

**Confirmation and Mechanism of Resistance to Glyphosate in Giant  
Ragweed (*Ambrosia trifida*) in Ontario**

by

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## **ABSTRACT**

### **Preliminary Investigation of the Mechanism of Resistance to Glyphosate in Giant Ragweed (*Ambrosia trifida*) in Ontario**

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The basis of resistance to glyphosate in two giant ragweed populations exhibiting different phenotypic responses to glyphosate is unknown. Population R1 exhibits a rapid necrosis of mature leaves followed by eventual regrowth of young tissue while population R2 shows general chlorosis and cessation of growth followed by growth in 21 to 28 days. The objectives were to determine the resistance index of the two populations and investigate mechanisms of resistance. The resistance index, based on LD<sub>50</sub> values, was 16 and 19 for R1 and R2, respectively. Accumulation of shikimate in leaf discs (an indicator of glyphosate target site sensitivity) occurred at lower glyphosate doses for S populations than in R1 and R2 suggesting differential target site inhibition. [<sup>14</sup>C]-glyphosate was used to measure absorption and translocation. Absorption levels of R1 and susceptible populations were similar. Less [<sup>14</sup>C]-glyphosate was translocated to the roots and above the treated leaf in the R1 population.

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## LITERATURE REVIEW

### Giant Ragweed

#### *Description*

Giant ragweed (*Ambrosia trifida* L), a member of the Asteraceae family, has very distinct vegetative features and is characterized by its great height. It is an erect summer annual herb that grows up to 6.0 m in height (Alex 1992; Alex et al. 1980; Bassett and Crompton 1982; Harrison et al. 2001). Its stems are unbranched to frequently branched (Bassett and Crompton 1982). The leaves are opposite, although they may be alternate at the top (Alex 1992). The leaves have long petioles and are palmately shaped with three to five lobes although they may be entire (Bassett and Crompton 1982). The first pair of leaves at the first node and those at the top of the plant are generally entire (Alex 1992). These vegetative characteristics distinguish giant ragweed from other species.

Giant ragweed is monoecious and cross pollinated, which results in high variation within the species (Johnson et al. 2007). Flower heads are green and are either male or female which are usually both present on individual plants (Alex 1992). The male flowers are grouped in raceme-like elongated clusters that are located at the end of the branches (Alex 1992). Male flowers have three strong black ribs on one side seen from above (Bassett and Crompton 1982). The female flower heads are located in groups of 1 to 4 in the axils of short narrow green bracts at the base of the cluster of the male flowers or in the spikes terminating the stems and branches (Bassett and Crompton 1982). In Canada, the plants typically flower from mid-July to October (Bassett and Crompton 1982). The fruit are 5 to 10 mm long with several ridges that end in short blunt spikes around the upper shoulder of the fruit that encircle a central beak (Alex 1992; Bassett and Crompton

1982). The seed itself is enclosed in a smooth black testa (Bassett and Crompton 1982).

The particular floral biology of *Ambrosia* species, including giant ragweed, may be the results of adaptations that force pollination among separate individuals. Earlier reports on giant ragweed suggest it is mostly cross-pollinated although, morphologically, it could self-pollinate (Bassett and Crompton 1982). More recent results with the related species common ragweed (*Ambrosia artemisiifolia* L.) show it has strong self-incompatibility mechanisms, thereby forcing cross-pollination (Friedman and Barrett 2008). It would be reasonable to assume that giant ragweed, like common ragweed, has strong self-incompatibility mechanisms. Overall, giant ragweed's reproductive characteristics contribute to its great variation.

