Investigation into the Molecular and Biochemical Mechanisms of Resistance in Two Biotypes of Glyphosate Resistant Giant Ragweed

by

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ABSTRACT

INVESTIGATION INTO THE MOLECULAR AND BIOCHEMICAL MECHANISMS OF RESISTANCE IN TWO BIOTYPES OF GLYPHOSATE RESISTANT GIANT RAGWEED

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Glyphosate resistant giant ragweed poses a significant threat to farmers in southern Ontario as interference from this species can result in massive yield loses. The objective of this study is to investigate two distinct mechanisms of glyphosate resistance present in giant ragweed biotypes. Target site sequencing along with inter-population crosses were performed to obtain insight into the pattern of inheritance. The role of H₂O₂ was examined using 3,3’-Diaminobenzidine staining and glyoxylate content was measured as a means of investigating glycolate oxidase activity. Sequencing data revealed no target site mutations while F1 survival data showed a decreased resistance level. DAB staining showed evidence of H₂O₂ accumulation. Glyoxylate levels showed no differences between biotypes. Results from this study will contribute to elucidating the mechanism of glyphosate resistance in giant ragweed.
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LIST OF ABBREVIATIONS

ALS ........................................... Acetolactate synthase
AMPA ........................................... Aminonmethylphosphonic Acid
DAB ........................................... 3,3’-Diaminobenzidine
DAA ........................................... Days After Application
EPSP ........................................... 5-enolpyruvylshikimate-3-phosphate
EPSPS ......................................... 5-enolpyruvylshikimate-3-phosphate Synthase
EXON ........................................... Expressed Region
GR ........................................... Glyphosate Resistant
HAT ........................................... Hours After Treatment
INTRON ..................................... Intervening Sequence
LD$_{50}$ ........................................ Lethal Dose 50%
PCR ........................................... Polymerase Chain Reaction
ROS ........................................... Reactive Oxygen Species
S ........................................... Susceptible
SR ........................................... Slow Response
RN ........................................... Rapid Necrosis
Introduction

Giant ragweed (Ambrosia trifida) is a major detriment to North American agriculture. Giant ragweed is one of the most common, and widely considered to be, one of the most problematic weeds in corn and soybean crops within the Corn Belt (Johnson, B. et al., 2007). Uncontrolled, full season interference from giant ragweed has been shown to cause yield losses of up to 90% in corn (Harrison et al. 2001), 99% in sweet corn (Williams and Masiunas, 2006) and 99% in soybean (Baysinger and Sims, 1994). While representing a significant threat to corn and soybean production, with the introduction of glyphosate resistant (GR) crops, control of giant ragweed has been possible through the application of glyphosate.

GR giant ragweed has steadily spread through the mid-western United States and southern Ontario. In 2004, GR giant ragweed was first reported in Ohio (Heap, 2013; Stachler, 2008). GR giant ragweed has now been confirmed in eleven states and one Canadian province (Heap, 2013). In Ontario, there are now at least 85 locations with confirmed infestations of GR giant ragweed (Follings et al. 2013). This spread of GR giant ragweed is concerning as resistant plants can be difficult to control.

Two different GR giant ragweed phenotypes have been documented in their response to glyphosate. The first phenotype is referred to as the “rapidly necrosing” response (Van Horn and Westra, 2013). Plants with this phenotype will rapidly curl their mature leafs after exposure to glyphosate. These leaves then become necrotic within 24-hours of application. The second phenotype is referred to as the “slow recovery” response (Van Horn and Westra, 2013). These plants appear to halt their growth for several days following glyphosate application. Occasionally chlorosis is observed along leaf margins. After several days the plants will resume their growth.
Both phenotypes have been observed in southern Ontario and, in view of their contrasting response to glyphosate, strongly suggest the existence of two distinct mechanisms of resistance.

Determining the underlying physiological and genetic mechanisms of these two phenotypes is of crucial importance. Current practices often solve resistance through the use of different herbicides. While short-term solutions will always be required as a means of protecting yields, long-term solutions must also be considered. Long-term solutions to resistance challenges require an understanding of the mechanisms conferring this resistance. This research contributes to determining the physiological and genetic basis for resistance to glyphosate in giant ragweed.
CHAPTER 1: LITERATURE REVIEW

1.1 GIANT RAGWEED

1.1.1 Taxonomy and Distribution of Giant Ragweed

Giant ragweed is a member of the Asteraceae family that is remarkable by its rapid growth and variability in size. Giant ragweed can be up to 5.6 m in height (Bassett and Crompton, 1981; Johnson et al. 2007). The root system is fibrous, with a relatively short taproot. The stem of the plant is rough, hairy, and green coloured with frequent branching points. Leaves are generally arranged in an opposite pattern and are lobed; ranging from one to seven lobes. Often the first leaves following the cotyledons are unlobed (Bassett and Crompton, 1981). The variation in giant ragweed size allows it to grow slightly taller than neighbouring plants.

1.1.2 Reproduction

Giant ragweed is a member of the Asteraceae family with a monoecious reproductive morphology. The plants produce separate unisexual male and female flowers on the same plant. Male flowers occur at the distal end as spikes, with the female flowers occurring at their base (Bassett and Crompton, 1981). This feature promotes cross-pollination, which is characteristic of plants in the Ambrosia genus. Self-pollinating would be possible but is prevented by physiological incompatibility, as is the case in Ambrosia artemisiifolia L. (common ragweed) (Friedman and Barrett, 2008). Giant ragweed, like other ragweeds, exhibits a high level of variability within populations.

Variability in giant ragweed is primarily evident in its leaf morphology and genome. Leaves are generally three-lobed, but vary from one to seven lobes. Leaf arrangement is mostly opposite but is sometimes alternate (Bassett and Crompton, 1981). In the related species
common ragweed variation at the ALS (acetolactate synthase) nucleotide sequence was observed at 12.5% (48 of 385 nucleotides) among 24 plants. In comparison, the self-pollinated Asteraceae common cocklebur (Xanthium strumarium L.) showed no variability within the ALS gene (Tranel et al., 2004). This indicates that the variability that exists within the Ambrosia genus manifests itself at the molecular level and is of particular importance in cross-pollinated species. The variability within giant ragweed contributes to its success as a weed.

1.1.3 Seeds and germination

Giant ragweed seeds are encased in a woody involucre that vary widely in size. Seeds vary from 4.75 mm to 11.11 mm in length and 3.18 mm to 6.35 mm in width. Seeds are generally 6 to 8 mm long with a structure consisting of one central spike surrounded by a circle of five or more smaller spikes; fruits have a tough woody texture. Within the outer covering is the true seed, which is contained within a black testa (Bassett and Crompton, 1981).

At maturity A. trifida seeds are dormant. If seeds fail to germinate at higher temperatures they may enter a secondary dormancy phase (Davis, 1930). Dormancy is also a variable trait in giant ragweed. Dormancy may break in seeds from some plants within three months while others may take one to two years (Davis, 1930). Dormancy in giant ragweed seeds along with variability in dormancy length contributes to the difficulty of controlling giant ragweed.

Giant ragweed seeds are capable of germinating over an extended period of time. In Canada, historically seedlings emerge between April and June with the majority of germination occurring within a few weeks from late April to early May (Bassett and Crompton, 1978). In Illinois emergence can begin as early as March (Abul-Fatih and Bazzaz, 1979a; Stoller and Wax
1974). An extended germination period makes control of giant ragweed more difficult as seedlings may emerge after herbicide application.

Seed germination is dependent on several factors including burial depth and soil temperature and moisture. After one winter burial period, 51% of seeds buried in the top 10 cm of soil germinated (Stoller and Wax, 1973). Optimal germination of giant ragweed occurs when soil moisture ranges between 26 to 33% and temperature between 10 and 24°C (Abul-Fatih and Bazzaz, 1979b). For optimal giant ragweed germination, seeds should be buried relatively shallow in cool, damp soil.

Giant ragweed is capable of producing a high number of seeds; up to 5100 per plant under optimal conditions (Baysinger and Sims, 1991). Of seeds collected in one year, only 56% of seeds were viable, 21% lacked embryos and the remaining were non-viable for various reasons (Harrison et al., 2001). In addition, over-winter mortality can range from 20 to 90% (Abul-Fatih and Bazzaz, 1980; Stoller and Wax, 1974; Harrison et al., 2001). Low viability and high over-winter mortality of seeds present a means for farmers to manage the weed by reducing the seed bank.

Continuous control of giant ragweed over a period of several years can deplete the seed bank. Greater than 98% of giant ragweed seeds will germinate over a seven season period (Harrison et al., 2007). A combination of no-tillage cropping and timely weed control to manage emerging seedlings that prevent additions to the seed bank is recommended. These measures taken over a four-year period is capable of reducing the seed bank by 90% (Harrison et al. 2007).
1.1.4 Effects on Crops

Giant ragweed is one of the most common and problematic weeds in corn and soybean production within the Corn Belt (Johnson et al., 2007). Giant ragweed outcompetes other annuals early on in its development by utilizing a very efficient physiology to create a high initial biomass during the seedling stage (Abul-Fatih, 1977; Abul-Fatih and Bazzaz 1979a). If left uncontrolled during the growing season, giant ragweed will become the dominant species in a field quickly establishing a canopy and blocking light to plants below (Abul-Fatih 1977).

Due to its rapid growth giant ragweed is capable of causing significant yield loses. In corn, giant ragweed at 1 plant per 10 m² caused 13.6% yield loss when the two species emerged simultaneously. The projected limit for yield loss under extremely high densities of giant ragweed is 90% (Harrison et al. 2001). In sweet corn, giant ragweed at 1 plant m⁻² reduced yield by 50%, while at higher densities, total crop failure was recorded (Williams and Masiunas, 2006). Giant ragweed at 2 plants per 9 m of row reduced soybean yield by 46%. Increasing this density to 16 plants per 9 m of row reduced yield by 92% (Baysinger and Sims, 1994). Giant ragweed, even at low densities, causes substantial yield losses in corn and soybean production.

