Programmed Cell Death and Altered Translocation Cause Glyphosate Resistance in Giant Ragweed (Ambrosia Trifida L.)

by

Mackenzie Lespérance

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Plant Agriculture

Guelph, Ontario, Canada

© Mackenzie Lespérance, December, 2015
ABSTRACT

Programmed Cell Death and Altered Translocation Cause Glyphosate Resistance in Giant Ragweed (*Ambrosia Trifida* L.)

Mackenzie Lespérance
University of Guelph
2015

Advisors:
F. J. Tardif
P. H. Sikkema

Mechanisms of resistance to the herbicide glyphosate in Canadian biotypes of giant ragweed are currently unknown. In Ontario, a glyphosate resistant (GR) biotype shows a distinct phenotypic response in the mature leaves characterized by a light-dependent, hydrogen peroxide induced, rapid-necrosing reaction to glyphosate, leaving meristems intact. To gain insight into the subcellular events leading to damage in the mature leaves, transmission electron microscopy was used to compare cellular morphology between GR and glyphosate susceptible (GS) biotypes. Morphological evidence of different programmed cell deaths (PCD) were observed between GR and GS biotypes and a rapid increase in starch accumulation was observed in the GR biotype. This evidence was accompanied by a time lapse quantification of $^{14}$C-
glyphosate through biological oxidation and liquid scintillation when application was specific to the apical meristem of GR and GS plants. At 24 hours after application, [14C]-glyphosate was shown to increase in the mature leaves and decrease in the apical meristem of GR plants in comparison to GS plants (P < 0.10). We propose that the translocation impairment of glyphosate in the GR biotype could be due to the combination of two distinct cellular events, PCD and the impairment of an active glyphosate transport system.
PREFACE

Hello/Bonjour/Sago!

My name is Mackenzie Anne Lespérance and I am a Haudenosaunee (Iroquois) woman. Throughout my growing years my spirit was fed and nurtured by two wonderful parents along the Trent Severn River near Healey Falls. I grew up in the middle of two siblings, an older brother and a younger sister. We share a mixed blood of Mohawk and Huron First Nations, English, Irish and French ancestry. “My face is white but my blood is red” I always say. I’ve been given a special gift my elders call ‘being a bridge’ because I’m able to navigate between the Western and Aboriginal ways of life. They say I am able to do this by blending in with my white skin and education while being known to my red community through my relations and ceremony. They say I can help both sides by being a bridge.

Although referred to as a bridge, I often feel as though I am two different rivers coming together. Where, ‘creativity of the mind’ and ‘connectedness to spirit and the land’ are the names of the two rivers. Coming from different places, yes, but beautiful when they meet. However, being infinitely creative has its costs on the land, as we can see with global environmental changes. So where is that balance? How do I respect the land and my relations while letting my mind learn and wander through the process known as the ‘scientific method’? It was simple, ask permission. We are a permissions people after all. So for the duration of my learning I asked permission, and this started with asking the right teacher.
For me, choosing the right teacher was just as important if not more important than picking what to learn. Which is why I spent a year working for my (eventual) supervisor, Dr. François Tardif. When he asked if I was interested in doing a Masters in science I was honest with him and told him my fears. Several years earlier, my confidence had been broken through a learning experience with another educator at the University of Guelph and to my knowledge, I wasn’t smart enough to go any further than my undergraduate degree. Shocked and surprised, he listened intently and eventually responded to my lack of confidence saying, “Well, we’ll have to change that now won’t we”. In saying that he gave me the verbal commitment I needed, indicating that learning with him would be much more than testing hypotheses. From that moment on, was when the teacher-learner relationship began. He gave me permission.

Choosing a project came easy, because I knew regardless of what I picked, I would learn a great deal from this teacher. None-the-less, I ended up choosing a herbicide resistance project on giant ragweed. I had always been fascinated with how the plant spirits could overcome human imposed adversity. Yes, I said plant spirits. They are my teachers too, as they are very much alive. Concurrent to this research I honoured the plant spirits in the traditional way of the Haudenosaunee people. I put down tobacco and cedar for all of the plants I killed, asking them permission and by doing so, thanking them for giving me knowledge I didn’t have before. I was also engaged in ceremony for the duration of the experimentation to honour and feed myself and others involved in the research.

As a scientific or western learner and an Aboriginal learner it is integral to identify all aspects of the knowledge attaining process as they are both important to my research. Being reflexive of
who I am and where I come from as an Aboriginal learner, I moved through my research using a reciprocal relationship with my supervisor. By using an open and respectful approach with one-another, this relationship allowed me to extend my unique learning style with others. With the help of another Aboriginal scientist, Cara Wehkamp, I was able to hold a sharing circle at one of our lab meetings to discuss the knowledge that I had gathered. Two eagle feathers were used to allow a discussion between me and my scientific peers that acknowledged equality and created intentional thinking. Although new to every one of my peers, the process of holding an intentional space for western dialogue was appreciated and welcomed. I also have permission from the department chair and my examination committee to use traditional medicines and two eagle feathers before and during (respectively) my defense examination.

These examples of integrating western and indigenous approaches to attain and share knowledge has been the highlight of my thesis and my time during graduate school at the University of Guelph. I want to acknowledge the cultural barriers in western education that have been challenged and broken through my relationship with my supervisor and peers. I am uplifted by the meaningfulness of my research and the sense of pride I have for honouring all parts of myself through this process.
ACKNOWLEDGEMENTS

Un gros merci à François Tardif pour m’avoir aidé à accroitre ma confiance en moi en ce qui a trait à mes habiletés de chercheuse scientifique! It is difficult to put into words how much you have helped me over the past several years, however, these are some of the highlights: 1. When we first met my confidence as a scientist had sadly been broken but by believing in me as you have, those negative thoughts have been mended and I feel encouraged to shine. 2. During my degree I had challenged you with many Aboriginal approaches to learning and researching, all of which you supported and worked with me to achieve; teaching me that barriers in education can be overcome. 3. And lastly, you always made me feel like my ideas were worth perusing, that money isn’t everything and that no one has all of the answers.

I also want to extend a huge thank you to my co-supervisor Dr. Peter Sikkema and my two committee members Dr. Mihai Costea and Dr. J. Chris Hall. Without your time, commitment, expertise, and resources none of this research could have been accomplished. Other contributors to this research were C. Susan Belfry at the University of New Brunswick who sectioned and imaged all of the TEM results and Dr. Sang Bog Kim and associates at Canadian Nuclear Laboratories (Deep River, Ontario) who essentially saved an entire chapter of my results when our biological oxidizer failed us. Much thanks and appreciation to Cora Loucks and Andrew McKenzie-Gopsill for helping with the statistical analyses of this research and for answering all of my ridiculous questions during the writing process.

On a more personal note, I want to thank my family, my partner Ben and special friends (Pete & Hugo) for emotional support and making sure I had enough money to eat and keep rent! And lastly, but definitely not least, my weeds lab family, my elders and my home-away-from-home, the Aboriginal Resource Centre.
LIST OF TABLES

Table A1. Analysis of variance of stomatal density per 10 mm$^2$ leaf area of giant ragweed (*Ambrosia trifida* L.) where four subsamples (1 mm$^2$ sections of leaf) were taken from ten plants of glyphosate resistant (GR) and glyphosate susceptible (GS) biotypes.

Table A2. The mean percentage and standard error of absorbed [$^{14}$C]-glyphosate within four different plant tissues of glyphosate susceptible and resistant giant ragweed (*Ambrosia trifida* L.) at various hours after treatment (HAT) when applied at the apical meristem, replicated three times.

Table A3. The mean percentage and standard error of absorbed [$^{14}$C]-glyphosate within four different plant tissues of glyphosate susceptible and resistant giant ragweed (*Ambrosia trifida* L.) at various hours after treatment (HAT) when applied at the mature leaf, replicated three times.

Table A4. Analysis of variance for percentage of absorbed [$^{14}$C]-glyphosate in the root from the apical meristem measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.)$^z$.

Table A5. Analysis of variance for percentage of absorbed [$^{14}$C]-glyphosate in the hypocotyl from the apical meristem measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.)$^z$.

Table A6. Analysis of variance for percentage of absorbed [$^{14}$C]-glyphosate in the mature leaf from the apical meristem measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.)$^z$.

Table A7. Analysis of variance for percentage of absorbed [$^{14}$C]-glyphosate in the apical meristem from the apical meristem measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.)$^z$.

Table A8. Analysis of variance for percentage of absorbed [$^{14}$C]-glyphosate in the root from the mature leaf measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.)$^z$.

Table A9. Analysis of variance for percentage of absorbed [$^{14}$C]-glyphosate in the hypocotyl from the mature leaf measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.)$^z$.

Table A10. Analysis of variance for percentage of absorbed [$^{14}$C]-glyphosate in the mature leaf from the mature leaf measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.)$^z$. 
Table A11. Analysis of variance for percentage of absorbed [$^{14}$C]-glyphosate in the apical meristem from the mature leaf measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.)$^x$. 


LIST OF FIGURES

**Figure 3.1.** Morphological classification of *Ambrosia trifida* L. leaves.

**Figure 3.2.** Micromorphological features of *Ambrosia trifida* L. leaf epidermis obtained using SEM and light microscopy.

**Figure 3.3.** Leaf venation pattern of *Ambrosia trifida* L. obtained using light microscopy proceeding basic fuchsin staining.

**Figure 3.4.** Light microscopy (A & B) and transmission electron micrographs (C to L) of leaf cells of *A. trifida* L. prior to treatment with glyphosate.

**Figure 3.5.** Transmission electron micrographs of bundle sheath cells of glyphosate susceptible *A. trifida* L. taken one week after glyphosate treatment.

**Figure 3.6.** Transmission electron micrographs of glyphosate resistant *A. trifida* L. leaves treated with glyphosate and sampled zero hours after treatment (A, C, E) and two hours after treatment (B, D, F).

**Figure 3.7.** Transmission electron micrographs of glyphosate resistant *A. trifida* L. leaves treated with glyphosate and sampled two hours after treatment (A, C, E, G, I) and three hours after treatment (B, D, F, H, J).

**Figure 3.8.** Transmission electron micrographs of glyphosate resistant *A. trifida* L. leaves treated with glyphosate and sampled three hours after treatment (A) and four hours after treatment (B).

**Figure 3.9.** Transmission electron micrographs of glyphosate resistant *A. trifida* L. leaves treated with glyphosate and sampled four hours after treatment (A, C) and six hours after treatment (B, D).

**Figure 4.1** Time course distribution of percentage absorbed [14C]-glyphosate in (A) mature leaves, (B) apical meristem, (C) stem, (D) roots between glyphosate resistant (●) and glyphosate susceptible (○) giant ragweed (*Ambrosia trifida* L.) when applied exclusively at the apical meristem. Data are means of three replications ± standard error where * represents $P \leq 0.10$

**Figure 4.2.** Time course distribution of percentage absorbed [14C]-glyphosate in (A) mature leaves, (B) apical meristem, (C) stem, (D) roots between glyphosate resistant (●) and glyphosate susceptible (○) giant ragweed (*Ambrosia trifida* L.) when applied exclusively at the mature leaf. Data are means of three replications ± standard error where * represents $P \leq 0.10$. 
ABSTRACT ........................................................................................................... ii

PREFACE ............................................................................................................. iv

ACKNOWLEDGEMENTS ...................................................................................... vii

LIST OF TABLES ................................................................................................... viii

LIST OF FIGURES ................................................................................................ x

TABLE OF CONTENTS ......................................................................................... xi

CHAPTER 1: LITERATURE REVIEW ..................................................................... 1

1.1 Giant Ragweed ............................................................................................... 1

1.2 Glyphosate ...................................................................................................... 3

    1.2.1 History ...................................................................................................... 3

    1.2.2 Chemical Properties ................................................................................... 4

    1.2.3 Absorption, Translocation & Mode of Action ............................................ 4

    1.2.4 Use and Importance of Glyphosate for Agriculture ............................... 6

    1.2.5 Morphological Symptoms of Glyphosate Treated Plants ....................... 8

1.3 Glyphosate Resistance .................................................................................... 8

    1.3.1 Overview .................................................................................................... 8

    1.3.2 Mechanisms .............................................................................................. 9

1.4 Glyphosate Resistance Mechanisms in Giant Ragweed ............................... 16

1.5 Programmed Cell Death ................................................................................ 17

    1.5.1 Overview of Programmed Cell Death ....................................................... 17

    1.5.2 Apoptosis-Like PCD ................................................................................ 18

    1.5.3 Necrosis .................................................................................................... 19

    1.5.4 Autophagy ............................................................................................... 20
CHAPTER 1: LITERATURE REVIEW

1.1 Giant Ragweed

Giant ragweed (*Ambrosia trifida* L.) is an annual weed species from the Asteraceae or Compositae family where seeds (technically fruits called cypselas) overwinter on the plant or in the soil (Bassett & Crompton, 1982). This dicot has righteously earned its name, growing up to 6 meters tall, which is its most defining feature (Bassett & Crompton, 1982). Other identifiable features of giant ragweed are the medium to large sized, varied shaped leaves with generally three prominent lobes. These leaves are arranged oppositely near the base and eventually become alternate towards the apex of the plant (Bassett & Crompton, 1982). Once it finishes its vegetative phase, these monoecious plants with separate male and female flowers are cross pollinated, predominantly by wind (Basset & Crompton, 1982). Eventually, it produces upwards to 5000 seeds per plant (Baysinger & Sims, 1991; Harrison et al. 2001). The biological features of giant ragweed make it easy to identify and also contribute to its genetic diversity.