### ***Origin and Distribution.***

Giant ragweed is native to North America and has been introduced to other regions of the world (Bassett and Crompton 1982; Hansen 1976; Washitani and Nishiyama 1992). It dispersed into Canada from the south following the retreat of the last glacier (Bassett and Crompton 1982). It is found in southern portions of every province in Canada, excluding Newfoundland (Bassett and Crompton 1982). It is most common in southern parts of Manitoba, Ontario and Quebec and in some areas of New Brunswick, Prince Edward Island and Nova Scotia (Bassett and Crompton 1982). It is found in the eastern two thirds of North America and most commonly found along the Mississippi River on alluvial terraces (Harrison et al. 2001; Miller and Miller 1999). It has been introduced into Europe and South America (Bassett and Crompton 1982; Hansen 1976). It has also been reported by Washitani and Nishiyama (1992) that it was introduced into Japan in the 1970s or 1980s. Giant ragweed is a prevalent weed in North America and is now becoming a

global invasive species.

Giant ragweed is most abundant on disturbed moist soils because it is essentially a flood plain species (Bassett and Crompton 1982). It has only become prevalent within the last 200 years, which coincides with the settlement of land by Europeans and the ensuing disturbance of land due to clearing and cultivating (Bassett and Terasmae 1962). Giant ragweed's habitat includes drainage ditches, open stream banks and low silty cultivated fields because of its preference for disturbed moist soils (Bassett and Crompton 1982). More recently, giant ragweed has become a common weed in no-till production systems in the US corn belt and southern Ontario (Johnson et al. 2009; Owen 2008).

### ***Competitiveness***

The growth and development characteristics of giant ragweed make it a very competitive weed. Because of its early germination and rapid growth, giant ragweed causes high yield loss in soybean and maize. Yield losses in soybean range between 45 to 77% at densities as low as 1 plant m<sup>-2</sup> (Webster et al. 1994). In comparison, giant ragweed is more competitive than another species of the same genus, common ragweed, which causes yield losses of only 15% at a similar density (Coble et al. 1981). In maize, yield losses ranged between 11 to 54% depending on giant ragweed density and relative time of emergence in relation to maize emergence (Harrison et al. 2001). Because of these high yield losses in maize and soybean, giant ragweed is a critical weed to control.

The high competitiveness of giant ragweed is due in part to its ability to establish early in the season and its ability to develop a dense canopy. It has an initial advantage over all other summer annuals because it is the first to emerge in the spring (Abul-Fatih and Bazzaz 1979; Bassett and Crompton 1982). In addition it has the capacity to develop

a high leaf area index (LAI, the sum of areas of all leaves per unit area of ground) (Connor et al. 2011). While LAI can reach values of 4.3 for maize and 2.4 for soybeans, giant ragweed can attain an LAI of 5 (Abul-Fatih and Bazzaz 1979; Sattin et al. 1994). As a consequence, giant ragweed reduces light intensity underneath its leaf canopy by 95% and it will exclude or suppress other annual weeds (Abul-Fatih and Bazzaz 1979). Giant ragweed's early germination and high LAI contribute to its competitive and aggressive nature. In order to minimize yield losses from this competitive weed it is imperative for farmers to manage giant ragweed in their fields.

### ***Management of Giant Ragweed in Field Crops***

While many methods can be used to manage giant ragweed, herbicides have been the tool of choice since the 1980s. In soybean, cloransulam provided at least 82% control when applied post emergence (POST) before giant ragweed reached a height of 25 cm while it provided 95% control when applied PRE (Franey and Hart 1999). Chlorimuron plus metribuzin applied PRE followed by (fb) chlorimuron applied early POST or imazaquin applied twice PRE and early POST provided 65 to 95% control (Baysinger and Sims 1992). Acifluorfen applied early POST followed by naptalam and 2,4-DB applied at late POST gave 84 to 96% control (Baysinger and Sims 1992). There are few herbicide combinations that provide consistent control of giant ragweed in soybean.