Giant ragweed’s competiveness may be enhanced by its capacity to respond to neighbouring plants. When competing with soybeans as the soybean canopy closes the bottommost leaves of giant ragweed plants abscise. In the void left, axillary leaves begin to grow. While the primary leaves appeared to be susceptible to shaded conditions the axillary leaves are shade tolerant (Webster et al., 1994). This ability combined with the weed’s ability to emerge early and maintain leaf area above the canopy makes giant ragweed a formidable weed (Webster et al. 1994; Johnson et al., 2007).
1.1.5 Management

Giant ragweed can be effectively managed with the judicious use of herbicides. However, between 1998 and 2000 four cases of resistance to ALS inhibitors were reported occurring in both soybean and corn production (Heap, 2013). The first case of glyphosate resistance in giant ragweed was reported in Ohio in 2004. In 2008, glyphosate resistant giant ragweed was confirmed in Ontario (Vink et al. 2012). Glyphosate plus 2,4-D applied pre-plant prior to soybean emergence provided 97 to 99% control of GR giant ragweed (Vink et al., 2012). Control of GR giant ragweed is still possible when mixing glyphosate with additional herbicides.

1.2 GLYPHOSATE HISTORY, MODE OF ACTION AND PROPERTIES

Glyphosate (N-(Phosphonomethyl)glycine)) is a white and odorless crystalline solid (Franz et al. 1997). It is a post-emergent, systemic, nonselective, and broad spectrum herbicide that provides control of perennial, biennial and annual weeds (Dill et al., 2010). A chemical derivative of the amino acid glycine, Dr. Henri Martin first synthesized the compound in 1950. After passing through several industries between 1950 and 1970 it was tested for herbicidal properties by Monsanto in 1970 eventually being sold as Roundup (Dill et al., 2010). The introduction of glyphosate to the agricultural industry was significant and is credited as one of the major turning points in weed management. Along with other herbicides introduced at the same time glyphosate has alleviated many of the constraints placed on farmers by nearly eliminating manual weed control (Baylis, 2000).

1.2.1 Mechanism of Action

Glyphosate’s lethal action on plants is a result of inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Figure 1-1) (Arhmein et al. 1980). Inhibition of this enzyme disrupts the shikimate biosynthetic pathway and prevents the synthesis of the aromatic amino
acids phenylalanine, tyrosine, and tryptophan (Dill, 2005). This enzyme is responsible for catalyzing the transfer of the enolpyruvyl functional group from phosphoenolpyruvate (PEP) to shikimate-3-phosphate (S3P) (CaJacob et al. 2003). Inhibition of EPSPS causes an accumulation of toxic intermediates of the shikimate pathway, diversion of carbon and other resources to the blocked shikimate pathway as well as starvation of the plant for the aromatic amino acids (De Nuria et al., 2006). These combined effects are lethal to the plant.

Glyphosate is a symplastically translocated herbicide which accumulates in the meristematic regions of the plant. Glyphosate is absorbed through the leaves and is loaded into the phloem passively (Gougler and Geiger, 1981; Sprankle et al. 1973). A slow acting herbicide glyphosate requires several days to kill the plant (Franz, 1997). Glyphosate is only translocated during active photosynthesis. Once the herbicide reaches the meristems the plant is starved of aromatic amino acids and growth is inhibited (Geiger et al., 1986).

Glyphosate is translocated using the phloem from mature leaf tissue to apical and root meristems (Amrhein et al. 1980; Shaner, 2009). The EPSPS enzyme is most heavily expressed in the meristems and least expressed in mature tissue (Weaver and Hermann, 1997). In addition meristematic tissue is more sensitive to glyphosate than mature tissue. Shoot and root meristems in velvetleaf (*Abutilon theophrasti* Medik.) are killed at 0.2 mg/kg concentrations of glyphosate while mature tissue can survive up to 8.4mg/kg concentrations (Feng et al. 2003). Glyphosate is absorbed into leaf tissue and is then translocated through the symplast to meristematic tissue.

1.2.2 Properties of Glyphosate

There are several properties of glyphosate that make it an ideal herbicide for weed management. First is glyphosate’s efficiency; the EPSPS enzymes of all higher plants appear
susceptible to glyphosate (Duke and Powles, 2008). Second is that glyphosate is systemic and phloem mobile (Siehl, 1997; Duke and Powles, 2008). Third glyphosate has no soil activity and is slowly degraded in soil (Williams, Kroes and Munro, 2000; Sprankle et al., 1975a). In addition glyphosate possess a low volatility \(2.59 \times 10^{-5} \text{ Pa at } 25^\circ \text{C}\) decreasing the risk of herbicide drift (Dill et al., 2010). Finally glyphosate is water-soluble making it simple to formulate (Franz, 1997). These properties contribute to making glyphosate the most widely used herbicide in the world.

While public perception of glyphosate and herbicides in general can be considered tolerated at best, glyphosate does have some properties that appeal to the public. First among these is glyphosate’s low toxicity in non-target organisms. The EPSPS gene is conserved only in plants, bacteria and fungi (Nandula, 2010). Glyphosate has a lower acute toxicity than sodium chloride or aspirin; its LD\(_{50}\) in rates has been reported at greater than 5 g kg\(^{-1}\) (Duke and Powles, 2008). This trait combined with its favourable environmental profile make glyphosate a safe choice for weed management with regards to human health (Williams et al. 2000).

### 1.3 Herbicide Resistance and Resistance to Glyphosate

Herbicide resistance mechanisms can be broadly classified as target-site and non-target-site based. No international standards have been passed regarding the terminology and definitions of target-site and non-target site resistance (Heap and LeBaron, 2001; Yu and Powles, 2010; Beckie and Tardif, 2012). This thesis will use the following definitions to describe target-site and non-target site resistance. Target-site resistance occurs when the herbicide reaches its target site at lethal doses but a change at the target site limits the herbicide’s impact. Non-target site resistance involves mechanisms that minimize the amount of active herbicide that reaches the target site.
Target site mutation can either prevent the herbicide binding to its target or increase the specificity of the target for its natural substrate (Stewart et al., 2010). Over production of the target site leaves the target site vulnerable but the target is present in high enough concentrations that normal cellular function is maintained (Gaines et al. 2009). Where a herbicide binds to the catalytic site of its target enzyme, target-site mutations are unlikely as mutations at these sites often eliminate enzyme functionality. Where herbicides bind to the non-catalytic sites mutations may evolve as enzyme functionality can be conserved (Yu and Powles, 2010).

Non-target-site based resistance includes a number of different mechanisms. Most mechanisms tend to fall into four categories: differential herbicide absorption, differential herbicide translocation, herbicide sequestration, or detoxification of the herbicide (Perez-Jones and Mallory-Smith, 2010). These mechanisms are explained in greater detail in section 1.3.2 below.

1.3.1 Glyphosate Resistance in Crops

Since 1996, many crops have been commercialized with glyphosate resistant traits. The first GR crops introduced were GR soybean and canola in 1996 (Dill et al 2008). Following this five other GR crops have been introduced (Duke and Powles, 2009). GR crop technology has become commonplace with approximately 80% of worldwide cropland devoted to GR genetically engineered crops (James, 2008).

The massive adoption of GR crops has had tangible, real-world benefits. GR crop technology has had significant economic impacts in decreasing the need for other more hazardous herbicides (Gianessi, 2008; Nelson and Bullock, 2003) and reducing weed management costs for farmers (Brookes and Barfoot, 2005, 2008). In addition GR technology
has decreased the use of fossil fuels by growers (Cerdeira and Duke, 2006). GR technology lowered emissions significantly in herbicide manufacturing, herbicide transport and general field operations (Bennett et al., 2004). GR technology has also decreased the need for multiple herbicide applications as well as the need for costly tillage (Gianessi et al., 2002; Olofsdotter et al., 2000). At face value the adoption of GR technology has been a major benefit to the agricultural industry.

While the adoption of GR technology has had many benefits its adoption has come at a cost; stemming from an overuse and overreliance on this technology. The major concern for GR crops is an unintended selection for GR weeds and volunteer GR crop plants. A common practice in the Midwestern United States is the continuous rotation of GR corn and GR soybean. Many of the same weeds infest both resulting in intense selective pressure for glyphosate resistance. The same is true for weeds infesting cotton crops in the United States and continuous no-till soybean production in Argentina (Duke and Powles, 2009). Over-reliance on glyphosate for weed management in GR crops places heavy selective pressure on weed species.

1.3.2 Glyphosate Resistance in Weeds

Over the 20-year period between 1976 and 1996 no glyphosate resistant weeds were reported in the literature. During this time glyphosate was available for use but GR technology was unavailable (Powles, 2008). In 1996 the first GR weed, Lolium rigidium Gaudin., was discovered (Powles et al., 1998). Since this time the number of GR weed species has steadily increased to 30 at the time of writing (Table 1-1) (Heap, 2014). Thus far, four mechanisms of glyphosate resistance have been identified and characterized: target-site mutation, target site overexpression, vacuole sequestration, and reduced translocation. A fifth mechanism has been recently reported,
though it has not been confirmed as a sole means of glyphosate resistance; glyphosate metabolism. Other mechanisms may exist, however, only the above have been identified and examined in the literature.

1.3.2.1 Target Site Modification

Mutations in the EPSPS gene are capable of conferring resistance to glyphosate. The first weed identified with an insensitive EPSPS enzyme was *Eleusine indica* (L.) Gaertn. (goosegrass), which had an 8- to 12-fold resistance level to glyphosate (Lee and Ngim, 2000). The population had a point mutation in *EPSPS* that coded for a Pro\textsubscript{106}Ser substitution. This amino acid substitution had the dual effect of preventing the enzyme from being inhibited by glyphosate while also increasing its affinity for its natural substrate (Stalker et al. 1985). Three other substitutions occurring at Pro\textsubscript{106} have been reported: Pro\textsubscript{106}Ala (Simarmata et al. 2008), Pro\textsubscript{106}Thr (Yuan et al. 2005), and Pro\textsubscript{106}Leu (Laundun et al. 2011). Since the identification of the Pro\textsubscript{106} mutation in *E. indica*, nine other species of weeds have been identified with *EPSPS* mutations leading to glyphosate resistance (Table 1-1) (Heap, 2013).