Biologically adaptable, giant ragweed can be found in a large geographical area. This species has been documented in the eastern two thirds of North America (Harrison et al. 2001). In Canada, it has been found in the southern parts of Manitoba, Ontario and Quebec, but has also been located in New Brunswick, Prince Edward Island and Nova Scotia (Bassett & Crompton, 1982). It is also commonly found in the eastern and Midwestern United States (Abul-Fatih & Bazzaz, 1980). The natural range of giant ragweed overlaps with important agricultural areas of North America.
The adaptability of giant ragweed makes it a potentially important weed in cultivated fields. Although it prefers a wet habitat, it is a good colonizer of disturbed sites and has become a serious problem in many agricultural systems (Washitani & Nishiyama, 1992). For example one giant ragweed plant in 1 m² land area reduced soybean (*Glycine max* (L.) Merr.) yield by 75% and corn (*Zea mays* L.) yield by 61% (Webster et al. 1994; Harrison et al. 2001). Giant ragweed is a good competitor because it emerges early, the seedlings have rapid growth, canopy closure is quick due to rapid leaf production and it is adaptable to local competition due to high plasticity in biomass allocation (Abul-Fatih & Bazzaz, 1980; Bassett & Crompton, 1982). In Canada, giant ragweed seeds germinate between late April and early May and but has been adapting to row crops and is now extending germination into June which makes it more difficult to control (Stroller & Wax, 1973; Bassett & Crompton, 1982; Norsworthy et al. 2010). Because of its highly competitive nature, there is a need to control this weed to reduce potential yield losses.

Different methods can be used to control giant ragweed, but the most effective is the use of herbicides. In corn and soybeans, acetolactate synthase (ALS)-inhibiting herbicides and glyphosate are generally very effective (Baysinger & Sims, 1992; Monsanto Canada Inc., 2015). The use of herbicide, while appealing to growers can often lead to selection of resistant biotypes.

The selection pressure imposed by herbicides, including glyphosate, favours the development of resistance in weeds. There are now 32 unique cases of weed species that have developed resistance to glyphosate worldwide (Heap, 2015). Resistance to glyphosate in giant ragweed has first been documented in Ohio in 2004 (Stachler et al. 2006). In Ontario, the first occurrence of
glyphosate resistant (GR) giant ragweed was documented in 2008 and, as of 2012, there were at least 82 confirmed occurrences in the province (Vink et al. 2012; Follings et al. 2013). Resistance to glyphosate in giant ragweed and other weeds reduces the usefulness of an otherwise very useful herbicide.

1.2 Glyphosate

1.2.1 History

Glyphosate (N-(phosphonomethyl) glycine) was first synthesized in 1950 by Dr. Henri Martin, a chemist who worked for a Swiss pharmaceutical firm Cilag (Franz et al. 1997). After coming to the conclusion that glyphosate had no pharmaceutical usefulness, further investigation on the molecule ceased (Franz et al. 1997). Almost ten years later in 1959, Johnson and Johnson Company purchased Cilag and sold research samples including glyphosate to Aldrich Chemical who then sold small amounts to Monsanto Company (Dill et al. 2010). By 1960, in Monsanto’s Inorganic Division, scientists such as Dr. Phil Hamm and Dr. John Franz continued research on glyphosate as a potential water softening agent followed by a potential herbicide on perennial weeds (Franz et al. 1997; Dill et al. 2010). A decade later (1971), Monsanto had successfully patented glyphosate as a herbicide and introduced it to the agrochemical market as Roundup® in 1974 (Powles & Preston, 2006; Székács & Darvas, 2012). Since glyphosate is non-selective, it could only be used in the absence of a crop such as in industrial areas or as a pre-plant or pre-emergence treatment in crops. Other in-crop uses included spot treatments, pre-harvest applications or post-harvest treatments. The ability to control annual and perennial weeds prior to planting with glyphosate contributed to the widespread adoption of no-tillage cropping systems (Ahrens, 1994). Since the late1990s, the introduction of GR crops such as canola, soybean, corn
and cotton has allowed the in-crop use of this herbicide (Padgette et al. 1996). This has resulted in an expansion of the use of glyphosate making it the most widely used herbicide in the world.

1.2.2 Chemical Properties

As a herbicide, glyphosate possess unique chemical properties. It is a derivative of the amino acid glycine that structurally consists of a basic amine group and two acidic moieties: a carboxyl and phosphate group (Székács & Darvas, 2012). This means that glyphosate is a highly polar molecule and is dissolvable in water (Franz et al. 1997; Székács & Darvas, 2012). However, commercially, glyphosate is formulated with a mixture of other additives to aid in herbicidal absorption and activity (Franz et al. 1997). These chemical properties and the way it is formulated impact its behavior on targeted plants.

1.2.3 Absorption, Translocation & Mode of Action

Glyphosate has unique absorption and translocation characteristics. When formulated glyphosate is applied to aerial parts of a plant such as leaves and stems, it diffuses through the leaf cuticle, potentially through an aqueous or hydrophilic pathway (Price & Anderson, 1985; Viougeas et al. 1995). Using a high to low concentration gradient, glyphosate travels between the surface of the cuticle to the aqueous apoplastic space (outside the cell walls) (Caseley & Coupland, 1985, Franz et al. 1997). Initially this process is quick but it eventually slows down due to the equilibrating concentration gradient (Franz et al. 1997). From the apoplast, glyphosate is able to get into the transpiration stream via the xylem and therefore travels apoplastically (Franz et al. 1997). Glyphosate can also penetrate the plasma membrane and travel symplastically (through cells) to the phloem (Jachetta et al. 1986; Franz et al. 1997). This means that glyphosate is ambimobile as
it can travel both in the xylem and the phloem (Franz et al. 1997). However, glyphosate is only slightly ambimobile because once it gets into the phloem retention is high and transfer back to the xylem happens only very slowly (Dewey & Appleby, 1983; Bromilow et al. 1993). The ability to remain in the phloem dictates the plant parts this herbicide accumulates in.

The phloem mobility of glyphosate allows it to reach various actively growing plant tissues. In the phloem, glyphosate travels acropetally (towards the apex) and basipetally (towards the base) to sink tissues, which are any meristematic tissues that utilize photoassimilates (Franz et al. 1997). The systemic action of glyphosate makes it able to translocate from its point of entry to actively growing tissues such as roots, rhizomes and shoots (Franz et al. 1997). The mode of glyphosate entry into those tissues is currently unknown, however, there are two proposed mechanisms (Shaner, 2009). The first scenario involves glyphosate transport at low concentrations, where it is actively transported across the plasma membrane, potentially through phosphate transporters (Denis & Delrot, 1993; Hetherington et al. 1998; Morin et al. 1997). In the second scenario, at high concentrations, glyphosate travels passively using a mass flow system (Denis & Delrot, 1993; Hetherington et al. 1998; Morin et al. 1997). Following glyphosate entry into the cytosol using one or both of these transport systems, it has to cross the double chloroplast membrane (Shaner, 2009). While there is knowledge on the way glyphosate passes through the plasmalemma towards the cytosol, there is no available information on its further entry into the chloroplasts.
Glyphosate has a unique target site relative to all other herbicides. Once glyphosate enters the chloroplasts, it competitively inhibits the enzyme 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS) in the shikimic acid pathway (Amrhein et al. 1980; Siehl, 1997). EPSPS catalyzes the synthesis of 5-enolpyruvoylshikimate 3-phosphate (EPSP) and inorganic phosphate from the condensation of shikimate-3-phosphate and phosphoenolpyruvate (PEP) leading to chorismate. Chorismate is a substrate for the biosynthesis of three essential amino acids; phenylalanine, tyrosine and tryptophan as well as other aromatic compounds involved in primary metabolism (Jensen, 1985; Franz et al. 1997). EPSPS inhibition causes a build-up of pre-cursor molecules upstream of the EPSPS catalyst site and aromatic amino acid starvation (Jensen, 1985; Siehl, 1997). Starvation of these three amino acids causes inhibition of secondary metabolites such as anthocyanins, flavonoids, hormones, growth promoters/inhibitors, lignins, lignans, and many phenols (Franz et al. 1997; Dewick, 1998; Herrmann & Weaver, 1999; Knaggs, 2003). These are compounds associated with many aspects of cell growth and defense. It has also been proposed that a carbohydrate drain from the continuous production of the upstream compound shikimate-3-phosphate contributes to plant death (Siehl, 1997). This is based on the observation that aromatic amino acid substitution only gave partial reversal of plant growth inhibition (Gresshoff, 1979; Rubin et al. 1984). Therefore, glyphosate death is a result of a carbohydrate drain away from photosynthetic carbon reduction and depletion of primary and secondary metabolites.

1.2.4 Use and Importance of Glyphosate for Agriculture

The systemic action of glyphosate along with its lack of selectivity have made it a very effective herbicide for the control of annual, biennial and perennial plant species regardless of if they are monocots or dicots (Franz et al. 1997). On the most recent Roundup WeatherMAX label
glyphosate can be used to control 49 annual and 24 perennial weed species in over 50 cropland scenarios (Monsanto Canada Inc., 2015). Other benefits of this herbicide are low soil residual activity and low toxicity to mammals and other higher life forms (Franz et al. 1997). This, undoubtedly coupled with the introduction of GR canola, soybean, cotton and corn in 1996, 1996, 1997 and 1998, respectively, are the reasons why glyphosate is the most widely labelled product in the United States and why it ended up becoming the first pesticide to reach one billion dollars (Franz et al. 1997; Young, 2006). Similar widespread use of glyphosate is also occurring in other jurisdictions including Ontario.

Although total pesticide usage (measured by total active ingredient weight) has been declining in Ontario, glyphosate usage has actually been increasing over the last two decades. According to the 1998 Survey of Pesticide Use in Ontario (Hunter & McGee, 1999) glyphosate made up 17% of the total herbicides used in field crops, fruit and vegetable crops, nursery crops, sod and ginseng. This number has increased to 64% according to the most recent survey in 2013-14 (OMAFRA unpublished data, 2015). While the areas devoted to corn and soybean production has been relatively constant between 1998 and 2013 in Ontario, the amount of glyphosate used in these crops has steadily increased (OMAFRA, 2015). This is most likely due to the widespread adoption of GR corn and soybean. The increased reliance on glyphosate leads to increase selection pressure for GR in weeds.
1.2.5 Morphological Symptoms of Glyphosate Treated Plants

Visible symptoms of glyphosate death appear rather slowly in comparison to other herbicides. For example, in GS giant ragweed plants, chlorosis, or yellowing of the apical leaves, begins to appear within 1 week after glyphosate application at 0.54kg ai ha\textsuperscript{-1} (data not shown). In soybean, visual evidence or glyphosate phytotoxicity can take up to three weeks to appear (Adcock et al. 1990). However, at a cellular level, changes in dicot species can occur very quickly after glyphosate application. Sublethal rates of glyphosate caused thylakoids in soybean cotyledon chloroplasts to become rearranged into circular shaped tubes 72 hours after application instead of the rectangular stacks observed in untreated plants (Vaughn and Duke, 1986). Similar effects occur in tomato (\textit{Lycopersicon esculentum} Mill.) 72 hours after sub-lethal glyphosate treatment (Mollenhauer et al. 1987). In addition, chloroplasts in tomato leaves closest to the plant apex, showed swelling, starch degradation and small remnants of thylakoid membranes and plastoglobuli in the stroma (Mollenhauer et al. 1987). Chloroplast degradation was also seen within 72 hours in the leaves of Palmer amaranth (\textit{Amaranthus palmeri} S. Wats.) when glyphosate was applied at a lethal rate (Lorentz et al. 2011). Other effects of glyphosate action such as shikimate accumulation also occur rapidly (Shaner et al. 2005). While external symptoms of glyphosate are slow to appear in glyphosate treated plants, it has rapid activity at the cellular level.

1.3 Glyphosate Resistance

1.3.1 Overview

Prior to the introduction of Roundup Ready crops in the late nineties, there had been no cases of GR weed species because it was an expensive herbicide that was not widely used (Heap, 2014).
When farmers began to use Roundup Ready, cropping systems, tillage systems declined and so did the diversity in weed management strategies (Duke & Powles, 2009; Heap, 2014). These reasons along with the massive adoption of Roundup Ready crops internationally (80% of all transgenic crops) caused increased selection pressure for the evolution of GR weeds (Duke & Powles, 2009; Heap, 2014). To date, there are 32 weed species that have evolved GR and Ambrosia spp. are among several GR species that pose serious economic threats to farmers using Roundup Ready cropping systems (Heap, 2015).

1.3.2 Mechanisms

1.3.2.a Target Site Modification

A number of weeds have developed GR due to target site modification, conferring around 2-10 fold resistance levels in comparison to their respective GS populations (Baerson et al. 2002; Ng et al. 2003; Perez-Jones et al. 2007). A herbicide target site is a specific enzyme essential for plant survival (Powles & Preston, 2006). Therefore, target site modification or resistance is a gene mutation that causes a structural change to a target site (enzyme) so that a herbicide can no longer inhibit said enzyme function (Powles & Preston, 2006). The most common mutation in the EPSPS gene conferring GR is at the proline 106 site (Pro\textsubscript{106}). To date, the Pro\textsubscript{106}Ser substitution is present in three populations of goosegrass (Eleusine indica (L.) Gaertn.) from Malaysia, a Californian population of rigid ryegrass (Lolium rigidum Gaud.) and Italian ryegrass (Lolium multiflorum Lam.) from Chile (Baerson et al. 2002; Ng et al. 2003; Simarmata & Penner, 2008; Perez-Jones et al. 2007). The Pro\textsubscript{106}Ala substitution has been reported in a South African population of rigid ryegrass and a Taiwan population of goosegrass (Yu et al. 2007).
Yuan et al. 2005). Thirdly, a leucine substitution; Pro106Leu, in another South African population of rigid ryegrass (Kaundun et al. 2011). Lastly, a Pro106Thr substitution has been found, in another Malaysian population of goosegrass (Ng et al. 2003). All these mutations have been selected by repeated glyphosate usage under field conditions.

