 Introduction to mechanisms of herbicide resistance

Herbicide resistance can be caused by a number of different mechanisms and those mechanisms are broadly grouped into two main categories namely, target site resistance and non-target site resistance. Target site resistance is the resistance caused generally by the changes to the target site through mutations resulting in the substitution of amino acids and overexpression of the target enzyme that allow the enzyme to continue to function even in the presence of the herbicide, (Nakka 2016). Target site resistance through mutations is the most common mechanism that confers resistance in plants (Tranel and Wright 2002).

Non-target site resistance generally occurs through enhanced herbicide metabolism, increased herbicide sequestration, decreased herbicide absorption or reduced herbicide translocation (Powles and Yu 2010, Bo et al. 2017). This effectively reduces the herbicide dose that reaches the target site and leads to a resistant phenotype. Enhanced herbicide metabolism among non-target site resistance mechanisms is the most common mechanism conferring resistance to ALS inhibitor herbicides in weeds (Dexter 2004, Kwon et al. 2015).

2.8.1 Target site resistance

Most, but not all herbicide modes of action involve binding to specific proteins or enzymes that are critical to plant processes and their inhibition ultimately leads to the death of the plant (Vencill et al. 2012). Insensitivity of the target site often is caused by one or more-point mutations (single nucleotide polymorphisms) that allows the target site to still perform its metabolic function while the herbicide can no longer bind to the enzyme or block the active site (Bo et al. 2017, Hanson et al. 2013).

The most common mechanism of herbicide resistance to different ALS inhibitors in weeds is due to target site mutations causing amino acids substitution resulting in the modification of the specific herbicide binding site of the ALS enzyme. There are many weed species with amino acid substitutions that confer resistance to different ALS inhibitors (Heap 2023). Depending on what type of substitutions they have, the resistance level varies to different families of ALS inhibiting herbicides. The *Amaranthus* species such as, redroot pigweed, smooth pigweed, waterhemp, powell amaranth, palmer amaranth with different amino acids substitutions (e.g., Ser653Asn, Ser653Thr, Trp574Leu, Asp376Glu, Pro197Leu) exhibited broad range of resistance to different ALS inhibitors (Heap 2023). In Pennsylvania, a Asp376Glu substitution was identified in smooth pigweed that conferred resistance (60 to 3200-fold) to four families of ALS inhibitors including the imidazolinones, sulfonylureas, triazolopyrimidine, and pyrimidinyl benzoates (Whaley et al. 2007). Some biotypes of waterhemp with mutations causing Ser653Asn and Ser653Thr substitutions showed resistance to imidazolinones (imazethapyr) (74 to 860-times) only (Patzoldt and Tranel 2007).

Target site resistance to ALS inhibitors is common in weed biotypes because the enzyme remains functional with one of a series of mutations in the separate five conserved domains of the ALS gene (Nakka 2016, Bo et al. 2017). The length of 5 highly conserved domains (A through E) varies and ranges from 12-57 base-pairs. It has been observed that most of the time the amino acid substitution caused by a single nucleotide change occurred in one of the 5 conserved regions of ALS. Domain A is the most prone to mutations conferring resistance to ALS inhibitors (Dexter et al. 2004). For instance, in domain A, 11 different amino acid substitutions at position 197 for Proline (Pro197) have been found to confer resistance to ALS-inhibitors in 91 weed species to date (Heap 2023).

Weeds often exhibit cross resistance through target site modifications to different active ingredients of the same herbicide family but can also show different patterns of cross resistance to active ingredients of other families of the ALS-inhibitors (Poston et al. 2000). As the binding sites for the five different families of ALS inhibitors are not the same and the herbicides could have some partial overlapping while interacting with the amino acids on the domains (McCourt et al. 2006), the pattern of cross resistance varies among the different herbicide families (Friesen et al. 1993). For instance, substitutions at Ser653 usually confer high resistance to the imidazolinones with no or moderate resistance to sulfonyluraes. On the other hand, Pro197 substitutions generally result in weed biotypes with high resistance to sulfonylureas and no to moderate resistance to imidazolinones (Tranel and Wright 2002, Heap 2023). Moreover, if a weed biotype is resistant to a specific active ingredient in one of the ALS inhibitor families, this resistance does not assure that the weed would be cross resistant to the other chemicals within that same herbicide family. For example, chlorsulfuron-resistant *Chlamydomonas reinhardtii* (P.A.Dang.) was cross resistance to imazaquin but did not show resistance to imazethapyr (Hall and Devine 1990).

Different substitutions at the same position of the ALS gene of plants caused various levels of resistance to different ALS inhibitors (Tranel and Wright 2002, Powles and Yu 2010). Some SNPs at the same position cause high resistance to many members of the ALS inhibiting herbicides while other SNPs cause lower levels of resistance to some chemical families of the ALS inhibitors (Heap 2023). For example, in a redroot pigweed biotype one mutation of cytosine to thymine (CCC to CTC) caused a Pro197Leu substitution which conferred an increased level of resistance to the weed biotype (up to 127-fold for sulfonylureas, 63-fold for imidazolinones, 35-fold for triazolopyrimidines) (Sibony et al. 2001). On the other hand, some populations of *Rapistrum rugosum* L. had a substitution Pro to Ser at the same position (197) which provided a lower level of resistance to the weed to Pyrimidinyl benzoates, Sulfonylureas, Triazolopyrimidine, and Triazolinones and were susceptible to imidazolinones herbicide of ALS inhibitors (Hatami et al. 2016). Sometimes, the same substitution at the same position of the ALS enzyme provides various levels of resistance to different families of ALS-inhibitors. In some biotypes of smooth pigweed, one mutation (AGC to AAC) causing substitution of serine to asparagine at position 653 conferred a high level of resistance of the weed biotypes to imidazolineones (imazethapyr) and, a pyrimidinyl benzoates family herbicide (pyrithiobac) but a low level of resistance to sulfonylurea herbicides (thifensulfuron and chlorimuron) and a Triazolopyrimidine herbicide (Chloransulam) (Whaley et al. 2006). This indicates that some herbicide families might bind differentially to the active site of the enzyme than others (Patzoldt and Tranel 2007).

Mutation in the target-site gene sequence is the main resistance mechanism also found contributing to the resistance to the ACCase-, PPO-, and PS II inhibitor herbicides (Powles and Preston 2006, Powles and Yu 2010, Heap 2023). Other types of target site resistance mechanisms like increased gene copy number or enzyme overexpression are rarely found among the ALS inhibitors. A few

cases have been reported where in barren brome (*Bromus sterilis* L.) target gene overexpression contributed to target site resistance to the ALS inhibitor herbicides (pyroxsulam, propoxycarbazone, sulfosulfuron, iodosulfuron and mesosulfuron) (Sen et al. 2021).

The ALS gene is nuclear inherited and redroot pigweed can self-pollinate and cross-pollinate (Costea et al. 2004). Cross pollination can assist gene transfer and movement of the resistant traits through pollen to susceptible populations from one field to another field (Tranel and Wright 2002, Kulakow 2018). High seed production and small seeds also make seed movement a prominent pathway for the spread of resistant individuals within and among fields (Khan et al. 2022).

ALS inhibitors are extensively used in crop production; thus, resistance to these herbicides due to target site mutations evolves frequently among the weed species. So far, 29 resistance-conferring amino acid substitutions at 8 positions (Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, Gly654) of the ALS gene have been identified (Heap 2023). Globally, to date, approximately 171 weed species have developed resistance to ALS inhibitors (Heap 2023).

2.8.2 Non-target site resistance

Non-target-site herbicide resistance (NTSR) mechanism in weeds can be defined as the alteration of one or more physiological processes that alter herbicide absorption, translocation, sequestration, or herbicide metabolism (Delye 2013, Jugulam and Shyam 2019). These mechanisms cause a reduction in the dose of the herbicide reaching the target site resulting in less herbicide activity and the survival of the plant (Powles and Yu 2010, Westerveld 2021). At least two groups of enzymes commonly are involved in enhanced herbicide metabolism, and these are: glutathione S-transferases (GSTs) and cytochrome P450 monoxygenases (Powles and Yu 2010, Nakka 2016, Jugulam and Shyam 2019).

Reduced herbicide absorption happens when herbicide absorption through the cuticle into the plant is reduced sufficiently such that the absorbed dose is low enough to no longer be lethal to the plant. Some populations of annual ryegrass (*Lolium multiflorum* Lam.) and *Palmer amaranth* have developed resistance to glyphosate due to reduced absorption (Nandula et al. 2008, Palma-Bautista et al. 2019). This mechanism of resistance is not very common to ALS inhibiting herbicides (Rey-Caballero et al. 2017).