In addition to the Pro\textsubscript{106} positon two other positions have been discovered and shown to confer resistance to glyphosate. The first is position Gly\textsubscript{101} where substitutions for Alanine have been shown to confer resistance (Devine and Preston, 2000). Another more recent discovery is a double mutation involving the Pro\textsubscript{106}Ser substitutions as well as a novel Thr\textsubscript{102}Ile mutation in *E. indica* (Jalaludin et al., 2013). While the most common point mutations conferring glyphosate resistance occur at position Pro\textsubscript{106}, other mutations are possible.
1.3.2.2 Vacuolar Sequestration

A second mechanism of glyphosate resistance is sequestration of the herbicide in the vacuole. Populations of Canada fleabane (*Conyza canadensis* (L.) Cronquist.) have been identified with this mechanism of resistance. Glyphosate enters the cytoplasm of resistant plants at the same rate as susceptible plants but is rapidly moved to the vacuole (Ge et al., 2009). Ge et al. (2009) suggested that sequestered glyphosate is either retained within the vacuole indefinitely or is slowly released at sub-lethal rates. Its accumulation in the vacuole restricts entry into the chloroplast where EPSPS resides. Differential vacuole sequestration provided resistant plants with a resistance index of 4.0 fold (Dinelli et al., 2006). Sequestration of glyphosate in the vacuole is capable of conferring resistance to otherwise lethal doses of glyphosate.

1.3.2.3 Gene Amplification

Overexpressed EPSPS enzyme, remains sensitive to glyphosate but is present in such quantities that the shikimate pathway is unaffected. GR palmer amaranth (*Amaranthus palmeri* S.Wats.) plants have been found with 5 to 160 times more copies of the *EPSPS* gene than susceptible plants. Increased copy number correlated with both increased mRNA and protein quantity. Increased copy number has been correlated with a 6 to 8-fold resistance to glyphosate (Gaines et al. 2010). Overexpression of the target site allows the plant to be affected by glyphosate but maintain normal cellular processes.

1.3.2.4 Reduced Translocation

Reduced translocation allows plants to survive glyphosate treatment by limiting the amount of herbicide that moves from the treated leaves to the meristems. This was identified in rigid
ryegrass (Lorraine-Colwill et al. 2003). Susceptible plants accumulated $^{14}$C glyphosate mostly at root tips while resistant plants accumulated it in their leaf tips. According to Heap (2013) seven different glyphosate resistant weed species use some form of altered or reduced glyphosate translocation (Table 1-1).

1.3.2.5 Glyphosate Metabolism

Glyphosate metabolism involves the metabolic breakdown of glyphosate into various substrates: aminomethylphosphonic acid (AMPA), glyoxylate and sacrosine. Two metabolic pathways have been identified for the degradation of glyphosate. The first uses glyphosate oxidoreductase and produces the compounds AMPA and glyoxylate. The second degrades glyphosate to sacrosine using C-P lyase (Duke, 2011). Both pathways’ metabolites have been reported in populations of Canada fleabane (*Conyza canadensis*) from Spain. In this case while the metabolites were detected their concentrations did not correspond to 100% of the glyphosate applied. In addition, resistant plants also differentially translocated glyphosate. The authors suggest that while glyphosate metabolism is occurring at a higher rate than susceptible plants, it is not solely responsible for the resistance to glyphosate observed (González-Torralva et al., 2012). Glyphosate detoxification in weeds species is rare in comparison to other mechanism.

Recently a GR population of sourgrass (*Digitaria insularis* L. Fedde) from São Paulo State in Brazil was found displaying hall marks of glyphosate metabolism (de Carvalho et al., 2013). GR sourgrass populations from Brazil contain AMPA and glyoxylate as well as sacrosine, indicating that glyphosate is being metabolized to some degree in GR sourgrass but the pathway in use remains unclear (de Carvalho et al., 2014). These results must be taken with caution as the amount of glyphosate applied to the plants does not equal the amount of glyphosate metabolites
recorded. Indicating that some glyphosate is being lost; possibly through the roots. In addition, the authors also note that microorganisms in the soils may be responsible for some of the metabolites found in the study (de Carvalho et al., 2014).

1.3.3 Inheritance of Herbicide Resistance and Glyphosate Resistance

Inheritance of herbicide resistance, and glyphosate resistance, is a subject that has been studied in some species of herbicide resistant weeds. In most cases of herbicide resistance, resistance is inherited as a single nuclear gene. This allows for rapid spread through the population via gene flow (Mithila and Godar, 2013).

Resistance is not always inherited as a single nuclear gene. Mechanisms granting resistance to Photosystem I and II inhibitors are often inherited through the cytoplasm. These herbicides target chloroplastic enzymes encoded in the chlorplastic genome. As such the traits are inherited only through the maternal plant, the plant that provides the chloroplasts (Mithila and Godar, 2013; Darmency and Gasquez, 1981). In addition, two cases of recessive inheritance have been reported. Trifluralin resistance has been shown to be inherited as a recessive trait in both green foxtail (Setaria viridis L. P. Beauv.) (Jasieniuk et al. 1994) and goosegrass (Eleusine indica L. Gaertn.) (Zeng and Baird, 1997). Finally one case of multi-gene based resistance has been reported in cases of chlorotoluron resistance occurring in Alopecurus myosuroides Huds. (Chauvel and Gasquez, 1994). While in most cases herbicide resistance is inherited as a single, semi-dominant, nuclear gene, this is not always the case.

With regards to glyphosate resistance many studies have shown inheritance occurring through a single, semi-dominant, nuclear gene. In cases where glyphosate resistance is due solely to EPSPS mutation, resistance is inherited as a single nuclearly encoded gene. Some cases of
reduced translocation are also inherited as single nuclear genes (Preston and Wakelin, 2008; Lorraine-Colwill et al., 2001; Wakelin and Preston, 2006). In addition vacuolar sequestration in *C. canadensis* is also inherited as a single nuclear trait (Zelaya et al., 2004). In the majority of cases glyphosate is inherited in a similar manner to other cases of herbicide resistance.

The inheritance of the *EPSPS* duplication mechanism in palmer amaranth (*Amaranthus palmeri* S. Wats.) does not indicate a single nuclear gene. The genome of *A. palmeri* likely originally contained two copies of *EPSPS* (Sammons and Gaines, 2014). However one loci has since been duplicated in GR populations (Gaines et al., 2013; Giacomini et al., 2013; Sammons and Gaines, 2014). Inheritance of this duplication event has varied from population to population (Sammons and Gaines, 2014). Populations from North Carolina followed an inheritance pattern consistent with polygenic traits (Chandi et al., 2012). Populations from Mississippi have shown some maternal influence with more copies being inherited when the maternal plant is the resistant parent (Ribeiro et al., 2014). In summary, while glyphosate resistance is often inherited as a single nuclear gene this is not always the case as glyphosate inheritance in *A. palmeri* appears to be quite complex.

Understanding the means by which herbicide resistance is inherited is essential in improving our understanding of how plants become resistant. In addition, understanding how these traits are passed between generations could help growers modify their weed management practices. More efficient weed management will allow farmers to eliminate resistant weeds from their fields faster, saving cost and labour.
1.4 GLYPHOSATE RESISTANT GIANT RAGWEED

1.4.1 History of Glyphosate Resistant Giant Ragweed

The first reported case of GR giant ragweed occurred in Ohio in 2004 (Heap, 2013; Stachler, 2008). Since 2004, GR giant ragweed has been confirmed in eleven US states and one Canadian province (Heap, 2013). In Ontario, there are now 84 locations with a confirmed infestation of GR giant ragweed (Follings et al., 2013).

Giant ragweed biotypes have varying levels of resistance to glyphosate. Populations of giant ragweed from Arkansas have a 2.3- to 7.2-fold resistance to glyphosate when compared to susceptible plants using LD$_{50}$ (Norsworthy et al., 2011), while populations from Tennessee have a resistance level of 5.3-fold, also using LD$_{50}$ (Norsworthy et al., 2010). For the purposes of this thesis the following definition for biotype recognized by the Weed Science Society of America will be used: a population within a species that has a distinct genetic variation (Ahrens, 1994). Vink et al. (2012b) found that GR giant ragweed in Ontario required a field rate 18 times greater than that of susceptible plants to attain effective control. In addition the recommended field dose of glyphosate provided only 57% control and resulted in significant soybean yield loss (Vink et al., 2012b). GR biotypes exhibiting the rapid necrosis response from southern Ontario had a resistance index to glyphosate ranging from 6.2 to 7.0. Biotypes exhibiting the slow response phenotype had resistance indexes of 3.8 to 4.3 (Green, 2014). In summary glyphosate resistance levels can vary between populations of giant ragweed.

1.4.2 Mechanisms of Glyphosate Resistance in Giant Ragweed

To date, GR giant ragweed from Ontario exhibits two phenotypes in response to glyphosate. The first, referred to as the rapid necrosoing (RN) response, is characterized by a very rapid change in appearance. Within 12 hours the plant’s mature leaves curl and after 24 hours become
completely necrotic. The young developing leaves remain unaffected and continue to grow. The second phenotype is referred to as the slow recovery (SR) response. Plants with this response appear to not respond when exposed to glyphosate and are difficult to distinguish from plants of susceptible populations until five to seven days after treatment. At this point susceptible plants begin to wilt and die, while SR plants exhibit some chlorosis at the leaf margins and eventually regrow at multiple growing points. Both phenotypes have been reported in southern Ontario, although the RN is more frequent than the SR.