Single site mutations other than Pro106 that confer GR are also possible and have been selected under laboratory conditions. An example is the Gly100Ala substitution in Roundup Ready crops (Funke et al. 2009). Other single site mutations have been found in plants and bacteria including, Thr42Met, Gly96Ala/Pro/Ser and at Pro101 with any residues smaller than leucine (He et al. 2003; Sost & Amrhein, 1990; Padgette et al. 1991; Eschenburg et al. 2002; Stalker et al. 1985; Healy-Fried et al. 2007). This illustrates the potential for a greater diversity of glyphosate resistance target site mutations than what has already been documented in the field.

While single mutations conferring GR are a concern, double mutations in the EPSPS gene can also confer GR and are of greater concern. Recently, a Malaysian goosegrass population was identified with the more common Pro106Ser substitution and an additional Thr102Ile substitution (Yu et al. 2015). The double mutation confers a very high level of resistance (180-fold) compared to any of the single mutations alone (Yu et al. 2015). The potential for other double mutations to be selected in the field is unknown, but if it were to occur, it could have important consequences on the usefulness of glyphosate.
1.3.2.b Target Site Overexpression

Target site overexpression in GR weeds or crops is when the EPSPS gene has a higher level of expression due to a higher copy number in the genome or increased transcription of EPSPS (Pline-Srnic, 2006). Increased EPSPS copy number has been reported in several populations of GR palmer amaranth (Gaines et al. 2010). These plants had relative genomic copy numbers of the EPSPS gene ranging from 5 to 160 more copies than the GS plants (Gaines et al. 2010). This resulted in increased EPSPS enzyme quantity which correlated with overall plant sensitivity to glyphosate. In addition, the absence of shikimic acid accumulation in response to glyphosate and the absence of any target site modification in the *epsps* gene confirm glyphosate resistance due to target site amplification (Gaines et al. 2010). Prior to this finding, others had used glyphosate selection pressure in tissue culture to generate target site overexpression resistance in several species (Pline-Srnic, 2006). For example, GR via target site amplification was found in cultivated carrot (*Daucus carota* L.), petunia (*Petunia hybrida* (Hook.) Vilm.) and tobacco (*Nicotiana tabacum* L.) (Nafziger et al. 1984; Steinrücken et al. 1986; Dyer et al. 1988; Suh et al. 1993); all showing increased EPSPS activity while maintaining enzyme sensitivity to glyphosate. Similarly, sensitive EPSPS enzyme and increase enzyme level were seen in cultured cell of the herbaceous species pink corydalis (*Corydalis sempervirens* Pers.) adapted to glyphosate. However this was not due to *epsps* gene amplification but rather to increased rate of EPSPS protein synthesis (Smart et al. 1985). This shows plants can increase their level of EPSPS activity using different mechanisms. Currently, factors affecting the transcription of EPSPS are poorly understood and require further research, however they illustrate the potential for glyphosate to have profound effect on EPSPS levels.
1.3.2. c Differential Translocation

Differential translocation as a GR mechanism is when a plant can survive a lethal dose of glyphosate due to impairment of transport via vascular tissues throughout the plant. This prevents glyphosate from reaching a high enough concentration in the chloroplast, resulting in EPSPS inhibition. Differences in glyphosate translocation within weedy species have been found in monocot and dicot species. For example, several populations of rigid ryegrass from Australia have evolved GR via differential translocation (Lorraine-Colwill et al. 2003; Wakelin et al. 2004). In both studies, [\(^{14}\)C]-glyphosate was absorbed equally in both GS and GR biotypes but over 48 hours, was predominantly detected in the treated leaf tip of the GR biotype. This was very different in comparison to the GS biotype, where most of the absorbed glyphosate was recovered in the root and shoot meristematic regions of the plant (Lorraine-Colwill et al. 2003; Wakelin et al. 2004). In addition to differences in [\(^{14}\)C]-glyphosate distribution, EPSPS sensitivity and expression was the same between GR and GS biotypes with no evidence of glyphosate metabolism (Lorraine-Colwill et al. 2003). Although the specific mechanism associated with altered translocation in rigid ryegrass hasn’t been determined, it has been proposed that a plasma membrane pump could be moving glyphosate into the apoplast where it can travel through the transpiration stream towards the leaf tip, or, that glyphosate cannot be loaded into the phloem (Lorraine-Colwill et al. 2003). Similar observations have been reported in Italian ryegrass, suggesting the symplastic mechanism involved in glyphosate import or retention is malfunctioning in the cells (Perez-Jones et al. 2007). Impaired glyphosate translocation is a mechanism of resistance often observed in ryegrass species.
Reduced glyphosate translocation has been observed in non-grass species. In the United States, GR horseweed (*Conyza canadensis* (L.) Cronq.), also referred to as Canada fleabane has been reported with differential translocation of glyphosate (Feng et al. 2004; Koger & Reddy, 2005). In GR horseweed, the majority of applied [\(^{14}\)C]-glyphosate remained in the treated leaf and very little was exported to other tissues (Feng et al. 2004). Interestingly, autoradiography showed reduced phloem loading in the GR biotype compared to the GS biotype over 48 hours (Feng et al. 2004). Other evidence supporting differential translocation in this case was confirmation of EPSPS sensitivity and similar [\(^{14}\)C]-glyphosate absorption between GR and GS biotypes (Feng et al. 2004). This evidence suggests that differential translocation of glyphosate in GR horseweed could be due to impaired phloem loading (Feng et al. 2004). Similar translocation results were obtained in hairy fleabane (*Conyza bonariensis* L.) but further evidence is still required to confirm this GR mechanism (Dinelli et al. 2008). While it is possible to observe reduced glyphosate movement in many plants species, the actual biochemical basis for this impairment remain unknown.

Several mechanisms of differential translocation have been suggested based on the current literature and knowledge of plant physiology; 1. reduced cellular uptake due to an altered phosphate transporter in the plasma membrane, 2. increased vacuolar uptake via an evolved tonoplast transporter, 3. increased cellular export into the apoplast via an evolved plasma membrane transporter or 4. increased chloroplast export via an evolved chloroplast envelope transporter (Shaner, 2009). Unfortunately, very little research has been done to elucidate the specific mechanisms of GR involving differential translocation.
1.3.2.d Vacuolar Sequestration

Sequestration of glyphosate in the vacuole is another exclusion mechanism conferring resistance. Populations of horseweed have the ability to accumulate glyphosate in the vacuole at a rapid rate after entry in the cytosol (Ge et al. 2010). This prevents the herbicide from entering the chloroplasts (where EPSPS is located) but more importantly, it also reduces the ability of the herbicide to enter the phloem, thereby impairing translocation. There are, therefore, two consequences of increased vacuolar sequestration: firstly, less glyphosate is translocated to sink tissues from source tissues when glyphosate is being sequestered in the vacuole and secondly, glyphosate is less toxic in sink tissues due to reduced quantities entering the chloroplast and binding to EPSPS (Ge, et al. 2010). Phosphorus-31 Nuclear magnetic resonance spectroscopy ($^{31}$P NMR) has been used to identify a pH-dependent chemical shift in glyphosate between the cytoplasm (PH~7.0) and the vacuole (PH~5.5) in plant cells (Ge et al. 2010, 2011). In addition to horseweed, four populations of ryegrass across Australia, Brazil, Chile and Italy showed GR via vacuole sequestration (Ge et al. 2012). It has been proposed that ATP-binding cassette (ABC) transporters on the tonoplast (vacuolar) membrane and/or tonoplast intrinsic protein (TIP) are somehow responsible in this GR mechanism (Ge et al. 2010, 2011, 2012; Yuan et al. 2010).

Interestingly, this GR mechanism has been shown to be temperature dependent. In two studies, when put into sub-optimal temperatures, GR Johnsongrass (Sorghum halepense L. (Pers.)), rigid ryegrass and horseweed, became susceptible to glyphosate (Ge et al. 2011; Vila-Aiub et al. 2012). Potentially, this could be a relatively easy way of screening for vacuolar sequestration as a method of GR.
1.3.2.e Metabolic glyphosate degradation

The metabolic degradation of glyphosate is another mechanism that can potentially allow weeds to survive glyphosate application. Glyphosate metabolism (degradation) occurs in soil fauna such as bacteria, actinomycetes and fungi (Duke, 2011). Currently, there are several known pathways by which glyphosate can be degraded; 1) conversion to sarcosine and inorganic phosphate through C-P lyase enzyme; 2) conversion to aminomethylphosphonic acid (AMPA) and glyoxylate through glyphosate oxidoreductase (GOX) enzyme; and 3) transformation to N-acetylglyphosate through glyphosate acyl transferases (GAT) (Duke, 2011). Although these genes or enzymes haven’t been found in plants, a GOX-like enzyme could be responsible for glyphosate degradation into AMPA as observed in GR soybean seeds (Duke, 2011). It is possible to confer GR via glyphosate metabolism and this was done in GR canola with the goxv257 transgene from *Ochrobactrum anthropi* (Duke, 2011). There have been some GR weeds documented with this resistance mechanism. For example, AMPA and sarcosine were detected in a Spanish population of GR horseweed 72 hour after glyphosate application (González-Torralva et al. 2012). Although, in this study, differential translocation of glyphosate was also found, suggesting two mechanisms could be responsible for overall resistance (González-Torralva et al. 2012). It is also possible that glyphosate is being metabolized in Brazilian GR sourgrass (*Digitaria insularis* (L.) Mez ex Ekman.) because AMPA, glyoxylate and sarcosine were found 168 hours after glyphosate application (de Carvalho et al. 2013). However, false positives of the hallmark symptoms of glyphosate metabolism can easily arise through microbial contamination (Duke, 2011). Therefore, there is a lot of skepticism in the scientific community surrounding methodologies that are used to confirm glyphosate metabolism in weed species so it is important to keep in mind that this mechanism of GR is still under scrutiny and may actually be quite rare.
1.4 Glyphosate Resistance Mechanisms in Giant Ragweed

Distinct GR giant ragweed biotypes from Ontario differ in their phenotypic response to glyphosate. The first, referred to as the rapid necrosing (RN) response, is characterized by a rapid change in appearance (Vink, 2012; Jeffery, 2014). Within 12 hours, the mature leaves curl and after 24 hours they 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 GS populations until five to seven days after treatment. At this point GS 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 (Vink, 2012). Plants with the RN response had a resistance index to glyphosate ranging from 6.2 to 7.0 while those with the SR response had resistance indexes of 3.8 to 4.3 (Green, 2014). The existence of two phenotypic responses and resistance level ranges suggest distinct mechanisms of resistance in these species.

Complete elucidation of the mechanisms of resistance linked to each phenotype has not been determined. However, more work has been done on the RN phenotype, and thus the biotype will be discussed further. Shikimate accumulation in leaf disks of RN giant ragweed occurs at a higher glyphosate dose than in leaf disks of GS plants (Green, 2014). While this could be interpreted as an indication of an insensitive EPSPS enzyme, sequencing of the epsps gene has revealed no mutation linked to resistance (Jeffery, 2014). Application of [14C]-glyphosate to the mature leaves has revealed that movement of glyphosate to the roots is reduced and more glyphosate remains in the necrotic mature leaves of the RN plants (Green, 2014). It appears that
the RN response would be in part responsible for reduced glyphosate translocation in the resistant plants.

The response to glyphosate in the RN plants is reminiscent of that caused by light activated herbicides such as paraquat or glufosinate. These light activated herbicides cause lethality through free radical mediated lipid peroxidation which destroys cell and organelle membranes (Hess, 2000). Indeed, the symptomology typical of RN plants is light dependent, as leaves that are maintained in the dark following glyphosate application do not exhibit any necrosis. However, the necrosis can appear if leaves are re-exposed to light within 24 hours (Tardif & Lespérance, 2013, unpublished). It has been further shown that the necrosis seen in the RN plants is caused by the rapid production of hydrogen peroxide (Jeffery, 2014). These observations would suggest that the onset of the RN response would take place primarily in the chloroplast. Investigation of the phenomenon at the sub-cellular level could provide further indication as to the basis for resistance in this phenotype.

1.5 Programmed Cell Death

1.5.1 Overview of Programmed Cell Death

Programmed cell death (PCD) is a genetically regulated and highly organized series of cellular events in eukaryotic organisms in response to external and internal stimuli related to development and/or defense. In plants, PCD events have been well documented during various stages of growth, development, reproduction and whole plant death (reviewed by Rantong & Gunawardena, 2015). For example, PCD in plants is responsible for events such as embryonic suspensor deletion, aerenchyma formation, tracheary element differentiation, root cap shedding,
leaf and flower abscission, incompatibility in pollen, leaf re-modelling and leaf senescence (Rantong & Gunawardena, 2015). In addition to developmental events, plants can also initiate PCD for survival to environmental stresses such as intense ultraviolet light, heat, hydrogen peroxide and pathogen attack (Rantong & Gunawardena, 2015). Although it is well accepted that plants undergo PCD, genetic and biochemical processes involved in plant PCD have been much less elucidated than in animals. Therefore, current research on plant PCD has been characterized based on its morphological similarities with animal PCD, where there is much controversy and overlap (Reape et al. 2008). Currently, three forms of plant PCD have been discussed; apoptosis-like PCD, necrosis and autophagy.

1.5.2 Apoptosis-Like PCD

Apoptosis-like programmed cell death (AL-PCD) is based on morphological traits seen in animal apoptosis. Animal apoptosis is defined by having three hallmark features: cell shrinkage while maintaining plasma membrane integrity, nuclear (DNA) fragmentation followed by eventual breakup of the cell into apoptotic bodies leading to phagocyte engulfment and degradation (Kerr et al. 1972). With the exception of apoptotic body formation (because plants don’t contain phagocytes), plants have shown similar morphological changes during PCD. For example, when subjected to a moderate heat treatment (55°C) carrot suspension cells display cellular (protoplast) retraction away from the cell wall without rupturing the plasma membrane (McCabe et al. 1997). DNA fragmentation is also observed, which produces multimers of 180-200 base pairs when PCD-activated nucleases cleave DNA at linker sites between nucleosomes in the genome (McCabe et al. 1997; Mittler & Lam, 1997). DNA fragmentation was not observed until 3 to 5 hours after heat treatment due to nuclease activation which is a slower, more organized
breakdown of DNA (McCabe et al. 1997; Reape et al. 2008). It has been suggested that differentiating between AL-PCD and other PCD mechanisms is best conducted within the first 6 hours of AL-PCD morphological change since it is a fairly rapid process (McCabe et al. 1997; Balk et al. 2003; Burbridge et al. 2006). Similar morphological results have also been reported in tobacco and soybean suspension cells, arabidopsis (Arabidopsis thaliana (L.) Heynh.) root hairs and tomato anthers (Levine et al. 1996; Yano et al. 1998; Burbridge et al. 2006; Senatore et al. 2009; Reape & McCabe, 2013). Therefore, AL-PCD has been characterized as an organized and timely set of morphological and nuclear changes in plants under moderate stress levels.