Reduced herbicide translocation occurs when movement of herbicide is reduced within the plant. This also causes a reduction in the dose of the herbicide active ingredient at the target site. This mechanism has been found in glyphosate resistant johnsongrass (*Sorghum halepense* L.) (Vila-Aiub et al. 2012). Although this mechanism is not very common among ALS inhibitors, it does occur. In corn poppy (*Papaver rhoeas* L.), for example, translocation was found a contributing mechanism along with TSR conferring resistance to tribenuron-methyl (Rey-Caballero et al. 2017). Riar et al. (2013) indicated that in Barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.), reduced translocation may also be a contributing mechanism to resistance to imazamox and bispyribac-sodium.

Herbicide sequestration occurs when the active ingredient accumulates in cell vacuoles or cell wall and as a result the active ingredient fails to reach the target site, again reducing the effective dose of herbicide that the target site exposed to (Vencill et al. 2012). In glyphosate resistant flaxleaf fleabane (*Conyza bonariensis* (L.) Cronq.) and horseweed (*Erigeron canadensis* L.) sequestration was found in contributing to the resistance to glyphosate (Jugulam and Shyam 2019). This mechanism is also rarely found to contribute to resistance to ALS inhibitors.

Metabolism of herbicides is a process where an enzyme uses the herbicide as a substrate and converts it to another form which is no longer toxic to the plant. Members of the diverse group of plant enzymes referred to as the cytochrome P450 monooxygenases are often involved in the process of herbicide detoxification by metabolizing herbicide active ingredients before the active ingredient reaches to the target site. Cytochrome P450s are prone to detoxify numerous herbicides with different modes of action. It could even metabolize the herbicides which have never been applied to these weeds including herbicides that have not yet been discovered (Nakka 2016). Enhanced herbicide metabolism causing resistance to ALS inhibitors has been found in some weeds including wild mustard (*Sinapis arvensis* L.) (Veldhuis et al. 2000), rigid ryegrass (*Lolium rigidum* Gaud.) (Han et al. 2013), redroot pigweed (Cao et al. 2021), flixweed (*Descurainia sophia* L.) (Yang et al. 2016) and late watergrass (*Echinochloa phyllopogon* (Stapf.) Koss.) (Yasuor et al. 2009, Yun et al. 2005). P450 monooxygenases can confer cross resistance or/and multiple resistance to active ingredients from one or more herbicide modes of action through enhanced metabolism (Beckie and Tardif 2012). Plants often evolve resistance to multiple herbicides through this metabolism process (Nakka 2016). It is considered a stress response as the plant protects itself from the phytotoxic effects of herbicide by preventing accumulation of the herbicide at the target site (Salas-Perez 2018).

There are three phases in the process of metabolism and detoxification of herbicides. In phase I, herbicide molecules are transformed into a hydrophilic metabolite with the help of P450 monooxygenases. In phase II, metabolites are conjugated to glutathione or glucose, and in phase III, primary conjugates convert to secondary conjugates in the process of detoxification and the conjugates are then stored in vacuoles or other compartments of the cell for final degradation (Hatzios 1997, Delye 2013).

Malathion, a common insecticide, is an inhibitor of P450 monoxygenases and can be used to reverse the effects of P450 metabolism-based herbicide resistance in plants (Beckie et al. 2012a, Breccia et al. 2017, Fu et al. 2017, Zhao et al. 2017). Other commonly used P450 inhibitors are piperonyl butoxide (PBO), and phorate (Jugulam and Shyam 2019). These P450 enzyme inhibitors are used to study and confirm this mechanism in many resistant weed biotypes. Malathion is applied prior to herbicide application to inhibit the activity of the P450 enzyme complex. Use of P450 inhibitors ensures that sufficient herbicide reaches the target site and if the herbicide resistance mechanism is because of the metabolism of the herbicide. Application of P450 inhibitors will help to revert the resistant phenotype to susceptible again (Gaines et al. 2020).

Nontarget-site resistance to ALS inhibitors has been identified in few eudicot weeds (Guo et al. 2015) and sometimes metabolism based non-target site resistance is found associated with target site resistance mechanism in some weed species. For example, non–target site based (enhanced herbicide metabolism) resistance to thifensulfuron-methyl in conjunction with target site–based (Trp-574-Leu mutation in ALS) resistance has been documented in redroot pigweed. The dry weight of a resistant redroot pigweed population showed a 44% reduction when thifensulfuron-methyl was applied with malathion compared with application of the herbicide alone which indicated that the cytochrome P450 enzyme complex was involved in metabolizing the herbicide (Cao et al. 2021). In a resistant population of flixweed, tribenuron-methyl plus malathion was applied and found around 79% dry weight reduction in comparison with tribenuron-methyl application alone. The findings also suggested metabolism-based resistance by P450 as partial resistance mechanism in addition to the target site mechanism (Pro-197-Thr) of resistance to the ALS inhibitor (tribenuron-methyl) (Yang et al. 2016). Guo et al. (2015) found Trp574Leu substitution in a tall waterhemp population from Illinois exhibited a high level of resistance to

some ALS inhibiting herbicides and concluded that enhanced metabolism was also involved in the resistance mechanism. Application of penoxsulam with malathion in some resistant populations of Barnyard grass caused > 94% dry weight reduction when compared with penoxsulam alone suggesting some level of resistance through P450 associated in those populations although they did not confirm any mutations in the ALS gene (Riar et al. 2012).

2.9 ALS resistant redroot pigweed in Canada

In Canada, the first redroot pigweed biotypes resistant to ALS inhibitors were found in a soybean and corn field in 1997 in Ontario. These biotypes were resistant to imazethapyr and cross resistant to thifensulfuron (Ferguson et al. 2001). In 2005, additional redroot pigweed populations at different locations in Ontario were found to be resistant to the two ALS inhibitors imazethapyr and thifensulfuron. Three specific amino acid substitutions in the ALS gene including Ala122Thr, Ala205Val, and Trp574Leu were found responsible to confer resistance in these redroot pigweed populations (McNaughton et al. 2005).

A population of redroot pigweed resistant to the ALS inhibitor florasulam was documented in Manitoba in 2002 in a wheat field (Beckie et al. 2008). In Quebec, the first resistant redroot pigweed to ALS inhibitors (imazethapyr) was found in 2009 in a soybean field. In Saskatchewan, the first ALS-resistant redroot pigweed showed resistance to thifensulfuron-methyl, and tribenuron-methyl in wheat field in 2010 (Heap 2023). The mechanisms conferring ALS resistance in these biotypes were not identified.

Chapter 3: Materials & Methods

3.1 Plant materials and growth conditions

Three redroot pigweed (*Amaranthus retroflexus* L.) populations suspected to be resistant to ALS inhibitor herbicides were collected from farmers' fields in Manitoba and denoted as ArMB1, ArMB2, and ArMB3. The precise locations of their origin in Manitoba are not known. Two control populations (ArSC1 and ArSC2) known to be susceptible to ALS inhibitors were collected at the University of Manitoba Ian N. Morrison Research station at Carman. Seeds were stored in seed storage room at 5 C in Plant Science department at University of Manitoba until further use.

To determine germinability of the seedlots, fifty seeds from each of the five populations (ArMB1, ArMB2, ArMB3, ArSC1 & ArSC2) were planted in trays (50.8 x 25.4 x 7.62 cm) (one tray per population to limit seed use) filled with a soil mixture of 40% sand, 40% topsoil, 20% peat & Sunshine-Mix (Sun Gro Horticulture, Canada Ltd., Vancouver, British Columbia, Canada). The soil mixture was fertilized with 53 kg P ha^{-1} & 11 kg N ha^{-1}. The germination percentage of the five populations was determined from the number of seedlings that germinated divided by the number of seeds that were planted, multiplied by 100. This test was conducted only once and therefore there was no replication for each population.

Seeds of ArMB1, ArMB2, ArMB3, ArSC1 & ArSC2 were germinated in small pots (10.2 x 7.6 x 10.2 cm) containing a mixture of 40% topsoil, 40% sand, 20% peat & Sunshine-Mix (Sun Gro Horticulture, Canada Ltd., Vancouver, British Columbia, Canada) fertilized with 11 kg N ha^{-1} & 53 kg P ha^{-1}. About 5-6 seeds per pot were planted into the soil mixture at a depth of 1 cm and thinned to one seedling per pot shortly after seedling emergence. The pots were kept under growth

room conditions (at 25/20 °C and 16/8 h day/night at a relative humidity of 75% throughout the study). The plants were watered daily as needed.