At the present time the mechanism of resistance in either phenotype is unknown. Several studies have concluded that resistant and susceptible biotypes absorbed the same amount of glyphosate, eliminating differential absorption as a mechanism (Norsworthy et al., 2011, Brewer and Oliver, 2009 and Hoss et al., 2003). Norsworthy et al. (2010) did note a difference in shikimate accumulation between susceptible and resistant biotypes from Tennessee. Both resistant and susceptible populations exhibited an increase in shikimate levels when compared to untreated checks, suggesting the \textit{EPSPS} target site remains sensitive to glyphosate. Determining the mechanism of resistance in either population will give valuable insight into how these traits developed and the most effective means of controlling them.
Figure 1-1. Glyphosate inhibits the shikimate pathway in the meristematic tissue of plants, bacteria and fungi. Adapted from Dill, 2005.
<table>
<thead>
<tr>
<th>Species</th>
<th>Common</th>
<th>Year</th>
<th>Locations</th>
<th>Mechanism(s)</th>
<th>Other MOA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amaranthus palmeri</em></td>
<td>Palmer Amaranth</td>
<td>2005</td>
<td>U.S.A</td>
<td>EPSP synthase amplification, <em>EPSPS</em> mutation, altered translocation</td>
<td>ALS Inhibitors, Photosystem II Inhibitors</td>
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<tr>
<td><em>Amaranthus quitensis</em></td>
<td>Smooth Amaranth, Smooth Pigweed, Red Amaranth, Slim Amaranth</td>
<td>2013</td>
<td>Argentina</td>
<td>Unknown</td>
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<tr>
<td><em>Amaranthus spinosus</em></td>
<td>Spiny Amaranth, Prickly Amaranth, Thorny Amaranth</td>
<td>2012</td>
<td>U.S.A</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td><em>Amaranthus tuberculatus</em> (syn. rudis)</td>
<td>Waterhemp</td>
<td>2005</td>
<td>U.S.A</td>
<td><em>EPSPS</em> mutation, <em>EPSPS</em> amplification</td>
<td>ALS Inhibitors, PPO Inhibitors, HPPD Inhibitors, Photosystem II Inhibitors</td>
</tr>
<tr>
<td><em>Ambrosia artemisiifolia</em></td>
<td>Common Ragweed</td>
<td>2004</td>
<td>U.S.A, Canada</td>
<td>Unknown</td>
<td>ALS Inhibitors</td>
</tr>
<tr>
<td><em>Ambrosia trifida</em></td>
<td>Giant Ragweed</td>
<td>2004</td>
<td>U.S.A, Canada</td>
<td>Unknown</td>
<td>ALS Inhibitors</td>
</tr>
<tr>
<td>Species</td>
<td>Common</td>
<td>Year</td>
<td>Location(s)</td>
<td>Mechanism(s)</td>
<td>Other MOA</td>
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<td><em>Bidens pilosa</em></td>
<td>Hairy Beggarticks</td>
<td>2014</td>
<td>Mexico</td>
<td>Unknown</td>
<td>Paraquat, ALS Inhibitors</td>
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<tr>
<td><em>Brachiaria eruciformis</em></td>
<td>Sweet Summer Grass</td>
<td>2014</td>
<td>Australia</td>
<td>Unknown</td>
<td></td>
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<tr>
<td><em>Bromus diandrus</em></td>
<td>Great Brome</td>
<td>2011</td>
<td>Australia</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td><em>Chloris elata</em></td>
<td>Tall Windmill Grass</td>
<td>2014</td>
<td>Brazil</td>
<td>Unknown</td>
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</tr>
<tr>
<td><em>Chloris truncata</em></td>
<td>Windmill Grass</td>
<td>2010</td>
<td>Australia</td>
<td>Unknown</td>
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<td><em>Conyza canadensis</em></td>
<td>Canada Fleabane, Horseweed, Coltstail, Marestail, Butterweed</td>
<td>2000</td>
<td>U.S.A, Brazil, China, Czech Republic, Spain, Italy, Poland, Canada, Greece,</td>
<td>Vacuole sequestration, altered translocation, <em>EPSPS</em> amplification</td>
<td>ALS Inhibitors, Bipyridiliums</td>
</tr>
<tr>
<td>Species</td>
<td>Common</td>
<td>Year</td>
<td>Location(s)</td>
<td>Mechanism(s)</td>
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<td><em>Conyza sumatrensis</em></td>
<td>Tall fleabane, White Horseweed</td>
<td>2009</td>
<td>Spain, Brazil, Greece</td>
<td>Unknown, hybridization with <em>C. canadensis</em></td>
<td>ALS Inhibitors</td>
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<tr>
<td><em>Cynodon hirsutus</em></td>
<td>Gramilla Mansa</td>
<td>2008</td>
<td>Argentina</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td><em>Digitaria insularis</em></td>
<td>Sourgrass</td>
<td>2008</td>
<td>Paraguay</td>
<td><em>EPSPS</em> mutation, altered translocation, glyphosate metabolism</td>
<td></td>
</tr>
<tr>
<td><em>Echinochloa colona</em></td>
<td>Junglerice</td>
<td>2007</td>
<td>Australia, Argentina, U.S.A</td>
<td><em>EPSPS</em> mutation</td>
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</tr>
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<td><em>Eleusine indica</em></td>
<td>Goosegrass, Wisegrass, Crowfootgrass</td>
<td>1997</td>
<td>Malaysia, Colombia, U.S.A, China, Argentina</td>
<td><em>EPSPS</em> mutation</td>
<td>ACCase Inhibitors</td>
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<tr>
<td><em>Kochia scoparia</em></td>
<td>Kochia, Burningbush, Summer Cypress, Mexican Firebrush</td>
<td>2007</td>
<td>U.S.A, Canada,</td>
<td><em>EPSPS</em> mutation,</td>
<td>ALS Inhibitors,</td>
</tr>
<tr>
<td><em>Leptochloa virgate</em></td>
<td>Tropical Spragletop</td>
<td>2010</td>
<td>Mexico</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Common</td>
<td>Year</td>
<td>Location(s)</td>
<td>Mechanism(s)</td>
<td>Other MOA</td>
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<td><em>Lolium multiflorum</em></td>
<td>Italian Ryegrass, Annual Ryegrass</td>
<td>2001</td>
<td>Chile, Brazil, U.S.A, Spain, Argentina, New Zealand, Japan</td>
<td>Altered translocation, <em>EPSPS</em> mutation,</td>
<td>ALS Inhibitors, ACCase Inhibitors, Glutamine Synthase Inhibitors</td>
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<td><em>Lolium perenne</em></td>
<td>Perennial Ryegrass, English Ryegrass, Winter Ryegrass</td>
<td>2008</td>
<td>Argentina, New Zealand</td>
<td>Unknown</td>
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<tr>
<td><em>Lolium rigidum</em></td>
<td>Rigid Ryegrass</td>
<td>1996</td>
<td>Australia, U.S.A, South Africa, France, Spain, Italy, Israel</td>
<td>Altered translocation, <em>EPSPS</em> mutation, Vacuole sequestration</td>
<td>ACCase Inhibitors, ALS Inhibitors, Dinitroanilines, Trizoles, Ureas, Isoxazolidiones</td>
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<td><em>Parthenium hysterophorus</em></td>
<td>Ragweed Parthenium, Santa Maria Feverfew,</td>
<td>2004</td>
<td>Colombia</td>
<td>Altered translocation</td>
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<td><em>Plantago lanceolata</em></td>
<td>Buckhorn Plantain, Ribwort Plantain, Lamb’s Tongue, Narrowleaf Plantain</td>
<td>2003</td>
<td>South Africa</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Common</td>
<td>Year</td>
<td>Location(s)</td>
<td>Mechanism(s)</td>
<td>Other MOA</td>
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<tr>
<td><em>Poa annua</em></td>
<td>Annual Bluegrass, Annual Meadow Grass</td>
<td>2010</td>
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<td>Unknown</td>
<td></td>
</tr>
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<td><em>Sonchus oleraceus</em></td>
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<td>ALS Inhibitors</td>
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<td><em>Sorghum halepense</em></td>
<td>Johnsongrass</td>
<td>2005</td>
<td>Argentina, U.S.A</td>
<td>Unknown</td>
<td></td>
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<td><em>Urochloa panicoides</em></td>
<td>Liverseedgrass</td>
<td>2007</td>
<td>Australia</td>
<td>Unknown</td>
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</tr>
</tbody>
</table>
1.6 HYPOTHESES AND OBJECTIVES

The hypotheses of this research were:

1. Glyphosate resistance in giant ragweed is not due to a mutation in the EPSPS enzyme at amino acid position 106.
2. Glyphosate resistance in giant ragweed is inherited by offspring as a single nuclear gene displaying incomplete dominance.
3. The rapid necrosis response involved in giant ragweed resistance to glyphosate is associated with an increase in the reactive oxygen species H$_2$O$_2$.
4. An overactive glycolate oxidase enzyme causes the accumulation of H$_2$O$_2$ in rapidly-necrosing glyphosate resistant giant ragweed plants.

The research objectives were:

i. To sequence a segment of the EPSPS gene in glyphosate resistant and glyphosate susceptible giant ragweed plants and compare the sequence across populations.

ii. Determine how each of the glyphosate resistance mechanisms is inherited when resistant plants are crossed against susceptible plants and resistant plants with the opposing mechanism.

iii. Examine the accumulation of H$_2$O$_2$ in the rapid desiccation of mature leaves in glyphosate resistant plants exhibiting the rapid necrosis response. To

iv. Measure the concentration of glyoxylate in mature plant tissue as a means of estimating the activity of the glycolate oxidase enzyme.
CHAPTER 2: 5-ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE SEQUENCING

2.1 ABSTRACT
GR giant ragweed represents a significant risk to agricultural productivity in southern Ontario; giant ragweed interference is capable of causing substantial yield losses. The objective of this study was to investigate the \textit{EPSPS} gene in susceptible and glyphosate resistant giant ragweed plants and determine if any mutations are present. DNA was extracted from eighteen populations of giant ragweed, from which a section of the \textit{EPSPS} gene was amplified and sequenced. Alignment of these sequenced areas showed alignment with the related \textit{Helianthus salicifolius} \textit{EPSPS} gene. Consensus sequences built from giant ragweed plants showed no mutations in the sequenced region when comparing plants with susceptible or resistant phenotypes. Previous work on giant ragweed was unable to conclusively determine if the \textit{EPSPS} target site was sensitive or insensitive to glyphosate. This work suggests the giant ragweed populations examined possessed a sensitive target site.

2.2 INTRODUCTION
Mutations in \textit{EPSPS} granting glyphosate resistance primarily occur at Pro\textsubscript{106}. Substitutions granting resistance at this position have been reported: Pro\textsubscript{106}Ala (Simarmata et al. 2008), Pro\textsubscript{106}Thr (Yuan et al. 2005), and Pro\textsubscript{106}Leu (Kaundun et al. 2011), and Pro\textsubscript{106}Ser (Lee and Ngim, 2000). At a second site, a Gly\textsubscript{101}Ala mutation has also been shown to confer glyphosate resistance (Devine and Preston, 2000). A third site has also been confirmed, Thr102, though only in conjunction with a mutation at Pro\textsubscript{106} (Jalaludin et al., 2013). Thus far, ten different GR weed
species have been found to harbor mutations leading to an amino acid substitution that grant resistance to glyphosate (Table 1-1).

Differences in resistance level to glyphosate have been noted between the various substitutions. Populations of *Lolium rigidum* harboring the Pro\textsubscript{106}Leu point mutation displayed only a 1.7-fold resistance to glyphosate (Kaundun et al. 2011). While populations of *Eleusine indica* with a Pro\textsubscript{106}Ser mutation had a 5.3-fold resistance increase when compared to susceptible plants (Baerson et al. 2002). These differences indicate that each amino acid substitution at Pro\textsubscript{106} has an impact on the level of resistance to glyphosate.