In maize, giant ragweed can be controlled by PRE and POST herbicides. Herbicide treatments that gave greater than 93% control included atrazine + acetochlor PRE; atrazine + metolachlor PRE; metolachlor + atrazine fb primisulfuron + prosulfuron POST; atrazine + simazine PRE fb primisulfuron + prosulfuron POST; dimethenamid + atrazine PRE fb dicamba POST (Ferrell and Witt 2002). Atrazine and

alachlor PRE can provide 85% control of giant ragweed (Webster et al. 1998). In the most recent study of control of giant ragweed in maize in Ontario, atrazine and dicamba provided the best and most consistent control when applied POST (82 to 94% control) (Soltani et al. 2011). There are more herbicide options available in maize than in soybean that provide acceptable control of giant ragweed.

The commercialization of glyphosate resistant (GR) soybean and canola (*Brassica napus* L.) in 1996, cotton (*Gossypium hirsutum* L.) in 1997, maize in 1998, sugar beets (*Beta vulgaris* L.) in 2008 and alfalfa (*Medicago sativa* L.) 2011 allowed for the POST in-crop application of glyphosate for broad spectrum weed control including giant ragweed (Armstrong and Sprague 2010; James 2011; Sidhu et al. 2000). Glyphosate consistently provided greater than 92% control of giant ragweed (Wiesbrook et al. 2001; Ferrell and Witt 2002). The commercialization of GR cropping systems gave soybean and maize producers an additional option for control of giant ragweed.

GR crops are resistant to glyphosate due to an altered 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme (*cp4 epsps*) isolated from *Agrobacterium tumefaciens* (Green 2009). GR soybean and sugar beet are resistant to glyphosate solely from the *cp4 epsps* (Green 2009). Canola with the GT73 (Roundup Ready, Monsanto) trait contains a *gox* gene (glyphosate oxidoreductase), *goxv247*, along with the *cp4 epsps* gene (Green 2009). This *gox* gene produces a modified-GOX enzyme that cleaves the C-N bond of glyphosate to produce  $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and glyoxylate (Barry et al. 1992; Green 2009). The combination of these genes gives the GR canola a higher level of resistance to glyphosate (Green 2009). GR maize possesses three complete and incomplete copies of a modified maize EPSPS gene known

as *zm-epsps* in the GA21 (Roundup Ready, Monsanto) and two copies of the *cp4 epsps* in the NK603 trait (Roundup Ready 2, Monsanto) (Green 2009). GR maize possessing the *zm-epsps* gene and a *gox* gene was developed but never commercialized (Nap et al. 2003; Pline-Srnic 2005). The *cp4 gene* has given farmers a significant tool for the use of an effective broad spectrum herbicide in GR cropping systems.

## **Glyphosate**

### ***History***

Glyphosate was not originally invented for herbicidal use; it was invented at the Swiss pharmaceutical company, Cilag, by Dr. Henri Martin however, glyphosate had no pharmaceutical application (Dill et al. 2010; Franz et al. 1997). It was later sold to Aldrich Chemical along with other research samples by Johnson and Johnson who acquired Cilag in 1959 (Dill et al. 2010). Aldrich sold glyphosate to many companies, but no biological activity was reported (Dill et al. 2010). Monsanto's Inorganic Division was synthesizing 100 related aminomethylphosphonic acid (AMPA) compounds as potential water softening agents (Dill et al. 2010). Two of these compounds showed herbicidal activity on perennial weeds in studies conducted by Dr. Phil Hamm, but activity was too weak to be used as a commercial herbicide (Dill et al. 2010). Dr. Hamm asked Dr. John Franz, a Monsanto chemist, to develop something that was more efficacious (Dill et al. 2010). Through his research Dr. Franz had reinvented glyphosate in May 1970 (Dill et al. 2010). It was commercialized as a herbicide in 1974 (Powles and Preston 2006). Now glyphosate is the most widely used herbicide in the world (Powles and Yu 2010).