3.2 Dose response assay

In order to confirm and determine the level of resistance to ALS inhibitors (imazethapyr or thifensulfuron-methyl), dose response assays were performed among the five populations. This experiment was arranged in a completely randomized design (CRD) with five replications and nine treatments. To determine the dose response to ALS inhibitors, 45 seedlings of each population of ArMB1, ArMB2, ArMB3, ArSC1 & ArSC2 were grown as described above and allowed to grow to the 4-6 leaf stage. Then Imazethapyr herbicide (Pursuit® 240, BASF Canada Inc., Mississauga, ON, Canada) from the imidazolinone family or thifensulfuron-methyl herbicide (Pinnacle SG, Du Pont Canada Inc. Missisauga, ON) from the sulfonylurea family were applied at various doses (0, 0.01, 0.1, 1, 10, 100, 1000, 2500, 5000, 10000 g ai ha^{-1}) to confirm and determine the dose response of these five populations to the two ALS inhibitors in separate assays. In Manitoba, the recommended field dose for postemergence application of imazethapyr is 50 g ai ha^{-1} and for thifensulfuron-methyl is 4.1 g ai ha^{-1} (Anonymous 2021a, 2021b). Herbicide treatments were applied to the plants using a spray cabinet (Pesticide Spray Chamber, Model 2, Agassiz Scientific Ltd. of Saskatoon, SK, Canada) equipped with a Teejet 80015VS nozzle (Spraying Systems Co., Wheaton, IL) calibrated to deliver 175 L ha^{-1} at 241.3 kPa. After treatment application, the plants were moved back to the growth room and maintained using the conditions as described above. Three weeks after herbicide application, the shoots of all plants (dead or alive) were cut at soil level and frozen. These shoots were then lyophilized and weighed. The lyophilized leaf samples were kept for DNA extraction at a later date. The experiment was conducted four times with imazethapyr and three times with thifensulfuron-methyl.

3.3 Malathion experiment to determine potential contributions of NTSR

To determine the presence of non-target site resistance, seedlings from all populations were treated with malathion which is known to inhibit cytochrome P450 enzymes in a separate experiment. The experiment followed a completely randomized design (CRD) with five replications and eight treatments and was repeated at least three times. A total of forty seedlings for each population were used in each of these experiments.

According to Ma et al. (2013), seedlings were treated with malathion insecticide (Hi-Yield Chemical Co., Bonham, TX) when redroot pigweed seedlings were at the 4-6 leaf stage. Malathion was applied at a single dose of 2000 g ai ha^{-1} one hour prior to treatment with the herbicides imazethapyr or thifensulfuron-methyl using the same spray cabinet and spray conditions as above. The eight treatments were comprised of three doses (10, 100 & 1000 g ai ha^{-1}) of imazethapyr or thifensulfuron-methyl with malathion or without malathion, malathion alone (2000 g ai ha^{-1}) and water. This experiment was run in conjunction with the dose response assay and the treatments were applied on the same day. Treated plants were placed into the growth room and maintained under the same conditions as described above. The shoots of all plants (dead or alive) were cut at soil level after three weeks of herbicide application, frozen, lyophilized and weighed.

3.4 ALS gene sequencing

A total number of forty lyophilized leaf samples from forty plants of the five populations (8 ,8, 12, 5 and 7 plants for ArMB1, ArMB2, ArMB3, ArSC1 and ArSC2, respectively) were ground into a fine powder by using a Savant Bio 101 Fastprep FP120 system (Savant Instruments, Inc., Holbrook, NY, USA). DNA was extracted from the forty samples following the protocol outlined in the DNeasy Plant Mini Kit (Qiagen Inc.). DNA quantity was determined by using an Epoch

Microplate Spectrophotometer (BioTek Instruments, Inc., Highland Park, USA) and 1% (wt/v) agarose gel electrophoresis was used to determine the quality of extracted DNA.

The ALS gene was sequenced from the forty samples of the five populations using a tiling approach on an iSeq 100 Sequencing System (Illumina, Inc., San Diego, USA). Fourteen primers pairs (Table 1) were developed to cover the 2065 bp length of the ALS gene using the tiling approach that ensured adequate coverage of the entire region of interest and covered all eight known sites of mutations for the ALS gene (that confer resistance to ALS inhibitor herbicides). The first 6 sets of primers were designed according to McNaughton et al. (2005) and remaining eight primer sets were designed using the IDT (Integrated DNA Technologies, Inc.Coralville, Iowa, USA) PrimerQuest^{TM} primer design tool. The reference sequence used for this experiment was *Amaranthus retroflexus* L., Accession AF363369.1(McNaughton et al. 2005). To facilitate barcoding of the plant samples for sequencing, Illumina adapter overhang nucleotide sequences were added to the 5'-end of each primer pair during primer design. These additional sequences were:

Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG- [locus specific sequence] as the forward primer overhang and 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG- [locus specific sequence] as the reverse primer overhang.

Table 1: Sequences of Pimers with annealing temperature used for PCR amplification and sequence of ALS gene.

Set	Primers	Annealing temperature	Nucleotide sequence[a]	Amplicon length (bp)
1	1F	59	5′-CTTCAAGCTTCAACAATG-3′	476
	1R		5′-CCAGTAGCACGAGCGTAG-3′	
2	2F	58	5′-GATGTYCTCGTYGARGCTCT-3′	456
	2R		5′-AAYCAAAACAGGYCCAGGTC-3′	
3	3F	53	5′-GTGTTAGATGTTGAGGATATTCC-3′	394
	3R		5′-GCATTCCCAACATATGAA-3′	
4	4F	56	5′-GTGGCTAGTACTTTAATGGGG-3′	539
	4R		5′-GAGGTCAGCCATTGGCGA-3′	
5	5F	59	5′-ATTCCTCCGCARTATGCSATT-3′	525
	5R		5′-CAGRTAWGGTCCTGGRGTATCC-3′	
6	6F	57	5′-TTCTTCCGAAATCTTCCC-3′	274
	6R		5′-CCTACAAAAAGCTTCTCCTCTATAAG-3′	
7	212F	57	5′-GAAATGTTCTTCCTCGACA-3′	206
	212R		5′-AGCATCAGTACCAATCATAC-3′	
8	222F	58	5′-TGACTCAGTCCCTCTTG-3′	199
	222R		5′-AACAGGTCCAGGTCTAC-3′	
9	311F	58	5′-GTAGACCTGGACCTGTTT-3′	243
	311R		5′-CGACAAATTTCCTCAATTCTTC-3′	
10	322F	57	5′-ACCCACTTATTCTGCTAAT-3′	256
	322R		5′-CTTATCAACCGCGTAATTC-3′	
11	412F	58	5′-GGGCTTTCCCTTGTACT-3′	245
	412R		5′-CACTTTAACATCACCACAAATC-3′	
12	421F	59	5′-GCTGAAATCGGGAAGAATAA-3′	225
	421R		5′-CTCGTCAAGAACCTGAATG-3′	
13	512F	58	5′-GGCTGCCCAATTCTATAAG-3′	198
	512R		5′-GGGAGATTCTCTACCCTAATC-3′	
14	521F	58	5′-CTATTGGAGCTGCTGTTG-3′	248
	521R		5′-GAGCATATCCGGGAAGAT-3′	

[a] Single letter abbreviations for mixed base positions: R=A, G; S=G, C; W=A, T; Y=C, T.

Amplicon PCR reactions were performed to amplify target fragments of DNA with the primers using T100TM Thermal Cycler (Bio-Rad, Hercules, CA). Each PCR reaction was comprised of 3 µl of genomic DNA (6-60 ng/ µl), 3 µl of forward and reverse primers (10µM) each, 2.75 µl of PCR grade water, 0.75 µl of DMSO and 12.5 µl PCR Master Mix (Phusion High-Fidelity PCR Master Mix with GC Buffer, Thermo Fisher Scientific, Inc. Winnipeg, MB, Canada). Amplicon PCR amplification was performed following 5 min incubation at 95 C; 35 cycles of 0.30 min at 95 C, 0.30 min at XC (where XC is the specific annealing temperature for each primer pair (Table 1)), and 1 min at 72 C; then 5 min at 72 C, and finally hold at 12 C for 10 min.

To visualize the PCR products, 1% (wt/v) agarose electrophoresis gels were run. The PCR products were purified using the AMPure beads XP (Beckman Coulter, Inc. Indianapolis, Indiana, USA) after PCR amplification to remove free primers, primer dimers and other contaminants. After DNA purification, DNA quantification was done by using a Qubit Fluorometer (Thermo Fisher Scientific, Inc. Waltham, MA USA) following manufacturer's instructions. The concentration of the PCR products was then standardized for each sample by calculating the amount of sample required to prepare a solution with a concentration of 50 ng in 60 µl. Then fourteen amplicons of each sample were pooled together into 0.2 ml tube in preparation for the labelling PCR reaction.

Then the second PCR reactions (Index PCR) were conducted with Index primers (IDT for Illumina DNA/RNA UD Indexes- Set A, Illumina, Inc., San Diego, USA) to add the indices with the sequencing adapters to the target amplicons so sequences could be assigned to individual plants after sequencing. Each labelling reaction contained 10 µl index primers, 25 µl Phusion Master Mix, 10 µl of PCR grade water and 5 µl of DNA (5 ng/ µl). The PCR conditions consisted of 95 C for 3 mins; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes; and hold at 4°C.