GR giant ragweed was first identified in Ontario from seed collected in 2008 (Vink et al. 2012). Two phenotypes have been identified. Plants exhibiting the rapid necrosis (RN) phenotype respond quickly, within 12 hours the plant’s mature leaves curl and after 24 hours become necrotic. The growing points remain unaffected and continue to grow. Plants exhibiting the slow recovery (SR) phenotype show chlorosis at the leaf margins within 5 to 7 days accompanied with a cessation in elongation. After 5 to 7 days susceptible plants die from exposure to glyphosate while SR plants resume their growth.

Previous work using radioactively labeled glyphosate has shown that reduced translocation of glyphosate occurs in RN plants (Green, 2014). Accumulation of shikimate within leaf disks is considered a hallmark of a susceptible EPSPS enzyme (Dill et al. 2010). Shikimate accumulates because the EPSPS enzyme is inhibited and the shikimate pathway is blocked (Figure 1-1). Shikimate accumulates in leaf discs of both GR phenotypes of giant ragweed. However this accumulation occurs at higher concentrations of glyphosate than susceptible plants, requiring considerably more glyphosate before the same accumulation is seen. In contrast, leaf disks from GR soybeans, which have an engineered glyphosate insensitive
EPSPS enzyme, show no accumulation of shikimate (Green, 2014). These results may mean that glyphosate’s entry into the chloroplast of resistant plants is impaired and occurs only at high concentrations, or that the EPSPS enzyme in these plants has a moderate level of resistance.

The objective of this study is to investigate the EPSPS gene in both resistant phenotypes to determine if any polymorphisms occur at Pro_{106}, Gly_{101}, or Thr_{102}. Based on previous studies we hypothesize that a mutation exists at one of these three positions.

### 2.3 MATERIALS AND METHODS

#### 2.3.1 Plant Material

Plants from four susceptible (S) and fourteen resistant (R) populations, seven from each of the two phenotypes [RN and SR] were used. The plants were grown in a growth room with a 16-h photoperiod at 26°C and a 8-h dark period at 22°C. Resistant plants were sprayed with glyphosate at 900 g a.e ha\(^{-1}\) when they had reached the two- to three-node stage (four to six fully formed mature leaves). Glyphosate was applied using a laboratory spray chamber with a single 8002 flat fan spray nozzle (TeeJet, Wheaton, IL). The spray nozzle was positioned 45 cm above leaves and calibrated to 210 L ha\(^{-1}\) of spray solution at 275 kPa. Five plants were selected from each of the resistant populations 14 days after treatment. Five plants were also randomly selected from the susceptible population. Leaf samples were frozen overnight at -80°C and then freeze-dried for 72 hours.

#### 2.3.2 DNA Extraction

DNA extractions were carried out using a high throughput protocol at the University of Guelph (Chris Grainger, Dept Plant Agriculture, *pers comm.*). Approximately 10 mm\(^2\) of freeze-dried
tissue was added to a 2 mL 96-well plate with a 2 mm steel grinding bead and 400 µL of lysis buffer (54 mM CTAB, 0.1 M Tris-HCl at pH 8.0, 1.4 M NaCl). A plate homogenizer then homogenized the samples at a frequency of 20 Hz for two 40 second cycles with a 180° rotation. Samples were then centrifuged for 1 min at 5000 g and then incubated for 1 hour at 65°C followed by a 5 min centrifuge step at 5000 g. A 100 µL aliquot of lysate was then combined with 200 µL of binding buffer (3.2 M GuSCN, 10 mM EDTA, 5 mM Tris-HCl pH 6.4, 4 mL Triton X-100, 110 mL H2O) and transferred to a DNA binding plate. The mixture was then vacuumed for 2 min at approximately 77.8 kPa. The samples were then washed using 400 µL of wash buffer (50 mL of binding buffer with 20 mL H2O) and vacuumed again as above for 3 min followed by a second wash with 750 µL of a second wash buffer (60% ETOH, 10 mM Tris-HC pH 7.4, 0.5 mM EDTA). This was followed by a third vacuuming as above for 10 minutes. The 96-well plate was then attached to a PCR plate and centrifuged for 1 min at 5000 g followed by incubation for 30 min at 56°C. Following this 100 µL of molecular grade water, pre-heated to 56°C, was added to the 96-well plate that was attached to a new PCR plate and centrifuged at 5000 g for 5 min to elute the DNA.

2.3.3 PCR Conditions and Sequencing

Eluted DNA was amplified by polymerase chain reaction for sequencing using primer sequences provided by Dr. Phil Westra and Christopher VanHorn from Colorado State University. Primer sequences were: forward 5’-ACATGCTTTGGGCTCTAAAGAA-3’, and reverse 5’-TTGAATTACCACCAGCAGCGGT-3’ and were expected to amplify a 192 base pair segment. The amplification reaction had a volume of 50 µL containing 35.6 µL molecular grade water, 5 µL 10x PCR Buffer (New England Labs catalogue #M0267S), 2.5 µL of each primer at 20 mM,
1 μL 10 mM dNTPs (Invitrogen catalogue #18427013), 0.4 μL of Taq DNA Polymerase (New England Labs catalogue #M0267S) and 3 μL of gDNA. Reaction conditions were: initialization at 95°C for 3 minutes, denaturation for 1 minute at 95°C, 55°C for 1 minute for annealing, extension 72°C for 1 minute, final elongation 72°C for 7 minutes, repeated for 35 cycles. Following PCR each sample was visualized on a 1.5% agarose gel with 1.69 μM ethidium bromide by electrophoresis running at 120V for 1 hour.

Sequencing of the PCR samples was carried out by the University of Guelph Genomics facility. Samples were prepared for cycle sequencing using the Life Technologies BigDye Terminator Sequencing Kit (catalogue #4337449). Samples were sequenced in both the 5’ and 3’ direction. Reaction conditions were per sample: 10 μL molecular grade H2O, 1 μL 5X sequencing buffer, 1 μL of either primer at 10 uM, 1 μL of BigDye terminator and 2 μL of PCR product. Cycle conditions were: 96°C for 2 minutes, 96°C for 30 seconds followed by 50°C for 15 seconds repeated for 30 cycles, final extension at 60°C for 4 minutes.

2.3.4 Analysis

Analysis of the sequenced products was done using the Applied Biosystems Sequence Scanner freeware version 1.0. Consensus sequences for each of the populations and each phenotype were constructed using the web-based software MultiAlin (Corpet, 1988). Sequences were input as FASTA files and run using default settings. Consensus sequences were aligned for analysis using the web-based software Clustal Omega using default settings (Sievers et al., 2011). Consensus sequences were searched against the NCBI database using the tBLASTx function and default settings (Altschul et al. 1990).
2.4 RESULTS AND DISCUSSION

The \textit{EPSPS} gene was PCR amplified in all susceptible, RN and SR giant ragweed plants. The amplified segment was 91 base pairs in length representing approximately 5\% of the total coding sequence length. The amplified area contained the three loci where mutations have been shown to confer glyphosate resistance, Gly\textsubscript{101}, Thr\textsubscript{102}, and Pro\textsubscript{106} a well conserved domain across bacteria, fungi and plants (Baerson et al. 2002).

A total of five plants from eighteen populations were amplified and sequenced. These sequences represented four S populations and seven populations from each of the RN and SR phenotypes. The five sequences from each population were combined to create a consensus sequence for that population. Following this three full consensus sequences were created using the consensus sequences from each population to create S, RN and SR consensus sequences.

When searched against the NCBI database the best match in all cases (S, RN or SR) when sorting BLAST results for total score was a partial coding sequence for \textit{EPSPS} from \textit{Helianthus salicifolius} A. Dietr.. In addition for all consensus sequences searched the top 25 alignments when sorting for Max score, total score or E value were all \textit{EPSPS} sequences from various plant species. These results suggest that the sequences amplified by PCR are in fact a segment of \textit{EPSPS}.

In comparing the consensus sequences against the \textit{H. salicifolius} one amino acid substitution was detected (Figure 2-1). At amino acid position 87 giant ragweed has a Lys\rightarrow Arg substitution when compared against the \textit{H. salicifolius} sequence. This mutation is conserved across all populations of giant ragweed sequenced and likely is not involved with resistance to glyphosate.
At the nucleotide level eight point mutations were sequenced when comparing giant ragweed to *H. salicifolius*. With the exception of the point mutation causing the above Lys$^{87}$Arg substitution all of these point mutations occur in wobble bases and have no effect on amino acid sequence. Interestingly, two of these silent mutations occur at the wobble bases of both the Gly$_{101}$ and Pro$_{106}$ sites. Across the sequence amplified the giant ragweed consensus sequences had a 91% homology to the *H. salicifolius* EPSPS sequence. Sequences from SR and RN populations were identical at the nucleotide level to S populations.

Given that most mutations granting resistance to glyphosate occur at Gly$_{101}$, Thr$_{102}$, and Pro$_{106}$ it appears unlikely that the basis for resistance in either giant ragweed phenotype is due to an altered target site. However, the sequenced area is only part of a larger gene and in the future it should be sequenced in its entirety to eliminate entirely the possibility that target site mutation is the case for resistance. Some technical challenges will need to be overcome in order to accomplish this. With no reference genome, or *EPSPS* reference sequence for giant ragweed primers designed to amplify the entirety of the gene are difficult to construct. Degenerate primers may be created using reference sequences from related species. Alternatively cDNA could be created from RNA extracts from giant ragweed. These cDNA segments could then be cloned into bacteria. Using the primers presented here bacterial colonies could be probed for more of the *EPSPS* sequence. At the present time, with the evidence at hand, it appears that target site mutation is not the mechanism granting resistance to glyphosate in the giant ragweed populations we were working with.
Figure 2-1. Clustal Omega alignment of Heliantus salicifolius slow response, rapid necrosis and susceptible consensus gDNA sequences with translated amino acids. Highlighted in bold is a domain heavily conserved across bacteria, fungi and plants. The Gly$_{101}$, Thr$_{102}$, and Pro$_{106}$ sites where mutations have occurred conferring glyphosate resistance are bolded and underlined. Comparing the giant ragweed consensus sequences to the H. salicifolius sequence one amino acid substitution is seen Lys$_{87}$Arg. All other nucleotide point mutations when comparing giant ragweed to H. salicifolius occur in wobble base positions and have no effect the on amino acid sequence.
CHAPTER 3: INHERITANCE OF RAPID NECROSIS AND SLOW RESPONSE PHENTOYPES IN F1 PLANTS

3.1 ABSTRACT
GR giant ragweed is a major concern for producers in southern Ontario as uncontrolled giant ragweed can be extremely detrimental to yields. The objective of this study is to investigate how the rapid necrosis (RN) and slow response (SR) mechanisms are inherited across one generation. Six total crosses were performed: both resistant phenotypes with susceptible plants in both directions (donating and receiving pollen) as well both resistant phenotypes with each other. F1 plants from all crosses showed a decrease in survival when compared with resistant lines. In addition, a parental effect was noted when SR plants crossed with susceptible plants. No data on resistance inheritance has been reported in the literature concerning giant ragweed; this work provides insight into how each of these phenotypes are inherited.