### ***Chemistry***

Glyphosate ([N-phosphonomethyl] glycine) is a phosphonomethyl derivative of the amino acid glycine (Dill et al. 2010). It is a white and odourless crystalline solid (Dill et al. 2010). It is comprised of one basic amino function and three ionisable acidic sites (Bromilow and Chamberlain 2000; Dill et al. 2010). Because it can react as a base or as an acid, it can be dissolved in dilute aqueous bases and strong aqueous acids to produce anionic and cationic salts, respectively (Dill et al. 2010). Glyphosate has strong intermolecular hydrogen bonding which makes it not very soluble in nonaqueous solutions (Dill et al. 2010; Knuuttila and Knuuttila 1979). Glyphosate converted to monobasic salts is more soluble in water compared to the free acid of glyphosate (Dill et al. 2010). Glyphosate is generally formulated in the form of a soluble monobasic salt (isopropylamine, sodium, potassium, trimethyl-sulfonium or ammonium) in concentrated water solutions (Dill et al. 2010). The chemical properties of glyphosate allow it to be dissolvable in aqueous solutions of varying pH.

### ***Mode of Action***

Glyphosate inhibits the enzyme EPSPS, which catalyzes a key step in the shikimic acid pathway (Amrhein et al. 1980; Haslam 1974; Steinrücken and Amrhein 1980). EPSPS catalyzes the condensation of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to produce 5-enolpyruvylshikimate 3-phosphate and inorganic phosphate (Herrmann and Weaver 1999). Glyphosate forms a ternary complex with EPSPS and the substrate S3P to become a competitive inhibitor to PEP (Boocock and Coggins 1983; Franz et al. 1997). Glyphosate does not bind significantly to the free EPSPS enzyme, but needs the S3P to be bound to the EPSPS first (Anderson et al. 1988). EPSPS plays a key role in the physiology of a plant and as a result makes it an ideal herbicide target site.

EPSPS is only found in plants and microorganisms (Haslam 1974; Steinrücken and Amrhein 1980). EPSPS is nuclear encoded and translocated into the plastid where the shikimate pathway is located (Della-Cioppa et al. 1986; Weaver and Herrmann 1997). Glyphosate also inhibits the importation of the precursor of the EPSPS enzyme into the chloroplasts when the EPSPS is present as the precursor, EPSPS-S3P-glyphosate ternary complex form (Della-Cioppa and Kishore 1988). Glyphosate inhibits both EPSPS in the shikimate pathway and the importation of the EPSPS into the plastid.

The shikimate pathway involves seven enzymatic reactions that primarily produce chorismate which is the precursor for the aromatic amino acids, tryptophan, phenylalanine and tyrosine, that are required for protein biosynthesis (Weaver and Herrmann 1997). Chorismate is also converted into quinones and indoles which along with aromatic amino acids are precursors of a host of secondary metabolites (Weaver and Herrmann 1997). One of the post-chorismate intermediates for phenylalanine and tyrosine is L-arogenate which gives negative feedback to the 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase-Mn (Gaines et al. 1982; Jensen 1985). DAHP synthase-Mn is used for the production of S3P and regulates the carbon flow in the shikimate pathway (Jensen 1985). Therefore when EPSPS is inhibited by glyphosate, the formation of chorismate is inhibited, causing an absence of L-arogenate (Amrhein et al. 1980; Hollander and Amrhein 1980; Jensen 1985). This results in no negative feedback on the DAHP synthase, which causes an accumulation of shikimate in the tissues and a carbon shortage for other pathways (Jensen 1985; Siehl et al 1997).

There has been some debate as to how the inhibition of EPSPS can lead to plant death. Some assume death occurs through insufficient aromatic amino acid production



from an inhibited shikimate pathway (Duke and Powles 2008). Another theory, supported by evidence, is that there is an increased carbon flow to the shikimate pathway when it is deregulated by the inhibition of EPSP causing a drain on the rest of the plant (Duke and Powles 2008; Siehl 1997). The primary mode of action of glyphosate is the inhibition of EPSPS, regardless of these two individual theories of how plants actually die once EPSPS is inhibited.