The PCR products were purified again after Index PCR amplification using the AMPure beads XP (Beckman Coulter, Inc. Indianapolis, Indiana, USA) to get rid of non-specific smaller fragments. Qubit fluorometric quantification was performed again after DNA purification. Then each of the forty DNA samples was diluted to a concentration of 1 nM by adding 10 mM Tris (pH 8.5) for normalization. Then 5 µl of diluted DNA from each library were pooled in a 0.5 ml tube. The pooled library was then diluted again by adding 10 µl of the library to 90 µl of 10 mM Tris (pH 8.5) to prepare the library loading concentration. To increase base diversity 1 nM 10% spike-in PhiX (Illumina, Inc., San Diego, USA) which is a ready to use for the Illumina library with balanced nucleotide representation was prepared. The PhiX was also diluted by adding 10 µl of 1 nM of PhiX to 90 µl of 10 mM Tris (pH 8.5) to prepare the PhiX loading concentration. For final library preparation 10 µl of PhiX from PhiX loading concentration was added to 100 µl of DNA library loading concentration. From there 20 µl of final library sample was loaded on a sequencing cartridge and the cartridge was placed on the iSeq 100 Sequencing System (Illumina, Inc., San Diego, USA) and run.

The resulting gene sequences were analyzed first using the Illumina Local Run Manager (LRM) software (Resequencing module) (Illumina, Inc., San Diego, USA). Quality metrics were checked using Sequencing Analysis Viewer (SAV) software (Illumina, Inc., San Diego, USA) and Integrative Genomics Viewer (IGV) software was used to visualize and compare the gene sequences of the five populations.

3.5 Statistical analysis

3.5.1 Target site resistance experiment

Statistical analysis was performed using SAS 15.3 (SAS Institute, Cary, NC 27513). Non-linear mixed model regression analysis was performed on percent of shoot dry weight (measured as percentage of the untreated control) in response to herbicide doses by using PROC NLMIXED in SAS (Seefeldt 1995). Experimental data was modelled to a 4-parameter log-logistic function (Equation 1) as it describes the dose response behavior more precisely (Bowley 2008; Seefeldt 1995).

[1] $$Y = C + \frac{D-C}{1+\exp\left(b(\log(x)-\log\left(GR50\right)\right)}$$

Where, Y is the percent of shoot dry weight, x is the herbicide dose, C is the lower limit, D is the upper limit, GR50 is the dose required to reduce plant dry weight by 50 percent between the upper and lower limits, and b is the slope of the curve at the inflection point (which in this case is the GR50). In this experiment, the herbicide treatments were considered as fixed effects and the experimental replication (run) was considered a random effect in the non-linear mixed model. By choosing a set of values minimizing the negative log likelihood initial parameters were optimized following the sample code described by Coffey (2016). For each parameter, a range of probable values was specified and within that range stepwise increases were used. To achieve convergence of the procedure, two strategies were followed as necessary: (1) to keep variance estimates greater than or equal to zero a bounds statement was invoked, and (2) the criterion of the relative gradient convergence was set to zero (Kiernan et al. 2012).

To compare the parameters single-degree-of-freedom estimates were used. For letter mean separation, Fisher's LSD at a significance level of 5% ($\alpha = 0.05$) was used and letter separation was based on the single-degree freedom estimates. To determine the level of resistance (resistance factors) GR50 value of the resistant population was divided by the GR50 value of the susceptible reference population (ArSC1).

3.5.2 Non-target site resistance experiment

In order to determine if NTSR mechanism was contributing to ALS resistance among the redroot pigweed populations, biomass data were used to determine percent biomass reduction in comparison with the untreated control by using the following formula (Chahal et al.2015):

$$[2]\ \text{Biomass reduction}\ (\%) = [(C - B)/C] \times 100$$

where C is the mean biomass of the untreated control and B is the biomass collected from treatments. Data from this experiment were subjected to analysis of variance (ANOVA) using PROC MIXED. Data were checked for Gaussian distribution by using the Shapiro-Wilk statistic (Littell et al. 2006). To determine if there were extreme outliers present in the data sets Lund's test (Lund 1975) was used. Visual inspection of residual versus predicted values (Kozak and Piepho 2018) were done to test homogeneity of variance and was corrected using the group option in the repeated statement to minimize the Akaike information criterion (Littell et al. 2006). To compare the mean biomass reduction of the herbicide treatment (imazethapyr or thifensulfuron-methyl) with malathion or without malathion, single-degree-of-freedom estimates were used.

Chapter 4: Results

4.1 Dose response

4.1.1 Dose response to imazethapyr

Based on the above ground dry biomass data at three weeks after treatment (WAT), the dose response curves for all three suspected resistant redroot pigweed (*Amaranthus retroflexus* L.) populations (ArMB1, ArMB2 & ArMB3) were different from the response curves of the two control populations of redroot pigweed (ArSC1 & ArSC2). In all cases, the dose response curves confirmed resistance to imazethapyr in the suspected resistant redroot pigweed populations (Figure 1). In the parsimonious model; C, the lower limit (16.77 +/-6.32) and D, the upper limit (98.37 +/-4.18) were held constant as no differences were found in these parameters among the populations. Despite modelling the *b* parameters individually in the parsimonious model, no differences were found among the *b* parameters of the populations which ranged from 0.47 to 1.08 among the five populations (Table 2). The *b* parameter represents the linear portion of the slope of the curve at the midway point before the curvilinear decline in the slope that results in the lower plateau. Differences among the curves were found among the GR50 parameters. The GR50 is the dose (g ai ha^{-1}) of imazethapyr that resulted in a 50% reduction in shoot dry weight. All resistant populations showed GR50 values many–fold greater than the two susceptible populations and the GR50 values of the ArMB1 and ArMB3 populations were 3 to 5-fold greater than the GR50 of the ArMB2 population.

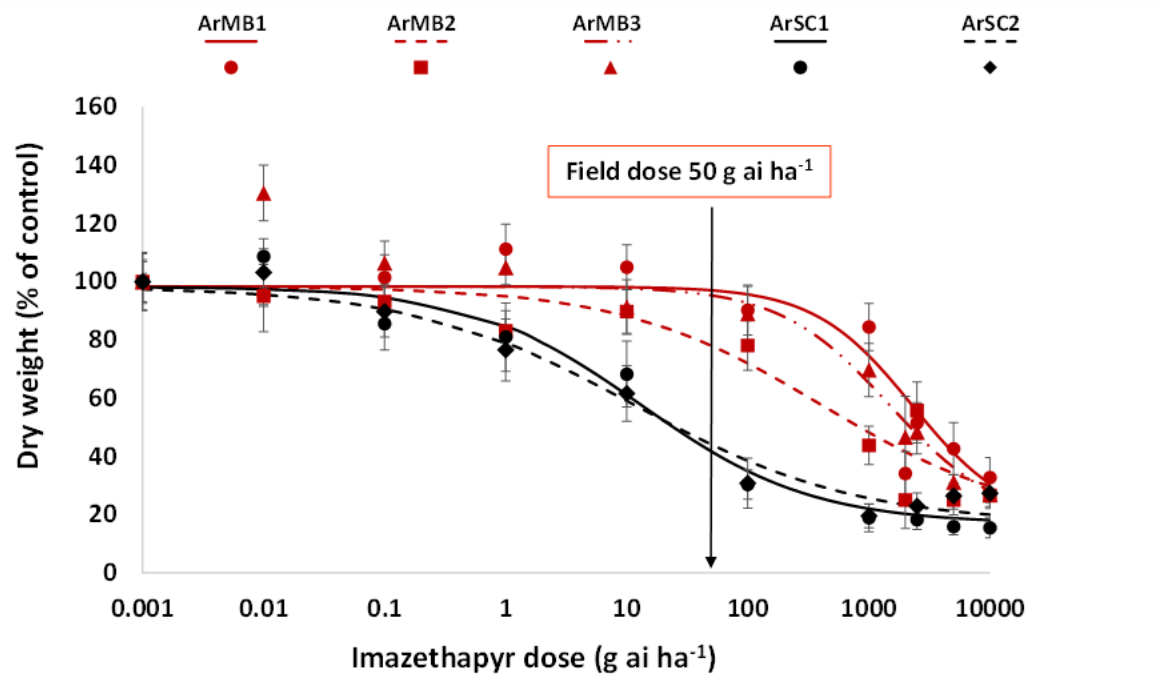

Figure 1: Dose-response curves of ArMB1, ArMB2, ArMB3, ArSC1 & ArSC2 redroot pigweed populations to imazethapyr. Dry weight was expressed as a percentage of the untreated control. Each symbol represents the mean of four experimental runs with five replicates each, and each line represents the log-logistic model. The vertical bars represent +/- one standard error of the mean.