3.2 INTRODUCTION
In most cases of herbicide resistance a single gene confers resistance; which is also generally encoded in the nucleus (Darmency, 1994). This is because most cases of herbicide resistance are due to a point mutation in target enzymes. This is the case in ALS-inhibiting herbicide resistance (Tranel and Wright, 2002), ACCase inhibiting herbicide resistance (Murray, Morrison, and Brûlé-Babel, 1995) and auxinic herbicide resistance (Preston et al., 2009). Incomplete dominancy is also a common trait of herbicide resistance cases. This is due to resistance occurring in wild populations where individuals freely cross. The result is F1 progeny that can be the result of crosses between susceptible and resistant individuals; inherited both a resistant (R) and susceptible (S) gene. Thus one copy of the enzyme is resistant to the herbicide while the
other is susceptible. Plants inherited a R and S gene would display incomplete dominance that is inherited as a single nuclear gene.

Three exceptions have been reported thus far. The first is maternal inheritance through the chloroplast genome granting Photosystem I and II inhibitor resistance (Mithila and Godar, 2013; Darmency and Gasquez, 1981). Chloroplastic inheritance occurs only through the plant donating the cytoplasm (the female plant) as no chloroplasts are inherited through pollen. Chloroplastic inheritance gene-flow is slower than nuclear inheritance as only some of the progeny will inherit the trait from a given cross (Mithila and Godar, 2013). This is the case with triazine resistance in *Poa annua*, where the resistance trait is inherited only through the maternal parent (Darmency and Gasquez, 1981).

The second is single nuclear recessive inheritance. Dominant resistance genes are favoured over recessive ones in out-crossing species; resistant phenotypes spread faster as heterozygotes will also display some level of resistance (Jasieniuk et al. 1994). This is not the case when plants primarily self. In highly selfing populations recessive genes can spread quickly as the number of homozygotes outnumbers the number of heterozygotes (Mithila and Godar, 2013; Jasieniuk et al. 1996). Recessive inheritance has been shown in green foxtail (*Setaria viridis*) to trifluralin (Jasieniuk et al. 1994; Zeng and Baird, 1997). The establishment of a recessive resistance trait in green foxtail is attributed to two reproductive traits. First is the heavy selfing nature of green foxtail; average selfing rates are greater than 99% (Jasieniuk, Brûlé-Babel and Morrison, 1996). Second is the high number of seeds that can be created from a single plant, up to 12 000 seeds (Douglas et al. 1985). These two traits combined can select for a recessive trait over a dominant trait.
Finally, multi gene inheritance has also been reported. Polygenic phenotypes are the result of the accumulation of many minor mutations with additive effects (Lande, 1983). These mutations are spread throughout a population. Individuals can possess any number of these mutations, increasing and decreasing the level to which they exhibit a given phenotype. Herbicides tend to target highly specific target sites that interfere with major enzymes and metabolic pathways. In addition repeated applications of high rates often cause between 95 and 99% mortality. This selects for only a few individuals who survive application. Polygenic herbicide resistance requires that many individuals survive herbicide applications, to ensure that all minor mutations are conserved in the population. Following this these individuals need to cross freely, resulting in progeny with a greater density of minor mutations; eventually leading to full herbicide resistance. As such the evolution of polygenic resistant phenotypes requires continuous weak selection (Jasieniuk et al., 1996). This ensures that individuals with no resistance contributing genes are eliminated from the gene pool. Individuals with one or more resistance contributing genes survive and cross. The resulting F1 population will likely possess a higher density of resistance contributing genes. Over several generations this can ultimately select for individuals resistant to high doses of a given herbicide. Considering current agronomic practices of high herbicide application rates polygenic inheritance is somewhat rare (Jasieniuk et al., 1996).

Polygenic herbicide resistance has, however, been reported. Using *Lolium rigidum* Neve and Powles (2005) were able to artificially select for resistance to the ACCCase inhibiting herbicide diclofop-methyl using continuous sub-lethal doses. A follow-up study concluded that the resistance phenotype was under the control of several genes (Busi et al., 2012).
Glyphosate resistance is often inherited following the traditional single nuclear gene model. Cases of *EPSPS* mutation, reduced and altered glyphosate translocation and vacuole sequestration of glyphosate have all been examined and are inherited as single nuclear traits. Inheritance of *EPSPS* duplication and over expression has been investigated though results have proven to be inconclusive thus far, showing only that inheritance of the trait is complex (Sammons and Gaines, 2014).

In Ontario there are now 85 locations with a confirmed infestation of GR giant ragweed (Follings, 2013). Two mechanisms of resistance to glyphosate have been identified in giant ragweed. The first, the RN phenotype, responds to glyphosate treatment very quickly. Within 12 hours the plant’s mature leaves curl and after 24 hours become necrotic. Meristematic tissue remains unaffected and the plant continues to grow. The slow response (SR) phenotype appears to have no effect when exposed to glyphosate and is difficult to distinguish from susceptible populations until 5 to 7 days after treatment. At this point susceptible plants begin to wilt and die. The SR plants however appear to be unaffected. The SR plants appear to halt their growth and can show some slight chlorosis at the leaf margins will continue their growth after exposure.

At present the genetic basis for either phenotype is unknown. The objective of this study is to investigate how the resistance trait is inherited when backcrossing plants from resistant populations into susceptible populations. We hypothesize that the trait will be inherited as a single gene displaying incomplete dominance, as is common with most cases of herbicide resistance.
3.3 MATERIALS AND METHODS

3.3.1 Plant Material

Seeds were stratified in wet Promix PGX soil (sphagnum peat moss and vermiculite, Direct Solutions) and stored in dark containers at 4°C for approximately 6 weeks until germination. Plants from three populations of giant ragweed were planted representing the SR phenotype, the RN phenotype and a susceptible (S) control. Germinated seedlings were transplanted and grown in a growth room with a 16-h photoperiod at 26°C and an 8-h dark period at 22°C. Light was provided by a mixture of fluorescent and compact fluorescent bulbs for a photosynthetic photon fluence rate (PPFR) of 300 µE m⁻² s⁻¹.

3.3.2 F1 Generation

At the two- to three-node stage (four to six mature leaves) plants from the resistant populations were sprayed with glyphosate at a rate of 900 g a.e. ha⁻¹ to confirm individual resistance. Glyphosate was applied using a laboratory spray chamber with a single 8002 fan flat spray nozzle (TeeJet, Wheaton, IL). The spray nozzle was positioned 45 cm above leaves and calibrated to deliver 210 L ha⁻¹ of spray solution at 275 kPa. After resistance was confirmed, based on survival at approximately 2 weeks after treatment, plants were moved to a greenhouse and placed in the same photoperiod as above. Greenhouse temperature was 25°C during the day and 18°C at night. High-pressure sodium lamps supplemented natural daylight for a maximum PPFR of 1200 µmol m⁻² s⁻¹. Once the plants reached maturity they were randomly assigned into pollen donor and pollen receiving groups. Receiving plants had six inflorescences covered in waxed crossing bags to prevent contamination. Male flowers were cut off in the bagged inflorescences prior to pollen expression. Pollen was then collected from each of the donor plants.
and applied directly to the female flowers of covered recipient plants. Donor and recipient plants were paired and each recipient received pollen only from its donor. Crosses performed were: susceptible pollinating both resistant populations, each resistant population pollinating the susceptible population, and both resistant populations pollinating each other for a total of 6 crosses. Each cross was performed with 10 pollen-receiving plants for a total of 60 pollen-receiving plants. After artificial pollination plants were grown for approximately 30 days. Once each pollen-receiving plant began to shed seed from un-bagged inflorescences the bagged inflorescences were harvested.

3.3.3 F1 Screening

F1 seeds along with seeds from each of the parental populations were germinated as above. Germinated seedlings were transplanted and grown in a growth room as above. Upon reaching the two- to three-node stage the plants from each crossing group as well as each parental population were sprayed with glyphosate at 900 g a.e. ha\(^{-1}\). Response to glyphosate was measured using survival and measured at 1, 7, 14, and 21 days after application (DAA). Survival was determined based on meristematic growth, where growth was present the plant was deemed alive. In addition to survival the F1 and parental plants were also screened for percent necrosis. Necrosis was measured visually on a scale from 0 to 5. The scale was measured based on percent leaf surface necrosis as follows: 0 – 0% necrosis, 1 – 1 to 25% necrosis, 2 – 26 to 50% necrosis, 3 – 51 to 75% necrosis, 4 – 75 to 99% necrosis, 5 – 100% necrosis.
3.4 RESULTS AND DISCUSSION

$F_1$ seeds were successfully obtained from all crosses with the exception of $S\delta^{g}xRN\varnothing$, where eight of ten crosses created seed though no seeds germinated from this cross. Survival of $F_1\ RN\delta^{g}xS\varnothing$ seeds was intermediate when compared against parental RN and S plants (Table 3-1). The average necrosis level of $F_1\ RN\delta^{g}xS\varnothing$ at 1 DAA was 2.00, which was also an intermediate response between both parental lines (Figure 3-1).

Survival of $F_1\ S\delta^{g}xSR\varnothing$ was 7% and that of $F_1\ SR\delta^{g}xS\varnothing$ was 31% (Table 3-1). Plants with a maternal SR plant showed a decreased survival while those with a paternal SR plant showed an intermediate survival when compared to parental S and SR lines. The SR survival trait appeared to be inherited only through pollen.