1.5.3 Necrosis

Necrosis has often been the term used for plant death that occurs under high stress and results in immediate cellular damage. However, depending on the author, morphological features of necrosis can be distinguished in two theories. Firstly, necrosis has been defined as plant death without a specific set of condensed morphology (Reape & McCabe, 2013). This definition is based on the acceptance of AL-PCD morphology as a unique classification of condensed morphology (Reape & McCabe, 2013). However, some morphological features have been reported in regards to necrotic plant death in acceptance of this classification. In the study mentioned above, when carrot cells were exposed to a high temperature treatment (85°C), cells had ruptured plasma membranes, protoplasts did not shrink and there were lysed mitochondria and nuclei (McCabe et al. 1997). This suggests that at higher levels of stress, carrot cells could not maintain a controlled series of events leading to death (Reape & McCabe, 2013). Additionally, rapid DNA fragmentation was observed presumably caused by intense heat damage rather than organized nuclease activity (McCabe et al. 1997). These findings are similar
to necrosis defined in animal PCD, where differentiation between apoptosis and necrosis is based on the timing and severity of damage (Lennon et al. 1991; Lockshin & Zakeri, 2004). This theory of necrosis considers time and severity of damage critical factors in determining PCD type.

The second classification of necrosis is based on the acceptance of autophagy (vacuolar) cell death instead of AL-PCD (van Doorn et al. 2011). Using this theory, key morphological features of necrosis are based on animal necrosis or ‘necroptosis’ where initially, there is swelling of the entire cell including various organelle swelling and early rupture of the plasma membrane (Kroemer et al. 2009; van Doorn et al. 2011). The features distinguishing necrosis in comparison to autophagic cell death are: the absence of lytic vacuoles, early rupture of the plasma membrane and shrinkage of the protoplast (van Doorn et al. 2011). Since there are no lytic vacuoles in this case, the corpse morphology appears ‘unprocessed’ looking, meaning that cells appear to have structurally intact organelles and other cellular contents (van Doorn et al. 2011). However, it has been argued that shrinkage of the protoplast can only occur with an intact plasma membrane, meaning that the plasma membrane is involved in the active retraction mechanism that is key to plant PCD events (Reape & McCabe, 2013). Therefore, this definition of necrosis can be subject to criticism based on the status of plasma membrane.

1.5.4 Autophagy

Autophagy is plant cell death that results in vacuolar engulfment of the cytoplasm and its contents leading to increased vacuolar volume and eventual rupture and release of hydrolases
Morphological features of autophagy could include invaginations of the vacuolar membrane (tonoplast), accumulation of autophagosomes (double membrane vesicles) and/or small lytic vacuoles, formation of actin cables, nuclear envelope disassembly and intact organelles until rupture of the tonoplast leading to an empty-walled cell corpse (van Doorn et al. 2011). Examples of autophagy cell death can be seen in the production of xylem fibers in hybrid aspen (Populus tremula L. x P. tremuloides Michx.), elimination of pistil primordia in corn tassels and senescing corolla in morning glory (Ipomoea purpurea L.) (Matile & Winkenbach, 1971; Cheng et al. 1983; Courtois-Moreau et al. 2009). Although autophagy has a unique set of morphological features leading to cell death, there is evidence of both autophagy and AL-PCD morphology existing simultaneously. For example, this has been seen in tracheary element formation in zinnia (Zinnia elegans L.) cells, aerenchyma formation in corn and the perforations developed in monstera (Monstera obliqua Miq.) leaf blades (Groover et al. 1997; Gunawardena et al. 2001; Gunawardena et al. 2005). Therefore, it is highly likely that autophagy and AL-PCD morphology are a result of synchronous or shared genetic and biochemical pathways leading to PCD.
1.6 Hypotheses & Objectives

General Hypothesis: The rapid-necrosis phenotype caused by hydrogen peroxide accumulation in GR giant ragweed prevents the toxic action of glyphosate in the meristematic tissues.

Null Hypothesis 1: There is no difference in subcellular damage caused by glyphosate between GR and GS biotypes of *Ambrosia trifida* L.

Null Hypothesis 2: There is no difference in the distribution of [\textsuperscript{14}C]-glyphosate when applied at the apical meristem of GR and GS biotypes of *Ambrosia trifida* L.

Null Hypothesis 3: There is no difference in the distribution of [\textsuperscript{14}C]-glyphosate when applied at the mature leaf of GR and GS biotypes of *Ambrosia trifida* L.

Objective 1: Determine the subcellular location of light-dependent, hydrogen peroxide damage using electron microscopy and characterize those ultrastructural changes in GR and GS biotypes of *Ambrosia trifida* L.

Objective 2: Determine the distribution of glyphosate over time using [\textsuperscript{14}C]-glyphosate when applied at two different locations; the apical meristem and the mature leaf in GR and GS biotypes of *Ambrosia trifida* L.
CHAPTER 2: MATERIALS AND METHODS

2.1 Plant material and growth conditions

One GR giant ragweed population was compared to two GS populations in these experiments. Each population came from a different location in Ontario, Canada. The resistant giant ragweed population was collected from an agricultural field near Windsor and is characterised by a rapid necrosing reaction followed by glyphosate application. This GR population was previously screened for resistance (Vink et al. 2012). The GS populations originated from Dresden and Petrolia and were combined in experimentation. Seeds for GR and GS populations were stored at 4 C in a seed storage facility in the Department of Plant Agriculture at the University of Guelph. Seed dormancy was broken using methods modified from Stachler (2008). Seeds were mixed with moist sand and were tightly packed into a fine nylon fabric in the shape of a sphere. These were then stored in plastic, non-lidded containers of moist sand for four to eight weeks in a refrigerator at 4 C. Seedlings were transplanted when they were at the radicle to cotyledon emergence stage.

When needed for experiments, plants were grown in a growth room under a 16 h light phase at 25 C and an 8 h dark phase at 20 C, under 75% relative humidity. They were watered as needed with water soluble 20-20-20 N:P:K fertilizer at 312 ppm.

2.2 Absorption and Translocation

To compare the amount of glyphosate that was absorbed and translocated in the GR and GS giant ragweed populations, radiolabeled \([^{14}C]\)-glyphosate was used to quantify if there were
differences. Plants were grown in a growth chamber under the same conditions as mentioned above. GR and GS giant ragweed were transplanted at the cotyledon stage in a baked clay medium in 9 x 9 x 13 cm plastic pots. Plants were watered with 312 ppm of water soluble 20-20-20 N:P:K fertilizer as needed throughout the experiment.

Cotyledons, first true leaves, and in some cases where plants had grown second true leaves, were excised from plants one day prior to glyphosate application to limit the amount of plant material to be biologically oxidized later in the experiment. At the time of glyphosate treatment, plants varied between the two to three-true leaf stage but were all uniform in terms of their source-sink tissue proportions (i.e. all treated plants had a set of developing leaves or primordia at the apex and a set of developed leaves a node below the apex). Plants were sprayed first with formulated glyphosate using a laboratory chamber track sprayer at 900 g a.e. ha\textsuperscript{-1}. Following this application, radiolabeled glyphosate-\textsuperscript{(phosphonomethyl-\textsuperscript{14}C)} (\textsuperscript{14}C-glyphosate) in the form HOOCCH\textsubscript{2}NHCH\textsubscript{2}PO\textsubscript{3}H\textsubscript{2} was spot applied to the adaxial surface of developing or mature leaves with five 2-μL droplets at the second or third node of the giant ragweed plants; \textsuperscript{14}C-glyphosate was applied with a 25-μL micropipet. The initial activity or activity concentration of the \textsuperscript{14}C-glyphosate was 1850 MBq mmol\textsuperscript{-1}. Prior to spot application \textsuperscript{14}C-glyphosate was dissolved into 9.5% (v/v) EtOH solution to achieve 182μM (equivalent to 3333 Bq (200,000 dpm) of radioactivity per 10μL). Three replicates of plants were harvested at 3, 6, 12 and 24 h after treatment.

At time of harvest, plants were dissected into four sections; developing leaves including the apical meristem (referred to as the apical meristem), the mature leaves, the stem and the roots.
The treated tissue was washed twice in two 20 mL vials containing 5 mL of aqueous 9.5% (v/v) ethanol containing 0.5% (v/v) Tween 20 to remove unabsorbed \(^{14}\)C-glyphosate. After washing the leaf, 15 mL of scintillation cocktail was added to each vial. Radioactivity of wash solution was quantified by liquid scintillation spectrometry (LSS) using a Beckman LS6K-SC scintillation counter. Following the leaf wash each portion was wrapped in tissue paper inserted into individual coin envelopes and dried at 60 C for 48 hours. Samples wrapped in tissue paper were then combusted to gaseous \(^{14}\)CO\(_2\) using two different biological oxidizers. Both oxidizers trapped the \(^{14}\)CO\(_2\) in a carbon-14 scintillation cocktail. Radioactivity was quantified by LSS using the Beckman or Tri-Carb B3110TR. \(^{14}\)CO\(_2\) recovery was > 94% determined by combusting known quantities of \(^{14}\)C-glyphosate. The experiment was repeated three times and means and standard errors were calculated.

2.3 Morphology and Micromorphology

Scanning electron microscopy (SEM) was conducted to examine the epidermal surface of the mature leaves of GR and GS giant ragweed. When the plants were at the four- to five-node stage, 2 mm x 4 mm rectangular segments were cut from the middle lobe of mature leaves, between two secondary veins. The hexamethyldisilazane (HMDS) treatment by Wright et al. (2011) was used to prepare the samples for SEM. Sections were passed through an ethanol dehydration series (30%, 50%, 70%, 85%, 95% and 100% (v/v); half hour each) and immersed into HMDS overnight. They were dried in the fume-hood and then mounted onto aluminum stubs with an equal amount of specimens showing abaxial and adaxial sides. Mounted samples were sputter-coated with 30 nanometers of gold using an Emitech K 550 sputter coater and then analyzed and photographed under a Hitachi SU1510 variable pressure SEM at 3kV.
2.4 Anatomy and Ultrastructure

Transmission electron microscopy (TEM) was conducted to examine the ultrastructural changes in the mature leaves of GR and GS giant ragweed over time after glyphosate treatment. When the plants were at the four-to five-node stage, a sublethal dose (6.66 mM equivalent 225 g a.e. ha\(^{-1}\)) of glyphosate was applied to a mature leaf at 1µL with a microliter syringe\(^{15}\). The glyphosate droplet was applied between two secondary veins in the middle lobe. Rectangular leaf segments were cut as above at 0, 2, 3, 4, 5, 6 and 9 hours after droplet application. To ensure that results would represent ultrastructural properties affected by the presence of glyphosate, segments were cut within the boundaries of the residue left by the droplet. Specimens were prepared for TEM using a modified Spurr’s Resin protocol (Ma & Peterson, 2000; Fineran, 1982). The specimens were fixed in 3% glutaraldehyde + 2% paraformaldehyde in 0.05 M sodium phosphate buffer at pH 6.8 overnight at 4 C. Leaf segments were washed three times in buffer followed by post-fixation in 1% buffered OsO\(_4\) for 1 h. After three more buffer washes, specimens were subjected to an ethanol dehydration series (5%, 15%, 30%, 50%, 70%, 95% and 3x 100%; twenty minutes each). Specimens were infiltrated and embedded in Spurr’s resin (Spurr, 1969) and polymerized at 70 C for 8-10 h. The embedded tissues were transversally cut with a diamond ultra knife\(^{18}\) at 0.5-1 µm thick for light microscopy (LM) and at 80 to 100nm for TEM. Sections were mounted on glass slides for LM observations and onto formvar and carbon-coated coppergrids for TEM observation. They were stained with Epoxy Tissue Stain (Electron Microscopy Sciences, catalogue number\(^{14}\)950; a mixture of toluidine blue and basic fuchsin; pH 8.2) and 5% uranyl acetate for 10 min and Reynolds lead citrate for 5 min (Reynolds, 1963), respectively. Observations were made on a JEOL 2011 Scanning Transmission Electron Microscope\(^{19}\) at 200...
kV, and images were taken with a Gatan Ultrascan digital camera\textsuperscript{20} and ‘Digital Micrograph’ software.

2.5 Statistics

2.5.1 Stomatal Density

A completely random design with subsampling was used to determine differences in stomatal density between GR and GS biotypes. The analysis considered the effect of plant biotype to be fixed and the effect of replicate within biotype to be random. The number of stomata per 10 mm\textsuperscript{2} leaf area served as the response variable with a Gaussian error distribution modelled using PROC MIXED in SAS version 9.3 (SAS Institute Inc., Cary, NC). Differences were considered significant at $P \leq 0.05$.