The ArSC1 and ArSC2 populations were sensitive to imazethapyr and the biomass of the plants were reduced by approximately 60% at the field dose (50 g ai ha^{-1}) three weeks after treatment (WAT) (Figure 1). All plants of these two susceptible populations (ArSC1 and ArSC2) became chlorotic and were controlled fully by imazethapyr at 3 WAT at a dose of 1000 g ai ha^{-1} (Figures 1, 2). On the contrary, the biomass reduction was only about 5%, 25% and 6% for the populations ArMB1, ArMB2 and ArMB3, respectively at the field dose of imazethapyr 3 WAT. In fact, plants these three suspected resistant populations (ArMB1, ArMB2 and ArMB3) were not controlled at a dose 1000 g ai ha^{-1} which is about 20 times the registered field rate (Figure 1). Moreover, two of the populations ArMB1 and ArMB3 had GR50 values greater than 1000 g ai ha^{-1}.

Table 2: Parameter Estimates and Resistance factors of dose response curves of three suspected resistant and two susceptible populations of redroot pigweed to imazethapyr. Standard Error (SE) is the Standard Error of the Estimate.

Population	**Parameter Estimates**[a]				**RF**[c]	
	Slope (*b*)	SE	GR50	SE	Compared with	
	Biomass (BM) reduction {% shoot BM (g ai ha^{-1})$^{-1}$}		----(g ai ha^{-1})----		ArSC1	ArSC2
ArMB1	1.08	0.34	2269 a[b]	540.6	171.3	194.0
ArMB2	0.52	0.15	408 b	193.1	30.8	34.9
ArMB3	0.96	0.33	1483 a	411.6	112.0	126.8
ArSC1	0.62	0.20	13.24 c	6.4		
ArSC2	0.47	0.16	11.69 c	7.7		

[a] Slope *b* represents the parameter of the log-logistic model (see equation 1) of the five populations (ArMB1, ArMB2, ArMB3, ArSC1 & ArSC2) of the best fitting curves through the GR50 where GR50 are the effective doses (g ai ha^{-1}) of imazethapyr required for 50% shoot dry weight reduction.
[b] Fisher's LSD at a significance level of 5% (α=0.05) was used to separate the means. When similar letters are shared between means within the column, these means are not significantly different from each other.
[c] Resistance Factor (RF) is calculated as a ratio of GR50 of a resistant population to GR50 of the susceptible population.

The two susceptible redroot pigweed populations ArSC1 & ArSC2 had GR50 values of 13.2 & 11.7 g a.i. ha^{-1} and these were not significantly different. Resistance factors were determined between each resistant population and each susceptible population. As expected, the differences between the resistance factor values (RFs) determined individually from the two susceptible redroot pigweed populations were subtle (Table 2). The highest GR50 value 2269 g ai ha^{-1} was found in ArMB1, which was much greater than that of the two susceptible populations (Figure 1, Table 2). Resistance factor (RF) values for ArMB1 were 171.3 and 194 compared with ArSC1 and ArSC2, respectively. The second highest GR50 value of 1483 g a.i. ha^{-1} was reported for ArMB3

which was 112-fold greater than ArSC1 & 126.8-fold greater than ArSC2. A GR50 value of 408 g ai ha^{-1} was recorded for ArMB2 which was 30.8 times & 34.9 times higher than ArSC1 & ArSC2, respectively. The RFs among the 3 resistant redroot pigweed populations ranged from about 30 to almost 200. Resistance to imazethapyr, however, was only significantly different between ArMB2 and the other two populations ArMB1 and ArMB3. The RFs of these two populations (ArMB1 and ArMB3) were not significantly different.

Figure 2: Response of redroot pigweed populations ArMB1, ArMB2, ArMB3, ArSC1 & ArSC2 to different doses of imazethapyr at 3 weeks after treatment (WAT).

The results of the two susceptible redroot pigweed populations ArSC1 and ArSC2 were not combined even though these two were not statistically different because ArSC2 showed low seed viability (or increased seed dormancy), and this population did not grow as well as all other redroot pigweed populations. The lowest germination percentage was observed for ArSC2 (Table 3) (although this germination test was conducted only once) and generally plants did not appear as vigorous as the plants from the ArSC1 population (visual observations). That's why it was thought

to be best to treat the two control populations separately in this analysis. Nevertheless, these two susceptible populations behaved the same in response to imazethapyr.

Table 3: Germination percentage of the five redroot pigweed populations (ArMB1, ArMB2, ArMB3, ArSC1 & ArSC2).

Population	Germination (%)
ArMB1	50
ArMB2	74
ArMB3	74
ArSC1	68
ArSC2	32

4.1.2 Dose response to thifensulfuron-methyl

The dose response curves for the five redroot pigweed populations to thifensulfuron-methyl were generated following the same Log-logistic model where *D* is the upper limit (99.90 +/- 3.01) & *C* is the lower limit (8.19 +/- 2.19) of this model and as in the imathezapyr experiments, the values also were consistent among the populations. The *b* parameters of the dose response curves ranged from 0.41 to 1.13 among the five populations and unlike the imazethapyr experiment, differences among the *b* parameters were observed in this experiment (Table 4). The *b* parameters or linear portion of the slope for ArMB1, ArMB2 and ArSC1 were the same and they were significantly greater than the *b* parameter of ArMB3 (Table 4). That means that in the three populations with greater slopes, the reduction in biomass per unit herbicide dose was greater compared with ArMB3 during the linear portion of the dose response curve. The parameter *b* of ArSC2 was intermediate and was not different from that of ArMB3 or that of the three remaining populations (Table 4).

Table 4: Parameter Estimates and Resistance factors of dose response curves of three suspected resistant and two susceptible redroot pigweed populations to thifensulfuron-methyl. Standard Error (SE) in here is the Standard Error of the Estimate.

Population	**Parameter Estimates**[a]				**RF**[c]	
	Slope (*b*)	SE	GR50	SE	Compared with	
	Biomass (BM) reduction {% shoot BM (g ai ha^{-1})$^{-1}$}		-----(g ai ha^{-1})-----		ArSC1	ArSC2
ArMB1	1.02 a[b]	0.32	0.48 b	0.12	0.53	15.94
ArMB2	1.13 a	0.26	0.55 b	0.13	0.61	18.30
ArMB3	0.41 b	0.06	6.68 a	2.83	7.35	220.6
ArSC1	0.75 a	0.15	0.91 b	0.28		
ArSC2	0.56 ab	0.12	0.03 c	0.01		

[a] Slope *b* represents the parameter of the log-logistic model (see equation 1) of the five populations (ArMB1, ArMB2, ArMB3, ArSC1 & ArSC2) of the best fitting curves through the GR50 where GR50 are the effective doses (g ai ha^{-1}) of thifensulfuron-methyl required for 50% shoot dry weight reduction.
[b] Fisher's LSD at a significance level of 5% (α=0.05) was used to separate the means. When similar letters are shared between means within the column, these means are not significantly different from each other.
[c] Resistance Factor (RF) is calculated as a ratio of GR50 of a resistant population to GR50 of the susceptible population.

Differences among the curves were also found among the GR50 parameters of the five populations. Population ArMB3 had the greatest GR50 (6.68 g ai ha^{-1}) while the GR50s of ArMB1 (0.48 g ai ha^{-1}) and ArMB2 (0.55 g ai ha^{-1}) were not different from the GR50 of ArSC1 (0.91 g ai ha^{-1}) (Table 4). This, together with similar *b* parameters, indicated that these populations which proved to be resistant to imidazolinine, were not resistant to thifensulfuron-methyl. The GR50 of the second susceptible redroot pigweed population (ArSC2) was significantly lower (0.03 g ai ha^{-1}) than that of ArSC1, although this had no practical implications. Taken together, with the smallest slope and

largest GR50, the dose response curve of ArMB3 was significantly different from all other populations (Figure 3).