Plants from crosses between the two resistant lines showed survival ranging from 60 to 80% (Table 3-1). This was lower than that of either resistant parental line. Yet considerably higher than any cross in which resistant plants were crossed with S. When comparing the two a similar increase in survival is seen when SR plants donate pollen as in the above SR$\delta^{g}xS\varnothing$ cross. In addition the rapid necrosis response is diminished when RN plants cross against SR plants, but the trait is inherited regardless of whether the RN plant is the pollen donor or receiver (Figure 3-1). In general $F_1$ plants from resistant by resistant crosses have an intermediate survival when compared against parental S, SR and RN lines.

Based on the information presented here new inferences can be made concerning the nature of each phenotype. The RN phenotype is a semi-dominant trait and is likely encoded in the nucleus. The trait can be inherited through pollen and individuals resulting from a cross between a resistant and susceptible plant show an intermediate level of resistance. The SR phenotype is also inherited through pollen but appears to not be inherited when the SR plant
receives susceptible pollen. Should wild populations exhibit this same trait this could be concerning for growers. Giant ragweed’s heavy outcrossing nature makes it possible for the rapid spread of the resistance trait through pollen, as both phenotypes can be inherited this way.

While some conclusions can be drawn from the data presented some limitations must be taken into account. Given that the mechanism for either phenotype is unknown it is impossible to know the zygosity of the parental plants. In addition crossing for several generations to ensure true breeding parental lines is unfeasible. It is therefore impossible to know if the parental lines were homozygous or heterozygous for either trait.
**Table 3-1.** Percent survival of F$_1$ progeny 21 days after application of 900 g a.e. ha$^{-1}$ glyphosate.

<table>
<thead>
<tr>
<th>Family</th>
<th>n</th>
<th>% Survival ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>15</td>
<td>13 ± 2.98</td>
</tr>
<tr>
<td>SR</td>
<td>15</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>RN</td>
<td>15</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>F$_1$ S♂ x SR♀</td>
<td>15</td>
<td>7 ± 2.98</td>
</tr>
<tr>
<td>F$_1$ SR♂ x S♀</td>
<td>35</td>
<td>31 ± 2.65</td>
</tr>
<tr>
<td>F$_1$ S♂ x RN♀</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F$_1$ RN♂ x S♀</td>
<td>15</td>
<td>47 ± 2.98</td>
</tr>
<tr>
<td>F$_1$ SR♂ x RN♀</td>
<td>20</td>
<td>80 ± 3.65</td>
</tr>
<tr>
<td>F$_1$ RN♂ x SR♀</td>
<td>20</td>
<td>60 ± 2.36</td>
</tr>
</tbody>
</table>
Fig. 3-1. Necrosis level of each cross at 1 DAA ± square root of the mean. For simplicity pollenating plants are denoted as ♂ while pollen-receiving plants are denoted as ♀. The scale was measured based on percent leaf surface necrosis and is represented in the Y-axis. Necrosis was measured as follows: 0 – 0% necrosis, 1 – 1 to 25% necrosis, 2 – 26 to 50% necrosis, 3 – 51 to 75% necrosis, 4 – 75 to 99% necrosis, 5 – 100% necrosis. Parental rapid necrosis plants had the highest amount of necrosis at 1 DAA. F1 plants inheriting the rapid necrosis trait appear to have an intermediate and less severe response.
CHAPTER 4: IN SITU VISUALIZATION OF HYDROGEN PEROXIDE AND GLYOXYLATE QUANTIFICATION IN RAPID NECROSIS TISSUE

4.1 ABSTRACT

Uncontrolled giant ragweed is capable of massive yield losses in corn and soybean production, and GR giant ragweed is a threat to agricultural productivity. The objective of this study is to determine the role of H$_2$O$_2$ and glycolate oxidase in the rapid necrosis (RN) phenotype seen in some populations of GR giant ragweed. Using a visual 3,3’-Diaminobenzidine stain H$_2$O$_2$ presence was investigated in mature leaf tissue. In addition, measuring levels of glyoxylate in mature leaf tissue investigated glycolate oxidase activity. Increased accumulation of H$_2$O$_2$ was detected in mature leaves of RN plants. No differences were detected in glyoxylate concentrations between sprayed and unsprayed tissue. Previous work on giant ragweed revealed that the rapid necrosis response is light dependent, this suggests that increased accumulation of H$_2$O$_2$ is involved in this response.

4.2 INTRODUCTION

The GR RN giant ragweed phenotype is light dependent (Lespérance and Tardif, unpublished). Droplets of glyphosate placed on mature giant ragweed leaves will only show a response when exposed to light. Leaves covered and left in the dark will not show any damage, until returned to the light. This rapid light dependent response is similar to the damage caused by some other light dependent herbicides, such as glufosinate and paraquat.

Given that the response is light dependent, it is possible that in RN plants photosynthesis is being disrupted causing the release of free radicals or reactive oxygen species (ROS).
Fahnensticher et al. (2008), using *Arabidopsis thaliana*, showed that overexpression of the enzyme glycolate oxidase lead to an increase in the H$_2$O$_2$ content of Arabidopsis rosettes, as well as an increase in glyoxylate levels. The overproduction of H$_2$O$_2$ had a distinctly negative effect on the Arabidopsis plants, retarding growth and creating necrotic lesions on the plant’s leaves. In addition this response was modulated with light intensity, getting more severe as light intensity increased (Fahnensticher et al. 2008).

Glycolate oxidase is a key enzyme in the salvaging of glycolate 2-P for photorespiration (Maurino and Peterhansel, 2010). The enzyme catalyzes the conversion of glycolate, imported to the peroxisome from the chloroplast, to glyoxylate (Akamatsu and Shimada, 1994). The reaction uses glycolate and oxygen to generate glyoxylate while also releasing the ROS H$_2$O$_2$ (see Figure 3-1) (Douce and Neuburger, 1999; Wingler et al. 2000; Maier et al. 2012). This reaction is coupled with the enzyme catalase to convert the H$_2$O$_2$ produced from the reaction into H$_2$O and $\frac{1}{2}$ O$_2$. In *A. thaliana* mutants harboring overly productive glycolate oxidase enzymes too much H$_2$O$_2$ is produced for the catalase to scavenge causing damage to leaves (Fahnensticher et al. 2008). Maier et al. (2012) attempted to use this pathway, working from the findings of Fahnensticher et al. 2008, to improve the growth of Arabidopsis plants. In order to counter act the negative effects of H$_2$O$_2$ build up they were forced to additionally transform their plants for increase catalase production (Maier et al., 2012).

It is possible that an increase in free radicals due to the presence of glyphosate is involved with the rapid necrosis response. The objectives of this study are to determine if H$_2$O$_2$ and glycolate oxidase are involved in the rapid necrosis response seen in RN giant ragweed plants. SR plants were used as a glyphosate resistant biotype lacking the rapid necrosis response. We first hypothesize that the rapid necrosis response will be coupled with an increase in H$_2$O$_2$.
presence in mature leaf tissue. In addition we also hypothesize that an over active glycolate oxidase enzyme is responsible for the accumulation of H$_2$O$_2$.

4.3 MATERIALS AND METHODS

4.3.1 Plant Material

Giant ragweed seeds were stratified in wet Promix PGX soil (sphagnum peat moss and vermiculite, Direct Solutions) and stored in dark containers at 4°C for approximately 6 weeks until germination. Plants from three populations of giant ragweed were planted representing the slow recover (SR) and RN phenotypes and a susceptible (S) control. Germinated seedlings were transplanted and grown in a growth room with a 16-h photoperiod at 26°C and an 8-h dark period at 22°C. Light was provided by a mixture of fluorescent and compact fluorescent bulbs for a photosynthetic photon fluence rate of 300 µE m$^{-2}$ s$^{-1}$.

4.3.2 In situ Localization of H$_2$O$_2$

Plants from each population were randomly selected and placed in three treatment groups: a negative control group left unsprayed; a positive control group sprayed with 1.3 kg a.i. ha$^{-1}$ of paraquat; and a test group sprayed with 900 g a.e. ha$^{-1}$ of glyphosate. Glyphosate was applied using a laboratory spray chamber with a single 8002 fan flat spray nozzle (TeeJet, Wheaton, IL). The spray nozzle was positioned 45 cm above leaves and calibrated to deliver 210 L ha$^{-1}$ of spray solution at 275 kPa. Plants were sprayed at the beginning of their photoperiod once they had reached the two- to three-node stage (four to six fully formed mature leaves). Mature leaves were sampled by cutting the petiole at the base with a razor blade 5 hours after treatment (HAT) for paraquat and 12 hours for glyphosate and untreated.

- 46 -
H$_2$O$_2$ was detected using a previously described protocol (Dutilleul et al., 2003; Thordal-Christensen et al., 1997) with modifications. A 1 mg/mL solution of 3,3’-Diaminobenzidine (DAB) was created in deionized H$_2$O. The pH of the solution was adjusted to 3.8 with the addition of 1 M HCl as the DAB dissolved. Immediately following sampling leaves were combined with 50 mL of DAB in a 50 mL syringe and hand vacuum infiltrated. Leaves were then allowed to incubate in the solution in a 50 mL tube for 16 hours in the dark at room temperature. Following incubation each leaf was boiled in 90% ethanol until all of the chlorophyll had been removed. H$_2$O$_2$ was visualized as a brown colour where the DAB polymerized in the presence of H$_2$O$_2$.

4.3.3 Glyoxylate Assay

Plants from each population were randomly selected and placed in two treatment groups: a negative control group left unsprayed and a treatment group sprayed with a 900 g a.e. ha$^{-1}$ of glyphosate applied as above. Mature leaves were sampled at 0, 2, 6, and 12 HAT as above. Immediately following sampling leaves were placed in aluminum foil, flash frozen in liquid nitrogen and stored at $-80^\circ$C prior to analysis.

Glyoxylate concentrations were determined using a protocol adapted from Fahensticher et al. 2008. Leaves were first ground in liquid nitrogen. Approximately 50 mg of ground tissue was homogenized by vortexing in a 1.5 mL micro centrifuge tube with a 2 mm steel ball in the presence of 500 µL of 0.1% phenylhydrazine and 100 mM HCl buffer. Samples were then incubated at 80°C for 5 min, chilled on ice for 5 min and centrifuged at 10 000 g for 5 minutes. The supernatant was then combined with 750 µL of 5.7 M HCl and 50 µL of 0.12 M K$_3$Fe(CN)$_6$. The samples were then centrifuged again at 10 000 g for 2 minutes. Finally samples were
transferred to a disposable cuvette and measured at 520 nm exactly 8 min after the addition of the K₃Fe(CN)₆. Blanks were prepared as above with no ground tissue and without the addition of K₃Fe(CN)₆.