### ***Cell Uptake and Translocation***

Cell uptake of glyphosate occurs in two different ways. At low concentrations, glyphosate is actively taken up by plant cells while at higher concentrations it passes through membranes via passive flow (Denis and Delrot 1993; Shaner 2009). The active transport is suggested to be via a phosphate transporter of the plasma membrane (Denis and Delrot 1993; Hetherington et al. 1998; Morin et al. 1997). This means that at low concentration, cell uptake is an energy dependent process while it is not at high glyphosate concentration. Alteration of any of these two mechanisms may have implication on whole plant response to glyphosate.

Glyphosate is an ambimobile herbicide due to both symplastic and apoplastic movement (Dewey and Appleby 1983; Gottrup et al. 1976; Gougler and Geiger 1981; Harvey et al. 1985; Jachetta et al. 1986). After initial absorption, glyphosate is transported in the apoplast until it enters into the symplast (Franz et al. 1997).

Glyphosate is translocated through the phloem to areas where the EPSPS synthase is most highly expressed which is in the sink tissues: the roots, meristems and flowers (Gougler and Geiger 1981; McAllister and Haderlie 1985; Weaver and Herrmann 1997).

Glyphosate is able to be phloem mobile due to its ionisable functionality of three

acid groups and one amine base (Bromilow and Chamberlain 2000). It was determined on an analogue of glyphosate that removal of one or more of these groups reduced phloem transport, thus it is the combination of all the ionisable functionality that confer this herbicide with symplastic mobility (Bromilow and Chamberlain 2000). The polarity of glyphosate allows it to be phloem mobile (Bromilow and Chamberlain 2000).

It is not known how glyphosate enters into the phloem, however, there are two possible scenarios (Shaner et al. 2012). It may enter into the mesophyll cells through a passive flow system and enter into the phloem companion cell through plasmodesmata and diffuse into the phloem (Shaner 2009). Alternatively, it may enter the mesophyll and/or companion cells through active transport and then diffuse into the phloem (Shaner 2009; Shaner et al. 2012). Therefore, glyphosate can be taken up actively and passively into cells and it is transported through the apoplast and then the phloem.

### ***Environmental and Toxicological Properties***

Glyphosate is considered to have a favorable environmental profile mostly due to its low toxicity. Glyphosate has low toxicity to the animal kingdom because the target site of glyphosate, EPSPS enzyme, is exclusive to plants and bacteria (Williams et al. 2000). Through many toxicological studies on laboratory animals, it has been determined that glyphosate has low oral, dermal and inhalation toxicity (Dill et al. 2010). There is no evidence of carcinogenicity, mutagenicity, neurotoxicity, reproductive toxicity or teratogenicity (Dill et al. 2010).

Other properties contribute to the favorable environmental profile of glyphosate. It has low volatility due to its strong molecular bonding and as it is a dense molecule ( $1.75\text{g cm}^{-3}$ ) it is not prone to evaporating from surfaces (Dill et al. 2010). While it is

stable in sterile soil and water, it is subjected to microbial degradation. Glyphosate is primarily degraded by microorganisms under both aerobic and anaerobic conditions (Dill et al. 2010). There are two degradation pathways present in the soil; the GOX pathway which degrades glyphosate into AMPA and glyoxylate; and a C-P lyase enzyme that degrades glyphosate to sarcosine and inorganic phosphate (Duke 2011). In addition, photodegradation of glyphosate can occur in solutions containing calcium ions under artificial light but this is not a major pathway for glyphosate degradation in the environment (Dill et al. 2010; Franz et al. 1997). As a result of its low mammalian toxicity, low volatility and microbial degradation, glyphosate is a relatively environmentally safe product.