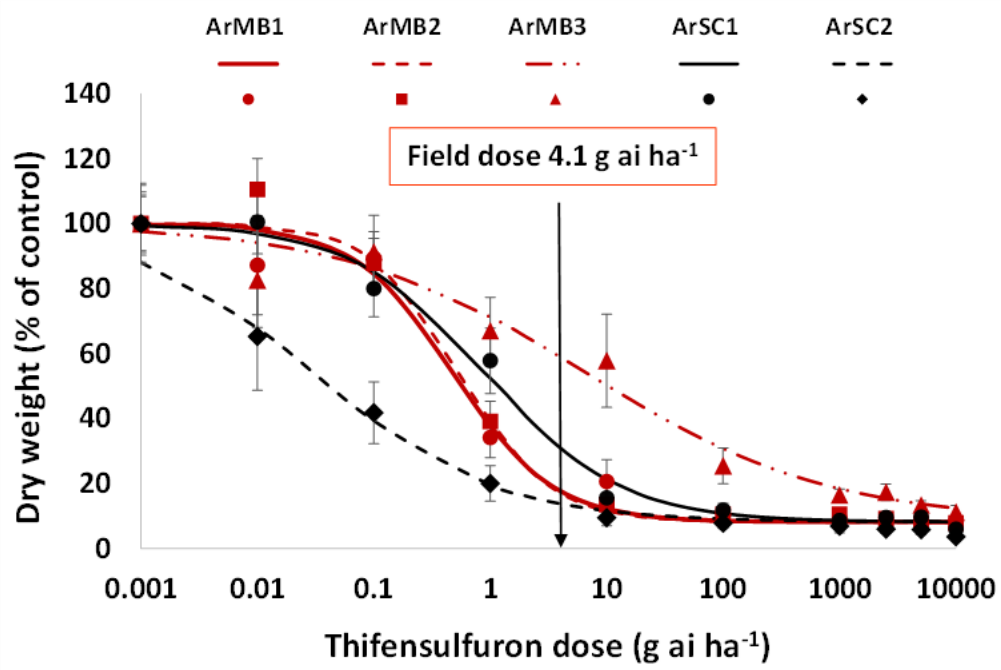

Figure 3: Dose-response curves of ArMB1, ArMB2, ArMB3, ArSC1 & ArSC2 redroot pigweed populations to thifensulfuron-methyl. Dry weight was expressed as a percentage of the untreated control. Each symbol represents the mean of three experimental runs with five replicates each, and each line represents the log-logistic model. The vertical bars represent +/- one standard error of the mean.

The redroot pigweed populations ArMB1, ArMB2, ArSC1 and ArSC2 were controlled at the field dose of thifensulfuron-methyl (4.1 g ai ha^{-1}). At the field dose, biomass was reduced by around 69-87% among these populations at 3 WAT (Figure 3), while biomass was reduced only by 41% in population ArMB3 at the field dose of thifensulfuron-methyl. ArMB3 showed a low level of resistance (RF 7.35) to thifensulfuron-methyl when using ArSC1 as the susceptible control, but a high level of resistance (RF 220.6) when using ArSC2 as the susceptible control, even though both

control populations were considered susceptible to thifensulfuron-methyl at the normal field dose. It is very prominent that the two susceptible populations responded differently to thifensulfuron-methyl (based on the GR50 parameter) (Table 4; Figure 3, 4) but not to imazethapyr (Table 2). Whether this was related to the poor germination and growth of ArSC2 (Table 3), or other factors is not known, however, the statistically different behavior of ArSC2 in this experiment contributes to the justification of keeping the susceptible control populations separate in the analysis of both dose response experiments. Based on the results of dose response experiments only ArMB3 showed a low level of cross resistance between the two tested members of the imidazolinone and sulfonylurea families of the ALS-inhibitors while the other two populations resistant to imidazolinone herbicides showed no level of cross-resistance between these two ALS inhibitors.

Figure 4: Response of redroot pigweed populations ArMB1, ArMB2, ArMB3, ArSC1 & ArSC2 to different doses of thifensulfuron-methyl at 3 weeks after treatment (WAT).

4.2 ALS sequence analysis

To investigate the mechanism of resistance to the ALS inhibitors (imazethapyr or thifensulfuron-methyl), the ALS gene (2065 bp in length) from all 5 redroot pigweed populations was amplified and sequenced. Sequence analysis revealed that the known susceptible populations ArSC1 and ArSC2, only showed the wild-type sequence (the reference sequence was *Amaranthus retroflexus* L., Accession AF363369.1) (Figure 5, Table 5). The experiments, however, identified point mutations (Ser653Asn/Ile) among the three resistant redroot pigweed populations that, to my knowledge, the substitution Ser653Ile has not been reported in redroot pigweed before. About 100% of the ArMB1 plant samples & 87.5% of the ArMB2 plant samples had a single nucleotide polymorphism resulting in a serine (AGC) to asparagine (AAC) amino acid substitution at position 653 when compared with ArSC1 or ArSC2. Other 12.5% ArMB2 plant samples did not show any substitutions at all the known codon positions on ALS gene (numbered relative to the ALS amino acid sequence of *Arabidopsis thaliana*). The substitution at position 653 of the ALS sequence in the ArMB3 population was not simple as in the other two ALS resistant redroot pigweed populations. About 8.3% of the plant samples had the same nucleotide polymorphism as ArMB1 & ArMB2 while a different set of ArMB3 plants (33.3%) showed a second nucleotide polymorphism that resulted in a serine (AGC) to isoleucine (ATC) substitution at position 653 (Figure 5, Table 5). About 58.4% of the ArMB3 plants had the wild-type gene sequence throughout all known sites that result in ALS-inhibitor resistance in this gene.

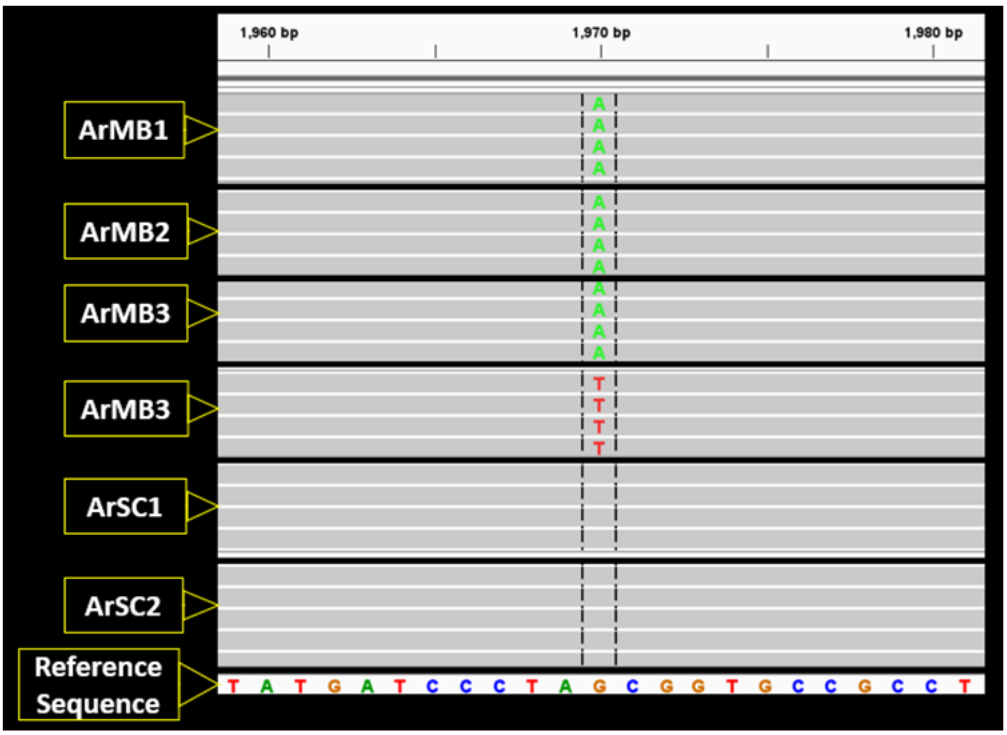

Figure 5: A piece of ALS gene sequence alignment from some of the samples of the five populations with mutations (in resistant populations ArMB1, ArMB2 & ArMB3) and without mutations (in susceptible populations ArSC1 & ArSC2) by using Integrative Genomics Viewer (IGV) software.

Table 5: Nucleotide bases & amino acid sequences of ALS gene at position 653 in each population. Wild type sequence and amino acid composition are indicated in black while mutations and substitutions are indicated in red or blue.

Population	Nucleotide substitution	Amino acid substitution	Total number of plants sequenced	Number of plants found with amino acid substitution	Percent of plants found with amino acid substitution
ArMB1	AAC	Asn	8	8	100
ArMB2	AAC	Asn	8	7	87.5
ArMB3	AAC / ATC	Asn / Ile	12	1+4	8.3+33.3
ArSC1	AGC	Ser	5	0	0
ArSC2	AGC	Ser	7	0	0

The sequence data revealed that zygosity of the resistant allele was not equally uniform among the resistant redroot pigweed populations. While 100% of the plants of the ArMB1 population were homozygous for the resistant ALS gene, only about 86% and 80% plants were homozygous and 14% and 20 % plants were heterozygous for the resistant allele in the ArMB2 and ArMB3 populations, respectively (Table 6). Homozygous alleles may confer an increased level of ALS-resistance than heterozygosity, however, the level of heterozygosity found in these experiments did not appear to affect the phenotype. The population ArMB1 had more homozygous individuals than ArMB3, yet these two populations had similar dose response curves. Overall, however, the results did confirm that target-site resistance mechanism is associated to the ALS-inhibitor resistance among the three resistant redroot pigweed populations.

Table 6: Percent of homozygous and heterozygous individuals found from ALS gene sequence of the three resistant redroot pigweed populations.