2.5.2 $^{14}$C-Glyphosate Translocation

The experiment investigating $^{14}$C-Glyphosate translocation through giant ragweed plants from the point of application was arranged as a completely randomized block design, blocked over time. The experiment was completed twice, once where glyphosate was applied to the plant apical meristem and once where it was applied to the mature leaf. In each experiment, there were three replications. Analysis of variance was completed using PROC MIXED in SAS version 9.3 (SAS Institute Inc., Cary, NC) with percentage of absorbed $^{14}$C-glyphosate found in the apical meristem, hypocotyl, mature leaves and roots as the dependent variable. Independent variables were partitioned into random variables (reps (blocks)) and fixed variables (biotype (GR or GS) and hours after application (3, 6, 12 and 24)). A SLICE option in the LSMEANS statement was
used to perform a means comparison between GR and GS biotypes at the four time points after $^{14}$C-glyphosate treatment, where there was a significant ($P \leq 0.10$) interaction. To test the model assumptions, residual plots were generated using PROC SGSCATTER and the Shapiro-Wilk statistic was used to test for a normal distribution using PROC UNIVARIATE.

### 2.6 Sources of Materials

1. Pro-mix PGX, Premier Tech Horticulture Inc., 1, avenue Premier, Rivière-du-Loup, Quebec, G5R 6C1
2. MVP, Turface Athletics, PROFILE Products LLC, 750 Lake Cook Rd, Suite 440, Buffalo Grove, IL, 60089800-207-6457
3. Plant-Prod 20-20-20 Classic, Plant Products Co Ltd, 314 Orenda Rd, Brampton, Ontario, L6T 1G1
4. Roundup WeatherMAX With Transorb 2 Technology Liquid Herbicide 540 g a.e./L, Monsanto Canada Inc., 900 – One Research Road, Winnipeg, Manitoba, R3T 6E3
5. PB600-1 Dispenser, Hamilton Company, 4970 Energy Way, Reno, NV 89502
6. Fisherbrand™ 20mL HDPE Scintillation Vials with Urea Cap, Fisher Scientific Company, 112 Colonnade Road, Ottawa, Ontario, K2E 7L6
7. Sunshine Mix 4, Planet Natural, 1251 N. Rouse Ave, Bozeman, MT 59715
8. Dry top dressing sand, Hutcheson Sand Mixes, 1265 Aspdin Rd, Huntsville, Ontario, P1H 2J2
9. Biological Material Oxidizer, Model OX-300, R.J. Harvey Instrument Coop., Hillsdale, NJ 07642
10. PerkinElmer Sample Oxidizer, Model 307, PerkinElmer®, 940 Winter St, Waltham, MA 02451
Ecolite, ICN Biomedicals Inc., 15 Morgan, Irvine, CA 92618

Scintillation counter, Beckman Instruments Inc. 2500 Harbor Blvd., Fullerton, CA 92634

Tissue paper, KimWipe, Kimberley Clark Inc., Rosewell, GA 30076

Tri-Carb B3110TR Liquid Scintillation Analyzer, PerkinElmer®, 940 Winter St, Waltham, MA 02451

10 μL, Model 701 N SYR, Cemented NDL 26s ga, 2 in, point style 2, Hamilton Company, 4970 Energy Way, Reno, NV 89502

K550X Sputter Coater, Quorum Technologies Ltd, South Stour Ave., Ashford, Kent, TN23 7RS, UK

SU1510 Variable Pressure SEM, Hitachi High Technologies America, Inc., 1375 North Avenue, Dallas, TX 75261-2208

Diamond Ultra Knife, DiATOME AG, P.O. Box 2501 Biel, Switzerland

JEOL 2011 Scanning Transmission Electron Microscope, JEOL Ltd., 3-1-2 Musashino, Akishima, Tokyo 196-8558, Japan

Gatan Ultrascan digital camera, Gatan, Inc., 5794 W. Las Positas Blvd., Pleasanton, CA 94588
3.1 Description of Untreated Plants

3.1.1 Leaf Morphology

Leaf blades for GS and GR biotypes can be ovate (egg shaped), elliptic (Figure 3.1 A & B) to round-triangular shaped. However, most leaf blades are palmately lobed with 3 to 5 lobes per leaf (Figure 3.1 C & D). The leaves are 40 to 150 mm in length by 30 to 70 mm in width but can grow as large as 250 mm long by 200 mm wide (data not shown). The bases of the leaves are truncate (perpendicular or 90° to the petiole) to cuneate (wedge shaped or 135° to the petiole). The leaf margins are usually toothed but can sometimes be entire (smooth). Adaxial (top) and abaxial (bottom) leaf surfaces can be scabrous (rough) and/or gland-dotted (semi-transparent dots).

3.1.2 Leaf Micromorphology

The leaf blade epidermis has both secretory or ‘glandular’ trichomes (Figure 3.2 A) and non-secretory trichomes (Figure 3.2 B). Secretory trichomes can be unicellular or multicellular (3 to 5 cells including a terminal secretory cell) with cylindrical shaped cells that are 40 to 60 μm in diameter and 40 to 50 μm long (Figure 3.2 C). Non-secretory hairs usually comprise two to three cells that are 100 to 200 μm long with the basal cell swollen and the terminal cells tapering gradually into a sharp point (Figure 3.2 C). The secretory trichomes develop on portions of the leaf epidermis located directly above (or underneath) a third or fourth order vein while non-
secretory cells are non-specific in their spatial arrangement and are spread over the entire epidermis. The leaf epidermis is smooth and devoid of epicuticular wax depositions, but the non-secretory trichomes have lignified cell walls with a verrucous surface (Figure 3.2 B & C).

The leaves are amphistomatic (stomata are present on both abaxial and adaxial epidermis). The stomata are “anomocytic”, meaning that they are surrounded by a limited number of cells that are indistinguishable from other epidermal cells (Farooqui, 1981). Their density, determined only on the abaxial epidermis is highly variable within both biotypes, ranging from 25 - 65 stoma/10mm² leaf area. On average, GR plants have a stomatal density of 4.24 ± 0.28 and S plants have 3.74 ± 0.28 (42 and 37 stoma/10mm²), however statistical analysis reveals these are not different at the P ≤ 0.05 level (Appendix, Table A1).

3.1.3 Leaf Venation Pattern

According to Hickey (1973), the general leaf venation pattern of A. trifida is “semicraspedodromous”, meaning that the secondary veins begin to branch within the margin area of the leaf blade and ultimately end at the margins edge (Figure 3.3 A). On average, six orders of veins were distinguished with some ending in the seventh order (Figure 3.3 B). The small areas between leaf veins, also known as areoles, have vein endings that are branched or unbranched (Figure 3.3 C). Ambrosia trifida tracheary elements (xylem) usually increase in diameter and number toward the end of the veins (Figure 3.3 D) and are not always accompanied by sieve tubes (phloem) at those endings (data not shown).
3.1.4 Anatomy and Ultrastructure of Photosynthetic Tissue, Stomata and Minor Veins

3.1.4.a Photosynthetic tissue

Although leaves are amphistomatic (having stomata on adaxial and abaxial sides), the mesophyll is dorsiventral (within the top half of a cross sectioned leaf). The palisade mesophyll consists of 1-2 layers of well-defined, rectangular-shaped, vertically oriented and tightly packed cells (Figure 3.4 A). Spongy mesophyll has 4-6 layers of branched/lobed or irregularly-shaped parenchyma cells, separated by large intercellular spaces (Figure 3.4 A). The bundle sheath cells that surround minor veins separate the vascular tissue from the intercellular spaces (Figure 3.4 B). The protoplast of mesophyll and bundle sheath cells have 1-2 large central vacuoles (data not shown) with peripheral cytoplasm containing the nucleus, chloroplasts, mitochondria, ribosomes and endoplasmic reticulum (ER) (Figure 3.4 C). Chloroplasts in the mesophyll and bundle sheath cells line the cell walls and have a lenticular shape, often with a concave face directed towards the cell wall (Figure 3.4 D). Chloroplast thylakoids are arranged in well-defined grana stacks, separated by lamellae with plastoglobuli and starch present (Figure 3.4 E). Since leaf samples were collected in the morning, the amount of starch in the chloroplasts is low (1 to 5% of the organelle’s volume), in both GS and GR plants (data not shown).

3.1.4.b Ultrastructure of stomata

Stomata of both GS and GR plants are identical structurally and ultrastructurally. Guard cells exhibit the characteristic cell-wall thickenings, mitochondria, ER, nucleus (Figure 3.4 F) and chloroplasts with starch grains (data not shown).
3.1.4.c Minor veins

Anatomy and ultrastructure of vasculature were examined in detail only in the minor veins of the third and fourth order. Minor veins of *A. trifida* consist of four types of cells: tracheary elements, sieve tube elements, companion cells (transfer cells), and phloem parenchyma cells (shown previously in Figure 3.4 B). Specifically, minor veins are termed “closed” which is characterized by a reduced number of plasmodesmata fields at the interface between the mesophyll and minor vein cells (Gamalei 1989, 1991). Gamalei (1991) further classifies this closed type of minor vein “type 2b” due to the presence of reticulate (finger-like) cell wall ingrowths in the transfer cells (Figure 3.4 G). More recently however, classification of this type of minor vein by Batashev et al. (2013) could be further categorized as “type 2IV” due to the presence of chloroplasts in the transfer cells and cell wall ingrowths in the phloem parenchyma cells (also shown in Figure 3.4 G). These ingrowths are frequent on the walls between transfer cells, phloem parenchyma and bundle sheath cells and can only be rarely observed at the contact interface of the sieve tube members (data not shown). This structural classification of *A. trifida* is important to note because plants with type 2 minor veins are always apoplastic (phloem) loaders. Which implies that, glyphosate travel at low concentrations could only occur through an apoplastic, phloem loading mechanism.

The cytoplasm of transfer cells is dense with numerous mitochondria, ER, ribosomes, and chloroplasts with well-developed grana (Figure 3.4 H). Phloem parenchyma cells are similar to transfer cells but have a less dense cytoplasm, fewer wall ingrowths, mitochondria, chloroplasts and ER (Figure 3.4 I). Plasmodesmata occur at a low frequency between transfer and phloem
Parenchyma cells (Figure 3.4 J) and they are absent between sieve tube members and other types of cells (data not shown).

Tracheary elements have spiral secondary cell wall thickenings and often exhibit grainy contains (Figure 3.4 K). Sieve tube elements, depending on their developmental stage, can contain mitochondria, ER, sieve element plastids, and filamentous proteins but are most often seen with very little cellular content (Figure 3.4 L). Sieve tube elements are located at the center of the vascular bundle and they never come into contact with bundle sheath cells or tracheary elements (shown previously in Figure 3.4 B). GS and GR plants have essentially the same leaf morphology, micromorphology, anatomy, and ultrastructure.

3.2 Observations on Treated Glyphosate Susceptible Plants from 0 to 1 Week after Treatment

No ultrastructural changes were observed in GS plants in the six hours after glyphosate application in this study (data not shown). Examination of GS plants one week after glyphosate application revealed drastic cellular changes indicative of the profound phytotoxic effects this herbicide has on plants. This is consistent with the phytotoxic action of glyphosate which generally produces observable symptoms between 7 to 10 days after application. A uniform level of degradation becomes apparent within all of the photosynthetic (i.e. bundle sheath and mesophyll cells) and vascular (i.e. sieve elements, tracheary elements, transfer cells and phloem parenchyma) tissues of GS plants. Temporally and spatially, consistent ultrastructural symptoms can be observed in all tissues.
Rupturing of the plasma membrane in all cells is a primary effect of glyphosate in GS plants (Figure 3.5 A). In addition, the protoplasts of GS cells do not collapse and remain unprocessed looking, meaning that most organelles are present and/or intact due to the absence of lytic vacuole degradation (Figure 3.5 B & C). In addition to these features, other ultrastructural characteristics are observed including swollen and ruptured chloroplasts with swollen plastoglobuli (Figure 3.5 B), which contrasts with normal chloroplast structure in untreated plants (shown previously in Figure 3.4 E). Also, in bundle sheath and mesophyll cells, many peroxisomes accumulate and are predominantly located along the inner cell wall amongst structurally intact mitochondria (Figure 3.5 C) which is not seen in bundle sheath cells of the untreated plants (shown previously in Figure 3.4 D). GS plants display several morphological “hallmark features” of necrotic cell death such as a ruptured plasma membrane and an unprocessed cell corpse.

These changes observed at the cellular level are consistent with reports published in other plants treated with glyphosate such as swelling of chloroplasts, disorganisation of grana and lamelae, and total loss of chloroplast integrity (Vaughn & Duke, 1986; Mollenhauer et al. 1987; Lorentz et al. 2011). The results of this study conclude that GS giant ragweed responds similarly to glyphosate as other plants species do.

### 3.3 Observations on Treated Glyphosate Resistant Plants from 0 to 6 hours

In contrast to GS plants, ultrastructural changes in GR plants occurred very rapidly following glyphosate treatment. As a rule, although we have examined leaf areas on which glyphosate was applied directly, the modifications in the tissues under the epidermis have a “patchy” character.
Therefore, the sequence of ultrastructural transformations is not uniform across the leaf section and/or within the same tissue. In order to rectify this issue, classifications of ultrastructural transformations will be based on what time they were first observed.

The most striking response observed in GR plants after the application of glyphosate was the rapid hyperaccumulation of starch in the chloroplasts at 2 hours after treatment (HAT). While chloroplasts of untreated plants appeared normal and functional (Figure 3.6 A), starch content increased remarkably 2 HAT in chloroplasts located in the mesophyll cells (palisade and spongy parenchyma) and bundle sheath cells (Figure 3.6 B). The rapid starch accumulation in chloroplasts of the mesophyll cells is so profuse that it is clearly visible even under a light microscope (data not shown).

In addition to starch hyperaccumulation, chloroplasts in all tissues began to show swelling at 2 HAT. For example, chloroplasts at 0 HAT appear normal in bundle sheath cells (Figure 3.6 C) and by 2 HAT, they show major swelling (Figure 3.6 D). Chloroplasts in the phloem parenchyma and transfer cells may have also developed a system of anastomosing tubules and saccules along their inner membrane called peripheral reticulum (PR). At 0 HAT, transfer cell chloroplasts have a normal double membrane (Figure 3.6 E) but by 2 HAT, these chloroplasts have accumulated a white blotted appearance inside their outer membrane (Figure 3.6 F). At this moment, however, other organelles in the vascular tissue remain largely unchanged (data not shown).