### ***Usage***

Glyphosate is a nonselective and nonresidual herbicide (Dyer 1994). Prior to the commercialization of GR crops, glyphosate was used in agriculture for weed control in field crops, intercrop rows and around perennial trees and vines (Powles and Preston 2006). Tree, vine, banana (*Musa* spp.) and coffee (*Coffea arabica* L.) crops, railroads and roadsides would get two to six applications annually (Bradshaw et al. 1997). Because it is a nonselective herbicide, use of glyphosate was limited since it could not be applied in-crop.

The use of glyphosate has increased dramatically with the introduction of GR soybean and canola in 1996, followed by cotton in 1997 and maize in 1999 (Owen and Zelaya 2005). Later, GR sugar beets were commercialized in 2008 and alfalfa was commercialized in 2005 which was then reregulated due to legal clearance and resumed commercialization in 2011 (Armstrong and Sprague 2010; James 2011). These GR crops

allowed for the POST in-crop application of glyphosate at high rates and at multiple times during the growing season without injuring the crop (Owen and Zelaya 2005).

### **Herbicide Resistance**

Herbicide resistance is the inherited ability of weed biotypes to survive application of herbicides that would normally be lethal as a result of selection (WSSA 1998). In the early- to mid-1950s, there was much debate as to whether herbicide resistance could develop in weeds. While some stated that herbicides could be effective selective agents and that weed populations had enough genetic diversity for HR weed populations to evolve, others pointed out that herbicides were different and that resistance would be unlikely to occur (Abel 1954; Blackman 1950; Harper 1956). However, herbicide resistance was first documented in the latter part of that decade in populations of wild carrot (*Daucus carota* L.) from Ontario, Canada, that were able to survive 2,4-D following selection with this herbicide (Switzer, 1957). This early case provided proof that herbicides could select for resistance.

The first case of high level herbicide resistance was in *Senecio vulgaris* L. in the 1960's. This weed, which normally was very susceptible to the triazine herbicides, developed high level resistance to the herbicide simazine (Ryan 1970; Scott and Putwain 1981; Souza-Machado 1982). This biotype has a target-site based resistance, in that the photochemical activity was not inhibited in the chloroplasts of the resistant plants (Radosevich and Devilliers 1976). This was a significant discovery because prior to this finding it was assumed that all living plants would die if herbicides could reach their target site intact (LeBaron and McFarland 1990).

### ***Herbicide Resistance Evolution***

Many factors affect the speed of herbicide resistance evolution. Firstly, herbicide resistance occurs from the repeated application of a herbicide or herbicides with the same mode of action, that will select for plants that possess alleles that will enable survival. The probability and rate of herbicide resistance depends on biological, herbicidal and operational factors (Powles and Yu 2010). Biological factors are species specific and include the genetics of resistance alleles. These genetic factors include the frequency, the number, the dominance, the mode of inheritance and the fitness cost of resistance alleles (Diggle and Neve 2001; Powles and Yu 2010). Other species-specific biological factors include cross pollination versus self-pollination, overall fecundity, persistence of a seed bank, and the capacity of seed and pollen to travel (Diggle and Neve 2001; Powles and Yu 2010). The herbicidal factors include the chemical structure, site of action, and the residual activity of the herbicide (Powles and Yu 2010). The operational factors include the herbicidal dose, the amount and frequency of application, the skills of the operator (efficacy of equipment, timing and environmental conditions) and agronomics (non chemical weed control, crop rotation) (Powles and Yu 2010). These numerous biological and operational factors contribute to the rate of herbicide resistance evolution in a particular weed.