Population	Homozygous (%)	Heterozygous (%)
ArMB1	100	0
ArMB2	86	14
ArMB3	80	20

4.3 Non-target site resistance

To determine non-target site resistance (NTSR) via cytochrome P450 monooxygenase, the three resistant redroot pigweed populations (ArMB1, ArMB2 & ArMB3) were treated with or without a single dose of malathion (cytochrome P450 inhibitor) 1 hour prior to imazethapyr or thifensulfuron-methyl herbicide application at three doses of each herbicide (10, 100 & 1000 g ai ha^{-1}). Based on the biomass reduction data, it was observed that regardless of herbicide dose there was no significant difference between the plants treated with imazethapyr plus malathion compared with no malathion among the three resistant redroot pigweed populations (Table 7). The biomass reductions ranged from -15.11% (+/-13.79) to 19.32% (+/-15.10) among the three resistant populations. Positive values indicate that biomass was reduced, and negative values indicate the biomass increased when herbicide was applied with malathion compared with no malathion. Therefore, significant positive numbers (i.e., a reduction in plant biomass when pre-treated with malathion compared with when treated with the herbicide alone) are indicative of non-target site resistance as herbicide function was regained. In some cases, the biomass reduction was quite large (e.g., 19.32% (+/-15.08) in ArMB3 at 1000 g ai ha^{-1}) but was not significantly different

from the untreated control plants. This appeared to be due to large variation within the treatments which may have been caused by differences among the plants in size at the time of treatment application. The malathion alone treated plants were also compared with the water treated plants of the three resistant populations. No significant differences in the shoot biomass were found between these two treatments (Table 7).

Table 7: Biomass reduction in plants treated with herbicide (imazethapyr or thifensulfuron-methyl) plus malathion compared with plants treated with no malathion in ArMB1, ArMB2 & ArMB3 populations.

	ArMB1		**ArMB2**		**ArMB3**	
Herbicide doses with malathion vs no malathion	**Biomass[a] reduction** ----------%	**SE[b]** ----------	**Biomass reduction** ----------%	**SE** ---------	**Biomass reduction** ----------%	**SE** ---------
Imazethapyr (g ai ha^{-1})						
10	10.82 n.s.	21.59	11.31 n.s.	12.42	-5.97 n.s.	18.40
100	8.24 n.s.	14.12	3.46 n.s.	14.96	5.86 n.s.	14.88
1000	6.19 n.s.	21.01	-15.11 n.s.	13.79	19.32 n.s.	15.08
Water vs Malathion	-10.80 n.s.	17.76	-14.17 n.s.	15.52	-16.75 n.s.	15.55
Thifensulfuron-methyl (g ai ha^{-1})						
10	8.82 n.s.	7.60	3.96 n.s.	6.99	22.71 n.s.	13.83
100	4.64 n.s.	4.37	2.64 n.s.	5.07	0.07 n.s.	5.53
1000	2.66 n.s.	3.27	4.17 n.s.	6.42	4.39 n.s.	3.85
Water vs Malathion	9.35 n.s.	33.85	-27.96 n.s.	15.38	-33.99 n.s.	20.76

[a]Positive values indicate the biomass was reduced and negative values indicate the biomass was increased after treatment with malathion. Significance of single degree freedom estimates is indicated at alpha=0.05 (n.s.= not significant). The data presented here was the combined results from three runs.
[b]Standard Error (SE) is the Standard Error of the Estimate.

As with imazethapyr, no significant reduction in shoot biomass was observed among the three resistant redroot pigweed populations when three doses of thifensulfuron-methyl were applied with malathion compared with no malathion (Table 7). The biomass reduction ranged from 0.07% (+/-5.53) to 22.71% (+/-13.83). The biomass reduction in plants treated with malathion alone also were not significantly different from the plants treated with water in the three resistant populations (Table 7).

The treatment standard errors around the biomass reductions were much greater in the imazethapyr experiments than the thifensulfuron-methyl experiments. This could be because compared with the field dose of each herbicide, the two doses (100 & 1000 g ai ha^{-1}) are much higher for thifensulfuron-methyl (around 25 and 250 times the field dose) than the imazethapyr (2 and 20 times). That means at these high doses of thifensulfuron-methyl all plants had died leaving only dead tissues at the time of sampling. The imazethapyr treated plants on the other hand, still had some live tissue at the time of sampling as the high doses were not as lethal for this active ingredient. The slower necrosis in the imazethapyr treated plants likely prolonged growth and may have contributed to the increase variation among the individual plants in these experiments. The NTSR analysis revealed that, regardless of the resistant line, no significant differences were found between the treatments of imazethapyr or thifensulfuron-methyl treated with malathion or without malathion. The data showed that ALS-resistance observed in the three resistant redroot pigweed populations was not associated with cytochrome P450 induced NTSR mechanism when plants were tested with the cytochrome P450 inhibitor, malathion.

Chapter 5: Discussion

The objectives of the research were to determine whether the three suspected redroot pigweed (*Amaranthus retroflexus* L.) populations from Manitoba are resistant to the ALS inhibitors imazethapyr and thifensulfuron-methyl, to quantify the resistance level and to determine the mechanisms of resistance. The results of the study revealed that the three suspected resistant populations (ArMB1, ArMB2 & ArMB3) have varying degrees of resistance (30.8 to 171.3-fold) to the herbicide imazethapyr while only one population (ArMB3) showed a low level of resistance (7.3-fold) to thifensulfuron-methyl compared with the susceptible reference population (ArSC1). Due to high variability and inconsistent performance in the other reference population (ArSC2) the results obtained using that population may be less reliable. The level of resistance generally is considered low to moderate resistance when the resistance factor in a biotypes is less than ten and considered high when the resistance factor is greater than ten (Heap 2023).

Redroot pigweed with high resistance factors to imazethapyr have been documented in several countries including Brazil, Canada, China, Italy, Serbia, Ukraine, and USA (Heap 2023). In my study, the resistance factors of the three resistant redroot pigweed populations were high and ranged from 30.8 to 171.3-fold in comparison with the susceptible population (ArSC1) to the imazethapyr. Similar results were found in Ontario, Canada where five redroot pigweed populations showed 33- to 168-fold resistance to imazethapyr compared with susceptible control plants (Ferguson et al. 2001). Some biotypes of tall waterhemp from Illinois were found with greater than 1000-fold resistance to imazethapyr (Foes et al. 1998). A biotype of powell amaranth from Perth County, Ontario was documented 109-fold resistance to imazethapyr (Diebold et al. 2003, Heap 2023). Waterhemp populations from Illinois exhibited 74- to 860-fold more resistance in comparison with the susceptible biotype to imazethapyr (Patzoldt and Tranel 2007). Whaley et

al. (2006) recorded 261- to 537-fold resistant smooth pigweed populations to imazethapyr from Virginia, Delaware and Maryland (USA). Green foxtail [*Setaria viridis* (L.) Beauv.], a grass weed exhibited 21- to 182-times more resistance to imazethapyr than the susceptible biotype in Ontario, Canada (Laplante et al. 2009). False Cleavers (*Galium spurium* L.) also was found highly (>100-times) resistant to imazethapyr (Beckie et al. 2012a). Many weed biotypes of different weed species showed various degrees of high resistance to imazethapyr herbicide.

The three resistant redroot pigweed populations showed high resistance to imazethapyr and the resistance level varied among the populations. The resistance factor for ArMB2 to imazethapyr was lower compared with the other resistant populations (ArMB1 and ArMB3). The variation among the three populations did not appear to be related to the zygosity of the ALS alleles as the most homozygous population (ArMB1) and least homozygous population (ArMB3) for the resistant allele showed same level of resistance to imazethapyr (Table 6). The data suggests that the number of heterozygotes in population ArMB1 is low (< 13%) and it may be that all individuals are homozygous. Different physiological basis for resistance, however, could be contributing to the varied response of the resistant populations to the imazethapyr. For example, absorption; translocation, sequestration, detoxification through ATP-binding cassette (ABC) transporter genes, glutathione S-transferase (GST) genes, glucosyltransferase (GT) genes could possibly be involved in the differential resistance of population ArMB2 and the other two resistant populations to the ALS inhibitor herbicide (imazethapyr). Cytochrome P450 induced non-target-site resistance was not observed as a contributing resistance mechanism in any of the three resistant populations, however, the populations were not evaluated for all possible NTSR mechanisms.

The most common reason why plants exhibit resistance to ALS-inhibitors is due to changes in ALS gene sequence due to single nucleotide polymorphisms that result in critical amino acid substitutions at specific locations on the ALS gene. These mutations reduce the affinity of herbicide to the enzyme and thereby enable the enzyme to continue to function even after exposure to herbicides. There are many *Amaranthus* species including palmer amaranth, smooth pigweed, and waterhemp with resistance to ALS-inhibitors as a result of amino acid substitutions at asp376glu, ala122thr, ser653asn or trp574leu and with these changes in ALS gene sequence those weeds showed a broad range of resistance (60-3200-fold) to ALS-inhibitors based on the specific substitutions (Patzoldt and Tranel 2007, Whaley et al. 2006, 2007, Berger et al. 2016). The three resistant populations in the current experiment had the Ser653Asn/Ile substitution in the ALS gene which confer a high level of resistance to imazethapyr and a low level of resistance to thifensulfuron-methyl compared with the susceptible populations. The two populations ArMB1 and ArMB2 had only the Ser653Asn substitution while ArMB3 had the Ser653Asn in only one individual and the Ser653Ile substitution in all other individuals where substitutions were found.