To create a standard curve a 1 M solution of glyoxylate monohydrate (Sigma catalogue #G4502) was created. A portion was diluted down to 0.5 M and then both were serially diluted down to 10 µM and 5 µM. An aliquot of 200 uL of each dilution was added to 500 uL of 0.1% phenylhydrazine and 100 mM HCl buffer and processed through the protocol as above.

4.4 RESULTS AND DISCUSSION

4.4.1 DAB Staining

No accumulation of H₂O₂ was detected in any leaves left unsprayed (Figure 4-2). In contrast leaves sprayed with paraquat showed an accumulation of pigment indicative of H₂O₂. Of plants treated with glyphosate only leaves from RN plants showed pigment accumulation. This indicates accumulation of H₂O₂ in those leaves, similar to those treated with paraquat.

The accumulation of H₂O₂ in glyphosate treated RN plants along with the rapidity of the response suggests that this response is in some way linked to the over production of ROS and, or, free radicals. Other ROS and free radicals will also need to be investigated to determine what, if any, other ROS or free radicals species are involved with the response, however the presence of H₂O₂ is an indication of ROS production. In addition other ROS and free radicals will need to be investigated over more time points. While it is possible that H₂O₂ is the first ROS created it is also possible that some other ROS or free radicle is being synthesized first and its presence is stimulating the release of others. Using time course experiments it should be possible to
determine which ROS or free radicals are the first generated. This knowledge will help too narrow the list of genes or gene products responsible for this response.

In order to fully understand the rapid necrosis response it is important to learn the source of this H$_2$O$_2$ accumulation. Two scenarios are possible with regards to the accumulation of H$_2$O$_2$. The first is that glyphosate is in some way stimulating the release of this H$_2$O$_2$. The second is that glyphosate is causing a cellular cascade culminating in the release of H$_2$O$_2$. In either case the source of this H$_2$O$_2$ accumulation needs to be investigated.

**4.4.2 Glyoxylate Assay**

Glyoxylate levels in S, RN and SR plants over a period of 12 hours did not change (Figure 4-2). No differences in glyoxylate levels were detected between sprayed and unsprayed plants, nor between plants exhibiting different phenotypes. While these results do not conclusively eliminate glycolate oxidase as the reason for an increase in H$_2$O$_2$ evolution (Figure 4-2) they do detract from its culpability. It remains possible that giant ragweed plants exhibiting the rapid necrosis response have both an over active glycolate oxidase gene induced by glyphosate as well as a means of eliminating excess glyoxylate.

In summary, the rapid necrosis response seen in some populations of giant ragweed is accompanied by an accumulation of H$_2$O$_2$ 12 hours after the application of glyphosate. In the future other ROS and free radicals will need to be investigated to determine if they play a role in this response. Time course experiments will also need to be run to determine at what point these ROS and free radicals are released. In addition despite similarities to *A. thaliana* mutants described by Fahrensticher et al. (2008), it appears that an over active glycolate oxidase enzyme is not responsible for the H$_2$O$_2$ accumulation.
Fig. 4-1. Subset of the photosynthesis light reactions with emphasis on the movement of metabolites from the chloroplast to the peroxisome and mitochondria. Outlined in black is the reaction creating glyoxylate from glycolate; catalyzed by enzyme (3) Glycolate Oxidase. The reaction causes the creation of H$_2$O$_2$. This creation is coupled with enzyme (4) Catalase that in wild type species scavenges the H$_2$O$_2$. In *Arabidopsis thaliana* mutants with an over active Glycolate Oxidase gene Catalase is unable to contend with this increase in H$_2$O$_2$ leading to H$_2$O$_2$ damage and high concentrations of glyoxylate in tissue.
A DAB stain detecting the presence of H$_2$O$_2$ was performed on plants exhibiting both resistance mechanisms as well as susceptible plants 12 hours after spraying. Three treatments were applied, an unsprayed negative control, a positive control using paraquat and the experimental treatment using glyphosate. Very little H$_2$O$_2$ was detected in unsprayed tissue while a substantial amount was stained in plants sprayed with paraquat. Susceptible plants and plants exhibiting the slow response mechanism showed very little staining when glyphosate was applied. Plants exhibiting the rapid necrosis response showed evidence of heavy accumulation of H$_2$O$_2$.
Fig. 4-3. Measurements of glyoxylate per 100 mg of ground tissue in two populations of glyphosate resistant giant ragweed and one susceptible populations. Mature leaves were sampled at 0, 2, 6 and 12 hours after spraying. No differences were seen between the populations nor within each population when comparing sprayed and unsprayed leaves.
CHAPTER 5: GENERAL DISCUSSION

5.1 Contribution

The first goal of this thesis was to determine whether rapid necrosis (RN) or slow recovery (SR) giant ragweed had an altered target site. This research confirmed that glyphosate resistance in both resistant phenotypes is not due to an altered target site in the \textit{EPSPS} coding sequence. These results are based on analysis of a highly conserved region of the \textit{EPSPS} gene, which revealed no mutations at amino acid position Gly\textsubscript{101}, Thr\textsubscript{102}, and Pro\textsubscript{106}. While only a portion of the gene was examined, the area sequenced represents a heavily conserved region and contains the only three reported loci where mutations have been shown to confer resistance. These findings confirm that a mutation in \textit{EPSPS} is not likely the cause of resistance in either phenotype. Previous research using leaf disks examining shikimate accumulation suggested the possibility of either altered target site or reduced cellular uptake as the mechanism of resistance (Green, 2014). It is now possible to eliminate target site mutation as a mechanism leaving only reduced cellular uptake as the most likely means of resistance. However, as this result has not been confirmed conclusively it is still possible that some other mechanism is responsible for resistance.

Determining the inheritance patterns of each resistant phenotype was the second objective of this study. In both populations, resistance is inherited following a pattern consistent with a nuclear encoded incompletely dominant gene, similar to the majority of herbicide resistance cases. In crosses between SR and susceptible (S) plants F\textsubscript{1} progeny display an intermediate survival when compared to parental SR plants. This suggests that the SR trait is nuclear and semi-dominant. The trait conferring the RN phenotype also appears to be semi-
dominant and nuclear. Plants from RN pollen donors and S receivers exhibit an intermediate survival response to glyphosate when compared against S and RN parents. Unfortunately, reciprocal F1 plants with RN ‘mothers’ could not be obtained, though this does not alter the above conclusion.

The third objective of this thesis was to confirm that the rapid necrosis response in RN plants was due in part to the action of reactive oxygen species (ROS), in particular H$_2$O$_2$. The results presented show a definitive involvement of H$_2$O$_2$ in response to applications of glyphosate. DAB staining results in RN plants after applications of glyphosate show a similar pattern to that of plants sprayed with paraquat. In addition, this accumulation of H$_2$O$_2$ following glyphosate treatment is seen only in RN plants and not in S or SR plants. This study represents the first time the involvement of H$_2$O$_2$ in the RN phenotype has been investigated and proven.

5.2 Limitations

Though amino acid positions Gly$_{101}$ and Pro$_{106}$ of the $EPSPS$ gene were sequenced the remainder of the $EPSPS$ gene was not. Though only mutations at position 101, 102 and 106 have been reported in the literature thus far as mechanisms for glyphosate resistance it is possible that another mutation could do the same. Sequencing of the entire $EPSPS$ would have been ideal. The $EPSPS$ gene is nearly 10kb long containing up to 8 expressed regions (EXONs) with intervening sequences (INTRONs) that vary considerably in length depending on species. Considering previous work done suggesting a sensitive target site and a lack of any other EPSPS sequence from giant ragweed the sequencing of the entire gene was deemed unnecessary.

The exact genotypes of parental lines used in chapter three is unknown. Given this, results reported here must be considered with care, as it is unknown if the parental plants were
homozygous or heterozygous for either resistant trait. Crossing of individuals from the same population for several generations could have been used to create nearly homozygous populations. Given the lack of previous work done in regards to giant ragweed breeding and the relatively long generation time for giant ragweed plants this was not feasible within the project time line. In addition F2 plants were not produced and thus the number of genes controlling the trait cannot be determined.

Results from Chapter 4 show an accumulation of \( \text{H}_2\text{O}_2 \) in plants exhibiting the rapid necrosis response. While \( \text{H}_2\text{O}_2 \) is an important ROS many others exist. These other species could have been investigated to determine if they too are involved in the response. While it is possible to implicate \( \text{H}_2\text{O}_2 \) in the RN response the cause of its presence remains unknown. It is unlikely that glycolate oxidase is the source of this \( \text{H}_2\text{O}_2 \) but this work does not definitively discount the possibility that this enzyme is not involved.

Finally, the bulk of the research done in this project centered on plants exhibiting the RN response. While the SR phenotype is not as eye catching as the RN response it is none-the-less a form of resistance in a potentially very detrimental species. Further work could have been done on the slow response mechanism to determine its means of resistance.

**5.3 Future Research**

The results presented in this study raise some interesting questions future researchers may seek to answer in order to learn more about the mechanisms of glyphosate resistance presented here. Results from Chapter 4 show an accumulation of \( \text{H}_2\text{O}_2 \). We hypothesize that this accumulation is coupled with an accumulation in other ROS and free radicals, which should also be investigated. In addition to this it should be determined if a release in ROS is required for resistance. It is
possible that if these ROS are free radicals are scavenged effectively, the plants may lose their resistance. This knowledge may lead to development of an adjuvant that acts as a free radical scavenger, which would allow growers to control glyphosate resistant giant ragweed with glyphosate and not requiring the further addition of other herbicides.

The rapid necrosis response occurs only in mature leaf tissue. Young leaves are not affected. This may reflect a significant physiological difference between the two growths. Determining why young tissue responds to glyphosate so differently when compared to mature tissue could assist in determining the mechanism causing the rapid necrosis.

Finally, further inheritance studies done using plants with known genotypes will conclusively determine how each of these traits is inherited. Using F1 and F2 generations both the number of genes and their dominance can be determined. This knowledge will help in the systematic elimination of potential gene candidates.
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