The influence of herbicide dose on the rate of evolution and level of resistance has been the subject of controversy. Some producers will try to reduce herbicide cost by applying less than the registered label rate. This reduction in herbicide rate will have no effect on selection pressure as long as there is no reduction in efficacy of the herbicide (Beckie 2006). However when the dose is reduced to the level that there is a reduction in

efficacy, this dose will impose a low selection pressure. Jasieniuk *et al.* (1996) suggested that the initial frequency of resistance alleles has a greater influence on the evolutionary process when herbicides impose a weak selection pressure versus a strong selection pressure. It has been argued by Gardner *et al.* (1998) that low herbicide doses favour the evolution of quantitative resistance traits and that major gene resistance arises more slowly than with high dose treatments. According to Neve and Powles (2005a) high herbicide doses will result in survival of individuals that possess resistant alleles that confer high levels of resistance and the frequency of these alleles will likely be low. As the dose is reduced, weaker resistance mechanisms will enable the plant to survive and the frequency of the resistance will be higher (Neve and Powles 2005a). In rigid ryegrass (*Lolium rigidum* Gaudin), application of diclofop-methyl at doses lower than the recommended field rate selected for plants accumulating weaker resistance genes (Neve and Powles 2005b). This however conferred the ability to rapidly evolve high levels of resistance under recurrent selection where multiple weaker mechanisms are selected for and enriched (Neve and Powles 2005a, 2005b). Herbicide dose has an influence on the level and the frequency of herbicide resistance.

It has also been debated whether adaptation occurs through genetic variability that is maintained in the population through a polygenic response or from the selection and fixation of novel mutations in a monogenic response (Hermisson and Pennings 2005; Neve 2007). It has been argued by McKenzie (2000) that if selection acts within the phenotypic distribution of susceptible individuals, a polygenic response is favoured. This goes along with Hermisson and Pennings (2005) who found that if the selection pressure is weak then existing variation is favoured. If selection acts outside of the phenotypic

distribution, a monogenic response with a rare mutation with a phenotype outside of the distribution is favoured (McKenzie 2000). Resistance through a polygenic or monogenic response is another factor that can be influenced by the herbicide dose exerting a strong or weak selection pressure. Whether this happened in any case of glyphosate resistance has yet to be demonstrated.

### **Glyphosate Resistance**

Prior to the introduction of GR crops, there were concerns that weeds may develop glyphosate resistance. Opinions varied with many stressing that glyphosate resistance would be very unlikely, if not impossible (Bradshaw et al. 1997). It had been used for over 20 years without weeds becoming resistant under field situations (Dyer 1994; Holt et al. 1993). It is the only herbicide that inhibits EPSPS with unique biochemical, chemical and biological characteristics (Amrhein et al. 1980; Anderson and Johnson 1990; Bentley 1990; Boocock and Coggins 1983; Bradshaw et al. 1997). Because glyphosate resistance development through molecular manipulation was so complex, it was thought that it would be very unlikely for weeds to develop resistance based on the same mechanisms (Bradshaw et al. 1997; Padgett et al. 1991). There had also been no documentation of plants being able to metabolize glyphosate as a mechanism of resistance (Bradshaw et al. 1997; Kaundun et al. 2011). However these seemingly reassuring predictions were soon proven wrong.

Glyphosate resistance was first documented in Australia in 1996 in rigid ryegrass (Powles et al. 1998). Resistant rigid ryegrass was found in an orchard in New South Wales where glyphosate had been applied for 15 years at rates between 720 to 1440 g a.e. ha<sup>-1</sup> two to three times a year (Powles et al. 1998). Plants had seven- to eleven-fold

resistance to glyphosate and also had cross resistance to diclofop (Powles et al. 1998). In 1997, GR goosegrass [*Eleusine indica* (L.) Gaertn.] was confirmed in Malaysia (Lee and Ngim 2000). The first dicot weed to become resistant was Canada fleabane [*Conyza canadensis* (L.) Cronq.] in Delaware in 2000 (VanGessel 2001). This was also the first occurrence in North America (VanGessel 2001). There are 27 species resistant to glyphosate belonging to the Amaranthaceae, Asteraceae, Brassicaceae, Chenopodiaceae, Plantaginaceae, Poaceae and Rubiaceae families in 24 countries (Heap 2014). Glyphosate resistance is widespread throughout the world and across many plant families.