In this experiment, the two populations (ArMB1 and ArMB2) showed high resistance to imazethapyr but did not show resistance to thifensulfuron-methyl. The substitution Ser653Asn is responsible for conferring high resistance to imazethapyr in these two populations. Similar results were found in some populations of waterhemp from Illinois which had the Ser653Asn substitution that conferred resistance to imazethapyr, but the plants were susceptible to chlorimuron, a sulfonylurea (SU) herbicide and Chloransulam, a member of the triazolopyrimidine (TP) family (Patzoldt & Tranel 2007). An accession of downy brome grass (*Bromus tectorum* L.) from Carter County, MT, USA with a point mutation that causes a Ser653Asn substitution showed a high level of resistance to the IMI herbicide imazamox, moderate resistance to the TP herbicide pyroxsulam

and the triazolinones (SCT) family herbicide propoxycarbazone and was susceptible to the SU herbicide sulfosulfuron (Kumar & Jha 2017). In many cases, the Ser653Asn substitution confers high resistance to the imidazoline (IMI) herbicides and moderate resistance to sulfonylurea (SU) herbicides (Heap 2023). For example, with the same substitution some smooth pigweed populations from Virginia, Delaware and Maryland (USA) exhibited a high level of resistance to IMI (imazethapyr) and the pyrimidinyl benzoates (PTB) family herbicide pyrithiobac but were only moderately resistant to two SU herbicides (thifensulfuron and chlorimuron) and a TP herbicide (Chloransulam) (Whaley et al. 2006). A biotype of green foxtail with substitution Ser653Asn also showed high resistance to IMI (imazethapyr) and PTB (pyrithiobac) but moderate resistance to SU (nicosulfuron) and SCT (flucarbazone) herbicide (Laplante et al. 2009). A serine substitution to Asn/Ile/Thr at position 653 confers various level of resistance to all five chemical classes of ALS-inhibitors (SU, IMI, SCT, TP and PTB) (Heap 2023). As the binding sites for IMIs and SUs are not same and herbicides in the SUs family bind more tightly and closely to the active site than the herbicides of IMIs family (Patzoldt and Tranel 2007) these may contribute to the differential response of the resistant populations to IMIs and SUs.

In addition to substitution Ser653Asn, another mutation causing substitution Ser653Ile was found in the ArMB3 population. To my knowledge, the Ser653Ile substitution has not been documented before in redroot pigweed or any other broadleaf species. As the substitution Ser653Ile has never been seen before in redroot pigweed it is likely that this substitution evolved here in Manitoba. Substitution Ser653Asn and Ser653Ile confer a high level of resistance to imazethapyr in population ArMB3 and the Ser653Ile substitution only likely was responsible for the low level of cross resistance to thifensulfuron-methyl in that population. Similar to my results, Laplante et al. (2009) described two populations of green foxtail one with a Ser653Asn and the other with a

Ser653Ile substitution. Both populations showed high levels of resistance to imazethapyr but the population with the Ser653Ile substitution showed low levels of resistance to nicosulfuron, a sulfonylurea herbicide. Therefore, substitution Ser653Asn/Ile appears to play the role in conferring resistance to imazethapyr but only the Ser653Ile substitution was the key substitution conferring resistance to thifensulfuron-methyl in ArMB3. The amino acid side-chain size of Ile could affect the binding of thifensulfuron-methyl and therefore, the resistance.

To study non-target site resistance as a potential resistance mechanism, malathion was used which is a cytochrome P450 inhibitor and is usually considered an indicator of metabolic resistance (Nakka et al. 2016). The analysis revealed that among the resistant populations cytochrome P450 enzyme induced non-target-site resistance was not present and the result is consistent with the findings reported by other researchers (Cechin et al. 2017, Silva et al. 2022). This might be because the enzyme P450 wasn't involved in metabolizing the herbicides (imazethapyr or thifensulfuron-methyl). That's why when malathion was applied with the herbicide imazethapyr or thifensulfuron-methyl to inhibit the enzyme, plant biomass wasn't significantly reduced indicating the absence of non-target site resistance mechanism through P450 enzyme.

Enhanced herbicide metabolism to ALS inhibitors is rarely found in dicotyledonous weeds (Guo et al. 2015). This might be because this mechanism often provides low level of resistance and as this mechanism can readily be masked by the high level of resistance provided via the target-site resistance mechanism (Délye et al. 2011), NTSR is thought to be underestimated as a contributing mechanism to ALS resistance might be because it often provides a low level of resistance (Nakka et al. 2016, Cao et al 2021). Nevertheless, NTSR has been found as a contributing mechanism in conjunction with target site resistance in some cases. One example is a redroot pigweed population from China where non–target site resistance via enhanced herbicide metabolism through P450

enzymes to thifensulfuron-methyl worked in conjunction with target site-resistance (Trp-574-Leu) (Cao et al. 2021). The two resistance mechanisms (TSR and NTSR) working in conjunction were not observed in any of my redroot pigweed populations.

Chapter 6: Conclusions

ALS-inhibitors are the group of herbicides with the largest number of active ingredients and are widely used all over the world (Bo et al. 2017). Widespread use of the herbicides with the same mode of action on different crop fields year after year has caused the evolution of ALS-herbicide resistant weed populations beginning in 1990 (Mykhalska and Schwartau 2022). In this study, the emergence of resistant redroot pigweed (*Amaranthus retroflexus* L.) populations to ALS inhibitors (imazethapyr or thifensulfuron-methyl) was associated with point mutations causing amino acid substitutions (Ser653Asn/Ile) in the herbicide target, the ALS gene. The emergence of cross resistance has also been identified in one population likely due to having the Ser653Ile substitution. Cytochrome P450 enzyme induced enhanced metabolism was not found to be a contributing factor to ALS-resistance in the three redroot pigweed populations in this study.

Redroot pigweed resistant to the ALS herbicides have been increasing over the last two decades in many countries (Heap 2023). Globally, resistance to other modes of action has also been reported in redroot pigweed including PSII-, PPO-, and EPSPS inhibiting herbicides (Heap 2023). Occurrence of ALS herbicide-resistant redroot pigweed in western Canada is not a major threat in most crops although in-crop herbicide options in pulse crops are very limited for ALS resistant biotypes. As it is a C4 weed and can grow quickly, farmers could have a challenge to manage this fast-growing weed.

Practical implications

The knowledge of my experiment on the mechanism of resistance can be very helpful to farmers. The sequence difference allows for the development of a PCR-based assay to distinguish wild-type (susceptible) from mutant (resistant) DNA. For example, by developing rapid detection tools

like primers we could discern if redroot pigweed plants from a field have the mutation conferring resistance by using such an assay. Such a test would be fast as it would only require a few hours and would be very cost effective as well. Therefore, before applying herbicide growers could take appropriate action to manage this weed. For example, if redroot pigweed plants from a field are found to be resistant to ALS inhibitors with this mutation, growers still could use alternative herbicides from other modes of action such as photosystem II inhibitors (e.g., atrazine) or synthetic auxins herbicides (e.g., 2,4-DB, dicamba). Some non-selective herbicides like EPSPS inhibitors (glyphosate) or glutamine synthase inhibitor (glufosinate) could also be used to control this weed in crops that are resistant to these non-selective herbicides. Sustainable and diversified management strategies for example, herbicide rotation and herbicide mixtures using multiple effective modes of action, crop rotation, and other cultural practices should be followed by the growers to minimize use of herbicide and to delay the evolution or selection of further of herbicide-resistant weeds in these agricultural systems.

Future research

In this experiment, even though cytochrome P450 induced non-target-site resistance was not found to be a contributing to the resistance of the three redroot pigweed populations, the possibility of involvement of the other non-target-site resistance mechanisms like reduced absorption, translocation or sequestration and detoxification through ATP-binding cassette (ABC) transporter genes, glutathione S-transferase (GST) genes or glucosyltransferase (GT) genes cannot be excluded. In future research, whether these mechanisms play a role in the differential resistance of the three populations to the ALS herbicide (imazethapyr) could be investigated. Application of other cytochrome P450 enzyme inhibitors like piperonyl butoxide (PBO) also could be tested to make sure this enzyme complex is not involved in metabolizing these herbicides. Identification of

candidate genes of P450, ABC, GST, & GT by RNA-sequencing could be conducted to investigate whether enhanced herbicide metabolisms through these genes are involved in the resistance among the three resistant redroot pigweed populations.

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