Later on, at 3 HAT, the changes in the photosynthetic tissues become more drastic. The hyperaccumulation of starch in the mesophyll and bundle sheath cells has stopped and in some
cells there are signs that some of the starch may be metabolised. This difference can be clearly observed in chloroplasts of bundle sheath cells between 2 HAT (Figure 3.7 A) and 3 HAT (Figure 3.7 B). Also, at 3 HAT, chloroplast damage begins within the photosynthetic tissues. While At 2 HAT, chloroplasts in bundle sheath cells have relatively organized thylakoid stacks or grana (Figure 3.7 C), at 3 HAT, the chloroplasts begin to lose organization in their grana and lamellae (Figure 3.7 D). Lastly, the mesophyll cells begin to show protoplast retraction away from the cell wall and these changes can be observed between 2 HAT (Figure 3.7 E) and 3 HAT (Figure 3.7 F).

In comparison to the photosynthetic tissues, the vascular tissues are only beginning to show ultrastructural changes at 3 HAT. Chloroplasts of the vascular cells, more specifically those of the phloem parenchyma, begin to show starch accumulation from 2 HAT (Figure 3.7 G) to 3 HAT (Figure 3.7 H). Phloem parenchyma also begins to show mitochondrial swelling and protoplast retraction from the cell wall between 2 HAT (Figure 3.7 I) and 3 HAT (Figure 3.7 J). Yet at this stage, transfer cells and sieve tube members are comparatively less affected (data not shown).

By 4 HAT, most of the organelle changes are underway and can be seen at various stages of swelling and internal damage as described above. However, all tissues, especially mesophyll cells have begun to appear osmophilic (darker stained due to the presence of lipids). This contrast can be seen especially in the palisade mesophyll between 3 HAT (Figure 3.8 A) and 4HAT (Figure 3.8 B). No appreciable changes were observed at 5 HAT compared to 4 HAT (data not shown).
At 6 HAT, large groups of cells within the mesophyll are nearly or entirely destroyed. Chloroplasts and other organelles have lost their integrity and the plasma membrane, while not ruptured, is detached from the cell wall and the entire protoplast collapses and retracts. These changes can be seen between 4 HAT (Figure 3.9 A) and 6 HAT (Figure 3.9 B). Digestion of organelles, potentially through vacuolar autophagy begins in the phloem parenchyma and transfer cells, especially of chloroplasts, mitochondria and smaller cytosol contents. For example, in transfer cells, vacuolar digestion can be observed between 4 HAT (Figure 3.9 C) and 6 HAT (Figure 3.9 D).

It can be concluded that over the six hour ultrastructural observations, sieve tube members and transfer cells show the highest resilience. This condensed morphology is consistent with an apoptosis-like programmed cell death and autophagy due to the maintenance of plasma membrane integrity and presence of autophagic structures and vacuolar degradation of organelles.

The application of glyphosate produced rapid ultrastructural changes in the leaves of GR plants. The general sequence of changes for GR treated plants are as follows; accumulation of starch in the photosynthetic tissues and swelling of chloroplasts in both photosynthetic and vascular tissues, accompanied by peripheral reticulum (but only in the transfer cells). Then, starch metabolism, loss of chloroplast structure with swelling of mitochondria. Eventually chloroplasts, mitochondria and other organelles show enhanced internal changes which lead to complete loss of organization/structure by 6 HAT (but plasma membrane and organelle membranes remain intact). The process appears first in the mesophyll, followed by bundle sheath cells and then
vascular cells. Within the vascular cells, the first impacted are the phloem parenchyma and the last are the transfer cells and sieve tube members.
Figure 3.1. Morphological classification of *Ambrosia trifida* L. leaves where, (A) is ovate, (B) is elliptic, (C) is palmate three-lobed and (D) is palmate five-lobbed.
Figure 3.2. Micromorphological features of *Ambrosia trifida* L. leaf epidermis obtained using SEM and light microscopy where, (A) represents secretory trichomes, (B) is a non-secretory trichome and (C) is a longitudinal section of a leaf showing the cells that make up secretory (open triangle) and non-secretory trichomes (black triangle). AdE, adaxial epidermis; AbE, abaxial epidermis.
Figure 3.3. Leaf venation pattern of *Ambrosia trifida* L. obtained using light microscopy proceeding basic fuchsin staining where, (A) is a portion of the leaf blade showing a secondary vein reaching the leaf margin, (B) classification of leaf venation from third to seventh order, (C) fourth, fifth and sixth order veins; sixth order veins ending in unbranched or branched (open black circle) forms, (D) sixth order vein increasing in diameter towards its apex.
Figure 3.4. Light microscopy (A & B) and transmission electron micrographs (C to L) of leaf cells of *A. trifida* L. prior to treatment with glyphosate. (A) Longitudinal-sectioned leaf showing palisade (Pa) and spongy (Sp) mesophyll layers. (B) Cross-sectioned leaf showing bundle sheath (BS) cells surrounding vascular tissues such as tracheary elements (TE), sieve elements (SE), transfer cells (TC) and phloem parenchyma (PP). (C) Protoplast of BS cells containing mitochondria (M), Chloroplasts (Ch), nucleus (N), endoplasmic reticulum (open triangles) and ribosomes (black arrows). (D) BS cells and mesophyll (Me) cells with concave shaped Ch’s. (E) Ch in a BS cell with organized grana stacks (open white triangle) separated by lamellae (open white arrow) with plastoglobuli (open black arrow) and starch (open black triangle). (F) Guard cell with a thick cell wall (CW), N, M and endoplasmic reticulum (open black triangle). (G) TC and PP have CW ingrowths (black triangles). (H) TC’s have a dense cytoplasm rich in ribosomes, Ch’s, M and endoplasmic reticulum (open black arrows). (I) PP has a less dense cytoplasm with fewer Ch’s, M and endoplasmic reticulum (open black triangles). (J) Plasmodesmatal connections (open white arrows) between the CW’s of a TC and PP. (K) Secondary CW thickenings (open black arrows) in a TE. (L) Plastids (P) and filamentous proteins (black arrows) found in a SE.
Figure 3.5. Transmission electron micrographs of bundle sheath cells of glyphosate susceptible *A. trifida* L. taken one week after glyphosate treatment. (A) Bundle sheath (BS) cell with a ruptured plasma membrane (black triangle) near the cell wall (CW). (B) BS cells with damage to the chloroplast (Ch) including swelling, a ruptured membrane (black arrow), swollen plastoglobuli (open triangles) and disorganized grana and lamellae. (C) BS cells with identifiable M and Ch. Also, there are numerous peroxisomes (open arrows) clustered near the inner CW.
Figure 3.6. Transmission electron micrographs of glyphosate resistant *A. trifida* L. leaves treated with glyphosate and sampled zero hours after treatment (A, C, E) and two hours after treatment (B, D, F). (A & B) Minor veins surrounded with bundle sheath (BS) cells and mesophyll (Me) cells; while at 0HAT there is very little starch in the chloroplasts (Ch), at 2HAT starch granules are very abundant in the chloroplasts of BS and Me cells. (C & D) Ch’s in BS cells at 0HAT show normal structure with some starch (open black triangle), while at 2HAT swelling and large starch (S) grains are evident. (E&F) Transfer cells (TC) at 2HAT have swollen Ch’s with peripheral reticulum (open black triangles) compared to Ch’s at 0HAT that show normal structure without peripheral reticulum.
Figure 3.7. Transmission electron micrographs of glyphosate resistant *A. trifida* L. leaves treated with glyphosate and sampled two hours after treatment (A, C, E, G, I) and three hours after treatment (B, D, F, H, J). (A&B) Starch (S) is abundant in bundle sheath (BS) cell chloroplasts (Ch) at 2HAT and soon becomes metabolised at 3HAT (double sided arrows). (C&D) At 2HAT, BS cell Ch’s have relatively organized grana stacks (open white triangles) and then they become indistinguishable by 3HAT. (E & F) Other photosynthetic tissue such as palisade mesophyll (Pa) goes from having protoplasts adhering to the inner cell wall at 2HAT to showing signs of retraction at 3HAT (double sided arrows). (G & H) Phloem parenchyma (PP) cells are void of starch at 2 HAT and begin to accumulate starch (open black triangles) at 3 HAT. (I & J) PP cells appear normal with structurally intact Ch’s and mitochondria (M) at 2 HAT, but at 3 HAT, Ch’s become disorganized, M begin to swell and the protoplast has retracted away from the cell wall (double sided arrows).
Figure 3.8. Transmission electron micrographs of glyphosate resistant *A. trifida* L. leaves treated with glyphosate and sampled three hours after treatment (A) and four hours after treatment (B). All tissues, especially palisade (Pa) mesophyll become more osmophilic (darker) from 3 to 4 HAT. AdE,: Adaxial epidermis.
Figure 3.9. Transmission electron micrographs of glyphosate resistant *A. trifida* L. leaves treated with glyphosate and sampled four hours after treatment (A, C) and six hours after treatment (B, D). (A&B) At 4HAT, most protoplasts are still adhered in palisade (Pa) and spongy (Sp) mesophyll, but by 6HAT almost all Pa and Sp mesophyll cells have retracted protoplasts (open black triangles). (C&D) At 4HAT, transfer cells have swollen mitochondria (M) and disorganized chloroplasts (Ch) but by 6HAT you begin to see these organelles digested within the vacuole (V). At 6HAT, the V can be observed with M at different stages of digestion, such as double membrane remnants (open triangle). The V is able to do this, potentially through autophagic structures (open black arrow).
CHAPTER 4: ABSORPTION AND TRANSLOCATION OF $[^{14}\text{C}]$-GLYPHOSATE IN GLYPHOSATE RESISTANT AND SUSCEPTIBLE PLANTS OF AMBROSIA TRIFIDA L. WHEN APPLIED AT SOURCE AND SINK TISSUES SEPARATELY

4.1 Results

There were differences in the distribution of $[^{14}\text{C}]$-glyphosate in GR giant ragweed in comparison to GS plants. However, these differences were only observed when $[^{14}\text{C}]$-glyphosate was applied at the apical meristem (Figure 4.1 A-B) and not observed when the treatment was applied at the mature leaf (Figure 4.2 A-B). When radioactivity was applied at the apical meristem, there were differences seen in the distribution of $[^{14}\text{C}]$-glyphosate at 24 HAT. For example, there was an increasing amount of $[^{14}\text{C}]$-glyphosate in the mature leaf tissue of the GR biotype in comparison to the GS biotype at 24 HAT (Figure 4.1 A). The percentage of absorbed $[^{14}\text{C}]$-glyphosate in GR mature leaves was 19.8% while in the GS it only reached to 1.4% (Appendix, Table A2). This coincided with a decreasing amount of $[^{14}\text{C}]$-glyphosate in the apical meristem tissue of GR in comparison to GS at 24HAT (Figure 4.1 B) where GR only had 68.2% in the apical meristem and GS remained fairly high at 93.2% (Appendix, Table A2).
Figure 4.1. Time course distribution of percentage absorbed $^{14}$C-glyphosate in (A) mature leaves, (B) apical meristem, (C) stem, (D) roots between glyphosate resistant (●) and glyphosate susceptible (○) giant ragweed (Ambrosia trifida L.) when applied exclusively at the apical meristem. Data are means of three replications ± standard error where * represents $P \leq 0.10$. 
Figure 4.2. Time course distribution of percentage absorbed $[^{14}\text{C}]$-glyphosate in (A) mature leaves, (B) apical meristem, (C) stem, (D) roots between glyphosate resistant (●) and glyphosate susceptible (○) giant ragweed ($\textit{Ambrosia trifida}$ L.) when applied exclusively at the mature leaf. Data are means of three replications ± standard error.
CHAPTER 5: DISCUSSION

The first objective of this research was to determine the subcellular location of light-dependent, hydrogen peroxide damage using transmission electron microscopy and then characterize those ultrastructural changes in GR and GS biotypes of *Ambrosia trifida* L. The results from this experiment showed two morphologically distinct PCDs over two vastly different time lines between GR and GS biotypes. Due to the current controversy in the literature surrounding the morphological classification of PCD it is challenging to put these results in finite groups of PCD. However, due to vast differences in the duration and severity of morphological responses to glyphosate, GR and GS biotypes are considered to have different PCDs.

In the GS biotype, several morphological traits were observed that have been classified as necrosis or necrotic PCD. This PCD response should not be confused with the name of the GR biotype (rapid-necrosing), as it was named prior to this research. The morphological or ‘hallmark traits’ of necrosis that fit with the results of the GS biotype are a ruptured plasma membrane or the absence of a controlled protoplast retraction (defined by Reape & McCabe, 2013) and a cell corpse that appears ‘unprocessed’ with the presence of visually intact organelles (defined by van Doorn et al. 2011). These results fit in both authors classification of necrosis, however other aspects of their classification were not observed in this research. For example, McCabe et al. (1997) define necrosis as a PCD that occurs under a high level of stress causing severe cellular damage such as lysed plasma membrane, mitochondria and nuclei within 24 hours. In the GS biotype, lysed mitochondria and nuclei were not observed.
and the observations were taken much later. Therefore, necrosis induced morphology according to McCabe (1997) could be seen after longer durations of time than previously reported. Swollen mitochondria as an early symptom of necrosis, which was mentioned by van Doorn (2011) was not observed in this study. However, swollen mitochondria could have been missed since our observations were taken at a later time on this course of defined PCD morphology.

Other ultrastructural changes in the GS biotype that are not associated with necrosis were observed. These observations were characteristic of glyphosate death morphology seen in other dicot species. These features included chloroplast swelling, starch degradation and chloroplast damage including disorganized grana and lamellae and ruptured chloroplast membranes (Vaughn and Duke, 1986; Mollenhauer et al. 1987; Lorentz et al. 2011). Although there have been studies that have classified ultrastructural changes caused by glyphosate, none of them have mentioned the status of the plant cell plasma membrane. Therefore, it remains unknown whether cell death following the application of glyphosate in dicot species can be distinctly classified as necrosis in all circumstances. Also, as mentioned previously, timing of necrosis morphology could be relevant in this classification so it is important for future research to consider this in their experimental design and methodology.

A particular result that was obtained in the GS biotype and not in the GR biotype or in other literature was the presence of swollen plastoglobuli. Plastoglobules are lipid bodies that are found on thylakoid membranes in the chloroplast (Austin II et al. 2006). Unfortunately, very
little research has been done on plastoglobuli and currently their function and origin is unknown. However, an enzyme involved in oxidative stress, tocopherol cyclase, has been localized to the inner surface of plastoglobules (Austin II et al. 2006). This indicates that plastoglobules could be involved in stress induced responses caused by glyphosate in the chloroplasts of GS plant cells.

Another result found in the GS biotype associated with oxidative stress was the increased presence of peroxisomes. Peroxisomes are single membrane bound organelles that contain many enzymes involved in oxidation such as the production of hydrogen peroxide (H$_2$O$_2$) (Cooper, 2000). However, since H$_2$O$_2$ is toxic in the cell, peroxisomes also contain antioxidant enzymes such as catalase which converts H$_2$O$_2$ into water (Cooper, 2000). The reason why peroxisomes are more numerous in the GS biotype is likely due to oxidative glyphosate stress. Similar proliferation of peroxisomes was observed in pea (Pisum sativum L.) plants under salt-induced oxidative stress (Hernández et al. 1995).

In the GR biotype, AL-PCD was observed and followed a very quick timeline relative to the GS biotype. Within 6 hours, hallmark features of AL-PCD were observed such as the controlled condensation or retraction of the protoplasm without rupturing the plasma membrane (Reape & McCabe, 2013). However, it is important to note that many other ultrastructural changes also occurred during this time frame and are not defined within the AL-PCD classification. For example, evidence of autophagic structures such as the invagination of the tonoplast was observed along with degradation of organelles in the
vacuole. These ultrastructural phenomena could be associated with autophagy, which is probable considering both types of cell death have been reported to occur simultaneously (Groover et al. 1996; Gunawardena et al. 2001; Gunawardena et al. 2005). Interestingly, swelling of mitochondria was observed, which is a morphological trait of necrosis defined by van Doorn et al. (2011). However, GR plants display a cell death response that includes morphological traits of autophagy which was not mentioned in van Doorn’s (2011) classification of necrosis. Therefore, PCD in GR plants is less likely to be necrosis-PCD and more likely to be AL-PCD.

Other morphological changes were observed in GR plants outside of specific PCD morphology such as peripheral reticulum in the chloroplasts of transfer cells and a massive increase of starch in the chloroplasts of photosynthetic tissues at 2 HAT. Very little research has been conducted on the function of peripheral reticulum, however it has been suggested they play a role in rapid transport of substances in and out of the chloroplast (Laetsch, 1968; Rosado-Alberio et al. 1968; Gracen et al. 1972). This could indicate that the chloroplasts in transfer cells of GR plants could have an early and unique response related to specific AL-PCD events. Especially since peripheral reticulum was not observed at 2 HAT in the GS biotype (data not shown). Secondly, the increased amount of starch in chloroplasts of photosynthetic tissues at 2HAT could be reminiscent of phloem transportation impairment. For example, the presence of glyphosate could trigger a halt in an active phloem transport mechanism, leading to starch build up in actively photosynthesizing tissues. Similar to glyphosate, if an active phloem transporter was impaired sucrose would also be denied entry into the phloem, leading to a build-up of sucrose in the form of starch. However, once the GR
biotype recognizes that it is no longer exporting sugars, that starch build-up ceases and is eventually degraded by 3 HAT.

The second objective of this research was to determine the distribution of glyphosate over time using radio-labeled glyphosate applied at the apical meristem and the mature leaf in GR and GS biotypes. The results of this study confirmed that glyphosate transport is impaired in the GR biotype in comparison to the GS biotype. However, differences in translocation were only observed when [$^{14}$C]-glyphosate was applied at the apical meristem. These results addressed criticism of previous research done on glyphosate translocation in the GR biotype (Green, 2014). For example, questioning why the developing leaves and apical meristem did not experience the same necrotic response that the older leaves do.

Currently the research suggests that there is an active glyphosate transport mechanism that is impaired in the GR biotype and that it is dependent on the presence of glyphosate. This would explain why the GR plants have a decreasing amount of glyphosate in their apical meristem in comparison to GS plants over 24 hours. Previous research on the GR biotype showed that the GR biotype could become GS at very high concentrations of glyphosate (Green, 2014). Specifically, a shikimic assay was used to show that an 80-fold increase in glyphosate concentration resulted in an increase in shikimic acid in the GR biotype, indirectly implying eventual EPSPS sensitivity (Green, 2014). Since this shows that EPSPS in not inhibited at low concentrations of glyphosate and it is recognized that glyphosate uses an active transport mechanism at relatively low concentrations of glyphosate, it is assumed that an altered
membrane transporter is impairing active entry of glyphosate in the cell or chloroplast, reducing the concentration at the EPSPS binding site (Denis & Delrot, 1993; Hetherington et al. 1998; Morin et al. 1997; Green, 2014). Supporting this theory is our observation of increasing translocation of glyphosate to the mature leaves in the GR biotype. Presumably, the $^{14}$C-glyphosate that was added to the apical meristem tissue was prevented from entering the phloem because of an altered membrane transporter and remained in the apoplastic space, eventually pulled through the xylem to the closest transpiring source leaves. Therefore, where glyphosate accumulates is where cell death occurs. Additionally, it could explain why there is rapid starch accumulation in the GR biotype 2 HAT. Since glyphosate and sucrose share similar transport systems, when glyphosate is detected, the active transport mechanism used to transport glyphosate and sucrose becomes impaired and causes a build-up of sucrose in the form of starch in the photosynthesizing cells of the mature leaves.

Taken together, these two experiments could indicate that a significant amount of glyphosate is sequestered in the collapsed protoplasts of mature leaves via the rapidly AL-PCD occurring in the palisade and spongy parenchyma cells. When glyphosate is applied to GR plants the xylem may be involved in the passive movement of glyphosate to mature leaves and then toward the margins of those leaves which is reminiscent of the necrosis pattern on leaf tissue (data not shown).
Contributions of this Research

This was the first ultrastructural investigation of *Ambrosia trifida* L., therefore many cellular aspects of leaf and minor vein morphology were identified for this plant species. By using a microscopical approach, a new mechanism of reduced translocation was illuminated (literally) through the identification of different PCDs in GR and GS biotypes. This study also addressed previous criticism surrounding the necrotic response in the GR biotype. It can now be affirmed that glyphosate induced cell death only occurs where glyphosate is accumulating, explaining why it is only visible in mature leaf tissue of GR plants over time. These experiments have shown that translocation impairment as a glyphosate resistance mechanism is complex. The involvement of other plant defense systems make glyphosate resistance possible for these plants.

Limitations of this Research

The biggest limitation of this research was not having enough replication in the translocation study. Due to equipment failure, only one replication of samples could be combusted locally. After several months searching for collaborative labs, Canadian Nuclear Laboratories in Deep River, Ontario, lent a hand but they only had enough time to process and evaluate two replications of the experiment. Regardless of good machinery efficiency, having only three replications of data on a highly variable weed species increased the standard error.

Other limitations of this research included the variability of the weed species itself. In the GR population, some individuals are susceptible to glyphosate. Since it is not always possible to pre-screen samples (e.g. spray them to verify for the GR phenotype) prior to doing a
microscopic analysis, I had to take a risk and chose a small amount of samples hoping they would encompass the GR phenotype. For example, during my ultrastructural investigation, I had to eliminate a 9 HAT observation because it displayed GS morphology. The reason why I could not increase my sample size was due to resources and time limitations. Embedding TEM samples takes weeks and is very expensive to do. Also, TEM imaging of my results was outsourced to a separate contractor who had the proper equipment, but was also a costly expenditure.

**Future Directions**

These results strongly suggest that treatment of glyphosate triggers a PCD pathway in GR and GS biotypes. A common phenomenon associated with PCD is internucleosomal DNA fragmentation which can be tested for through analysis of DNA fragmentation using agarose gel electrophoresis (Ryerson & Heath, 1996). Since the timing of DNA laddering is of considerable importance in PCD, a time lapse experiment after glyphosate treatment would provide insight on the initiation of PCD in GR and GS biotypes. Also, as more biochemical and genetic analyses of PCD emerge, early events in PCD can be examined for as well. For example, one of the molecular markers for the mitochondrial-dependent PCD pathway is the release cytochrome c (cyt c) from the inner membrane of the mitochondrion to the cytosol (Robson & Vanlerberghe, 2002). Performing an immunoblot analysis probed with a cyt c antibody would be a good way of suggesting PCD events after glyphosate application to the GR or GS biotypes (Robson & Vanlerberghe, 2002). To determine whether cyt c release is a key event preceding the death of glyphosate-treated plants, a time course experiment can be
performed to determine the cyt c level in leaf mitochondria using immunoblots probed with cyt c antibody.

The cell death by glyphosate treatment may occur through a chloroplast-dependent pathway of PCD. The anti-apoptotic protein Bcl-2, which is involved in animal PCD, performs similar function in plants but is localized to the chloroplast as well as the mitochondria (Dickman et al. 2001). If the GR or GS biotype have a mitochondrial-independent pathway or a chloroplast-dependent pathway of PCD, a similar time course experiment can be performed to determine the Bcl-2 level in leaf chloroplasts and mitochondria using immunoblots probed with Bcl-2 antibody (Chen & Dickman, 2004).

In order to confirm with more confidence and a lower probability rate, more replications of the translocation study should be done to reduce standard error. In addition to this, advanced research on glyphosate translocation mechanisms could be looked into such as phosphate transporters associated with active phloem import of glyphosate.
LITERATURE CITED


Investigating the mechanisms of glyphosate resistance in _Lolium multiflorum_. Planta. 226:
395-404.

Pline-Srnic, W. 2006. Physiological mechanisms of glyphosate resistance. Weed Tech. 20:
290-300.


Price, C. E. and N. H. Anderson. 1985. Uptake of chemicals from foliar deposits: effects of


Reape, T. J., E. M Molony and P. F. McCabe. 2008. Programmed cell death in plants:

Reape, T. J. and P. F. McCabe. 2013. Commentary: the cellular condensation of dying plant

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron opaque stain for

alternative oxidase have increased susceptibility to mitochondria-dependent and -independent pathways of programmed cell death. ASPB. 129: 1908-1920.


Rubin, J. L., C. G. Gains, R. A. Jensen. 1984. Glyphosate inhibition of 5-
enolpyruvylshikimate 3-phosphate synthase from suspension-cultured cells of _Nicotiana
silvestris_. Plant Physiol. 75: 839-845.

fragments during cell death induced by fungal infection or by abiotic treatments. Plant Cell.
8: 393-402.

programmed cell death leading to anther dehiscence in tomato. Plant Phys. 149: 775-790.

shikimate accumulation assay with excised leaf discs. Weed Sci. 53: 769-774.


Stachler, J. M. 2008. Characterization and management of glyphosate-resistant giant ragweed (Ambrosia trifida L.) and horseweed [Conyza canadensis (L.) Cronq.]. Ph.D. Dissertation. The Ohio State University: Columbus, OH.


CHAPTER 7: APPENDIX

Table A1. Analysis of variance of stomatal density per 10 mm² leaf area of giant ragweed (*Ambrosia trifida* L.) where four subsamples (1 mm² sections of leaf) were taken from ten plants of glyphosate resistant (GR) and glyphosate susceptible (GS) biotypes.⁷

<table>
<thead>
<tr>
<th>Covariance Parameters</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Z-Value</th>
<th>Pr &gt; Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant(Biotype)⁷</td>
<td>248.74</td>
<td>86.4047</td>
<td>2.88</td>
<td>0.0020</td>
</tr>
<tr>
<td>Residual</td>
<td>41.6385</td>
<td>7.6021</td>
<td>5.48</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num df</th>
<th>Den df</th>
<th>F-Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotype (GR vs. GS)</td>
<td>1</td>
<td>18</td>
<td>1.65</td>
<td>0.2157</td>
</tr>
</tbody>
</table>

⁷ Experiment was designed as a completely random design.

Table A2. The mean percentage and standard error of absorbed [¹⁴C]-glyphosate within four different plant tissues of glyphosate susceptible and resistant giant ragweed (*Ambrosia trifida* L.) at various hours after treatment (HAT) when applied at the apical meristem, replicated three times.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Glyphosate Susceptible Biotype</th>
<th>Glyphosate Resistant Biotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 HAT</td>
<td>6 HAT</td>
</tr>
<tr>
<td>Root</td>
<td>2.9 ± 1.95</td>
<td>3.6 ± 1.95</td>
</tr>
<tr>
<td>Hypocotyl</td>
<td>1.6 ± 3.42</td>
<td>3.1 ± 3.42</td>
</tr>
<tr>
<td>Mature Leaf</td>
<td>1.8 ± 4.63</td>
<td>10.1 ± 4.63</td>
</tr>
<tr>
<td>Apical Meristem</td>
<td>93.8 ± 7.06</td>
<td>83.2 ± 7.06</td>
</tr>
</tbody>
</table>
**Table A3.** The mean percentage and standard error of absorbed $[^{14}\text{C}]$-glyphosate within four different plant tissues of glyphosate susceptible and resistant giant ragweed (*Ambrosia trifida* L.) at various hours after treatment (HAT) when applied at the mature leaf, replicated three times.

<table>
<thead>
<tr>
<th>Plant Tissue</th>
<th>Glyphosate Susceptible Biotype</th>
<th>Glyphosate Resistant Biotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 HAT</td>
<td>6 HAT</td>
</tr>
<tr>
<td>Root</td>
<td>6.0 ± 3.81</td>
<td>6.3 ± 3.81</td>
</tr>
<tr>
<td>Hypocotyl</td>
<td>6.0 ± 1.68</td>
<td>7.7 ± 1.68</td>
</tr>
<tr>
<td>Mature Leaf</td>
<td>83.7 ± 13.92</td>
<td>78.4 ± 13.92</td>
</tr>
<tr>
<td>Apical Meristem</td>
<td>4.4 ± 14.44</td>
<td>7.7 ± 14.44</td>
</tr>
</tbody>
</table>

**Table A4.** Analysis of variance for percentage of absorbed $[^{14}\text{C}]$-glyphosate in the root from the apical meristem measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.).

<table>
<thead>
<tr>
<th>Covariance Parameters</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Z Value</th>
<th>Pr Z</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep (Block)</td>
<td>5.3642</td>
<td>6.1192</td>
<td>0.88</td>
<td>0.1903</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>5.9872</td>
<td>2.2629</td>
<td>2.65</td>
<td>0.0041</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotype</td>
<td>1</td>
<td>14</td>
<td>0.05</td>
<td>0.8200</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAT</td>
<td>3</td>
<td>14</td>
<td>1.53</td>
<td>0.2514</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotype*HAT</td>
<td>3</td>
<td>14</td>
<td>0.17</td>
<td>0.9139</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Footnote: Experiment was designed as a completely randomized block design, blocked over time.
Table A5. Analysis of variance for percentage of absorbed $[^{14}\text{C}]$-glyphosate in the hypocotyl from the apical meristem measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.)$^7$.

<table>
<thead>
<tr>
<th>Covariance Parameters</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Z Value</th>
<th>Pr Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep (Block)</td>
<td>9.6228</td>
<td>12.8679</td>
<td>0.75</td>
<td>0.2273</td>
</tr>
<tr>
<td>Residual</td>
<td>25.5083</td>
<td>9.6412</td>
<td>2.65</td>
<td>0.0041</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effects</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotype</td>
<td>1</td>
<td>14</td>
<td>2.68</td>
<td>0.1242</td>
</tr>
<tr>
<td>HAT</td>
<td>3</td>
<td>14</td>
<td>0.38</td>
<td>0.7671</td>
</tr>
<tr>
<td>Biotype*HAT</td>
<td>3</td>
<td>14</td>
<td>0.72</td>
<td>0.5575</td>
</tr>
</tbody>
</table>

$^7$ Experiment was designed as a completely randomized block design, blocked over time.

Table A6. Analysis of variance for percentage of absorbed $[^{14}\text{C}]$-glyphosate in the mature leaf from the apical meristem measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.)$^7$.

<table>
<thead>
<tr>
<th>Covariance Parameters</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Z Value</th>
<th>Pr Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep (Block)</td>
<td>5.2309</td>
<td>12.9124</td>
<td>0.41</td>
<td>0.3427</td>
</tr>
<tr>
<td>Residual</td>
<td>59.0149</td>
<td>22.3055</td>
<td>2.65</td>
<td>0.0041</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effects</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotype</td>
<td>1</td>
<td>14</td>
<td>1.85</td>
<td>0.1955</td>
</tr>
<tr>
<td>HAT</td>
<td>3</td>
<td>14</td>
<td>1.43</td>
<td>0.2753</td>
</tr>
<tr>
<td>Biotype*HAT</td>
<td>3</td>
<td>14</td>
<td>2.58</td>
<td>0.0953</td>
</tr>
</tbody>
</table>

$^7$ Experiment was designed as a completely randomized block design, blocked over time.
Table A7. Analysis of variance for percentage of absorbed [$^{14}$C]-glyphosate in the apical meristem from the apical meristem measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.).

<table>
<thead>
<tr>
<th>Covariance Parameters</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Z Value</th>
<th>Pr Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep (Block)</td>
<td>49.7105</td>
<td>62.3552</td>
<td>0.80</td>
<td>0.2127</td>
</tr>
<tr>
<td>Residual</td>
<td>99.7318</td>
<td>37.6951</td>
<td>2.65</td>
<td>0.0041</td>
</tr>
<tr>
<td>Effects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotype</td>
<td>1</td>
<td>14</td>
<td>3.73</td>
<td>0.0740</td>
</tr>
<tr>
<td>HAT</td>
<td>3</td>
<td>14</td>
<td>1.58</td>
<td>0.2380</td>
</tr>
<tr>
<td>Biotype*HAT</td>
<td>3</td>
<td>14</td>
<td>2.75</td>
<td>0.0821</td>
</tr>
</tbody>
</table>

Experiment was designed as a completely randomized block design, blocked over time.

Table A8. Analysis of variance for percentage of absorbed [$^{14}$C]-glyphosate in the root from the mature leaf measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.).

<table>
<thead>
<tr>
<th>Covariance Parameters</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Z Value</th>
<th>Pr Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep (Block)</td>
<td>10.1299</td>
<td>12.8880</td>
<td>0.79</td>
<td>0.2159</td>
</tr>
<tr>
<td>Residual</td>
<td>21.7371</td>
<td>8.2158</td>
<td>2.65</td>
<td>0.0041</td>
</tr>
<tr>
<td>Effects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotype</td>
<td>1</td>
<td>14</td>
<td>17.67</td>
<td>0.0009</td>
</tr>
<tr>
<td>HAT</td>
<td>3</td>
<td>14</td>
<td>2.27</td>
<td>0.1250</td>
</tr>
<tr>
<td>Biotype*HAT</td>
<td>3</td>
<td>14</td>
<td>2.08</td>
<td>0.1494</td>
</tr>
</tbody>
</table>

Experiment was designed as a completely randomized block design, blocked over time.
Table A9. Analysis of variance for percentage of absorbed $[^{14}\text{C}]$-glyphosate in the hypocotyl from the mature leaf measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (Ambrosia trifida L)$^2$.

<table>
<thead>
<tr>
<th>Covariance Parameters</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Z Value</th>
<th>Pr Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep (Block)</td>
<td>0</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>Residual</td>
<td>8.4471</td>
<td>2.9865</td>
<td>2.83</td>
<td>0.0023</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effects</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotype</td>
<td>1</td>
<td>14</td>
<td>29.38</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HAT</td>
<td>3</td>
<td>14</td>
<td>0.84</td>
<td>0.4962</td>
</tr>
<tr>
<td>Biotype*HAT</td>
<td>3</td>
<td>14</td>
<td>1.03</td>
<td>0.4113</td>
</tr>
</tbody>
</table>

$^2$ Experiment was designed as a completely randomized block design, blocked over time.

Table A10. Analysis of variance for percentage of absorbed $[^{14}\text{C}]$-glyphosate in the mature leaf from the mature leaf measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (Ambrosia trifida L)$^2$.

<table>
<thead>
<tr>
<th>Covariance Parameters</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Z Value</th>
<th>Pr Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep (Block)</td>
<td>191.28</td>
<td>240.7</td>
<td>0.79</td>
<td>0.2134</td>
</tr>
<tr>
<td>Residual</td>
<td>389.71</td>
<td>147.3</td>
<td>2.65</td>
<td>0.0041</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effects</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotype</td>
<td>1</td>
<td>14</td>
<td>0.90</td>
<td>0.3590</td>
</tr>
<tr>
<td>HAT</td>
<td>3</td>
<td>14</td>
<td>1.29</td>
<td>0.3158</td>
</tr>
<tr>
<td>Biotype*HAT</td>
<td>3</td>
<td>14</td>
<td>1.27</td>
<td>0.3213</td>
</tr>
</tbody>
</table>

$^2$ Experiment was designed as a completely randomized block design, blocked over time.
Table A11. Analysis of variance for percentage of absorbed $[^{14}\text{C}]$-glyphosate in the apical meristem from the mature leaf measured at 3, 6, 12 and 24 hours after treatment (HAT) in glyphosate resistant and susceptible biotypes of giant ragweed (*Ambrosia trifida* L.)$^z$.

<table>
<thead>
<tr>
<th>Covariance Parameters</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Z Value</th>
<th>Pr Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep (Block)</td>
<td>318.76</td>
<td>357.41</td>
<td>0.89</td>
<td>0.1862</td>
</tr>
<tr>
<td>Residual</td>
<td>306.88</td>
<td>115.99</td>
<td>2.65</td>
<td>0.0041</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effects</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotype</td>
<td>1</td>
<td>14</td>
<td>0.90</td>
<td>0.3585</td>
</tr>
<tr>
<td>HAT</td>
<td>3</td>
<td>14</td>
<td>0.81</td>
<td>0.5078</td>
</tr>
<tr>
<td>Biotype*HAT</td>
<td>3</td>
<td>14</td>
<td>0.70</td>
<td>0.5660</td>
</tr>
</tbody>
</table>

$^z$ Experiment was designed as a completely randomized block design, blocked over time.

SAS Code

Stomatal Density

data density;
input bio plant subsam dens;
cards;
7 1 1 4.87
7 1 2 4.70
7 1 3 4.68
7 1 4 4.51
7 2 1 5.16
7 2 2 6.11
7 2 3 5.14
7 2 4 4.76
7 3 1 2.46
7 3 2 2.97
7 3 3 2.70
7 3 4 3.03
7 4 1 3.22
7 4 2 3.11
7 4 3 2.95
7 4 4 2.68
7 5 1 3.27
7 5 2 3.38
7 5 3 2.84
7 5 4 3.49
7 6 1 3.76
7 6 2 3.68
7 6 3 3.60
7 6 4 3.70
7 7 1 3.43
7 7 2 3.08
7 7 3 3.43
<table>
<thead>
<tr>
<th>bio</th>
<th>plant</th>
<th>subsam</th>
<th>dens</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>7</td>
<td>4</td>
<td>3.35</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>1</td>
<td>4.46</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>2</td>
<td>4.30</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>3</td>
<td>4.22</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>4</td>
<td>3.95</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>1</td>
<td>3.41</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>2</td>
<td>2.95</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>3</td>
<td>3.19</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>4</td>
<td>3.35</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>1</td>
<td>3.89</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>2</td>
<td>4.08</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>3</td>
<td>3.41</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>4</td>
<td>4.35</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
<td>4.22</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>2</td>
<td>3.84</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>3</td>
<td>4.51</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>4</td>
<td>4.38</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>1</td>
<td>4.89</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>2</td>
<td>4.03</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>3</td>
<td>4.81</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>4</td>
<td>4.51</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>1</td>
<td>6.49</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>2</td>
<td>6.03</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>3</td>
<td>5.87</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>4</td>
<td>5.35</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>1</td>
<td>3.46</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>2</td>
<td>3.35</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>3</td>
<td>3.35</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>4</td>
<td>3.97</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>1</td>
<td>4.70</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>2</td>
<td>5.30</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>3</td>
<td>4.81</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>4</td>
<td>3.87</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>1</td>
<td>2.54</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>2</td>
<td>3.14</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>3</td>
<td>2.89</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>4</td>
<td>2.65</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>1</td>
<td>3.95</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>2</td>
<td>3.73</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>3</td>
<td>3.00</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>4</td>
<td>3.60</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>1</td>
<td>4.87</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>2</td>
<td>6.03</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>3</td>
<td>4.70</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>4</td>
<td>4.97</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>1</td>
<td>4.60</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>2</td>
<td>4.54</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>3</td>
<td>4.95</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>4</td>
<td>4.27</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>1</td>
<td>3.14</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>2</td>
<td>3.49</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>3</td>
<td>3.35</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>4</td>
<td>3.43</td>
</tr>
</tbody>
</table>

;  

proc mixed covtest data=density;  
class bio plant subsam;  
model dens=bio /outp=second residual;
random plant(bio);
lsmeans bio; run;
proc sgscatter; plot studentresid*(bio pred);
proc univariate normal;
histogram /normal kernel;
var resid;
run;

Translocation

Title "Translocation Experiment" ;
Data First;
Input ID Biotype$ Rep HAT Root Meri Mat Hypo;
Cards;
1  R 1 3 5.44 86.04 3.35 5.17
2  R 2 3 2.50 92.37 1.29 3.85
3  R 3 3 0.30 99.31 0.16 0.23
4  R 1 6 1.66 94.59 1.70 2.04
5  R 2 6 6.68 74.38 13.32 5.62
6  R 3 6 0.38 99.21 0.20 0.21
7  R 1 12 1.65 78.32 18.40 1.62
8  R 2 12 1.35 65.29 9.80 23.56
9  R 3 12 2.04 96.37 0.75 0.85
10  R 1 24 1.15 63.94 33.36 1.55
11  R 2 24 10.97 45.67 25.13 18.22
12  R 3 24 3.04 94.90 0.91 1.15
13  S 1 3 1.50 95.67 1.23 1.60
14  S 2 3 6.26 89.47 2.30 1.97
15  S 3 3 0.96 96.11 1.73 1.20
16  S 1 6 1.80 81.42 11.79 4.99
17  S 2 6 7.94 88.55 0.80 2.71
18  S 3 6 0.95 79.73 17.64 1.69
19  S 1 12 1.83 85.18 11.19 1.80
20  S 2 12 1.30 93.94 2.22 2.54
21  S 3 12 0.44 95.23 3.98 0.36
22  S 1 24 1.65 95.30 1.35 1.71
23  S 2 24 9.21 85.40 2.65 2.74
24  S 3 24 0.54 98.85 0.33 0.29
;
Title "Apical Meristem Application";
Proc Mixed Covtest Maxiter=2000;
class ID Rep Biotype HAT;
model Root= Biotype HAT Biotype*HAT / residual outp=second;
Random Rep;
lsmeans Biotype*HAT/ pdiff tdiff Slice=HAT;
ods output diffs=ppp lsmeans=mmm;
Run;
%include "C:\Users\mlespera\Downloads\pdmix800.sas";
%pdmix800 (ppp,mmm,alpha=0.05,sort=no,slice=HAT);
Run;
proc sgscatter; plot studentresid*(Rep Biotype HAT pred);
proc univariate normal;
histogram /normal kernel;
var studentresid;
run;