### **Mechanisms of Glyphosate Resistance**

Multiple mechanisms confer resistance to glyphosate in weeds. These mechanisms include target site mutation, increased EPSPS production, reduced translocation, decreased spray retention and foliar uptake, rapid vacuolar sequestration and increased metabolism (Baerson et al. 2002; de Carvalho et al. 2012; Dinelli et al. 2006; Ge et al. 2010; Lorraine-Colwill et al. 2003; Michitte et al. 2007; Ng et al. 2003; Yu et al. 2007). There have been multiple mechanisms of glyphosate resistance that have evolved in weeds.

#### ***Target Site***

Target site resistance is defined as “resistance that is provided by gene mutation conferring a change to a target site enzyme such that the herbicide no longer effectively inhibits the normal enzyme function” (Powles and Preston 2006). This gene mutation is normally a specific nucleotide substitution within a specific coding region that will encode a different amino acid that will result in a structural, charge, or hydrophobicity changes in the target site enzyme (Powles and Preston 2006). These changes will make



the herbicide unable to inhibit the target site enzyme which will confer resistance (Powles and Preston 2006).

Glyphosate target site resistance was first documented in goosegrass from Malaysia (Lee and Ngim 2000). This resistant goosegrass had eight- to twelve-fold resistance when comparing the LD<sub>50</sub> (dose that is lethal to 50% of the population) of resistant to susceptible goosegrass (Lee and Ngim 2000). The half of the maximum inhibitory concentration of glyphosate on the EPSPS enzyme was five-fold higher in the resistant than in the susceptible goosegrass (Baerson et al. 2002). There were four single nucleotide polymorphisms identified when sequencing the cDNA, two of which resulted in amino acid substitutions in the EPSPS enzyme (Baerson et al. 2002). One substitution did not contribute significantly to the resistance (Baerson et al. 2002). However the Pro<sub>106</sub>Ser substitution did contribute to resistance (Baerson et al. 2002). This substitution corresponds with the same substitution found in the GR EPSPS enzyme from *Salmonella* Typhimurium and was additionally found later in rigid ryegrass (Baerson et al. 2002; Bostamam et al. 2012; Comai et al. 1983; Stalker et al. 1985). The Pro<sub>106</sub>Ser substitution in goosegrass was the first case of target site resistance which conferred a resistance index of eight- to twelve-fold.

Later, three other point mutations were found that conferred resistance at position 106. One coded for a Pro<sub>106</sub>Thr substitution in goosegrass and rigid ryegrass, while the other two were a Pro<sub>106</sub>Ala and a Pro<sub>106</sub>Leu substitution in rigid ryegrass ((Bostamam et al. 2012; Jasieniuk et al. 2008; Kaundun et al. 2011; Ng et al. 2003, 2004; Yu et al. 2007; Wakelin & Preston 2006). Interestingly, the Pro<sub>106</sub>Leu substitution was previously identified in a GR mutant of rice (*Oryza sativa* L.) EPSP synthase that had been selected

through a directed evolution strategy (Zhou et al. 2006). In total there are four Pro<sub>106</sub> substitutions that confer glyphosate target site resistance.

Why is it that four different substitutions with proline can have such an effect on glyphosate bonding with the EPSP synthase? The four amino acids taking the place of proline are quite different from each other. Serine and threonine are polar amino acids with hydroxylic side chains of different sizes (Ng et al. 2003). Alanine is a simple amino acid that only has a methyl group as its side chain and is much less hydrophilic and reactive than serine and threonine (Berg et al. 2002). Leucine has a large hydrocarbon side chain that is hydrophobic (Berg et al. 2002). The main difference between the four substituted amino acids is that proline is cyclic and is the only cyclic amino acid (Yu et al. 2007). The cyclic nature is caused by its pyrrolidine side group and it gives it conformational constraints (Berg et al. 2002; Ng et al. 2003). When proline is in a peptide bond in an  $\alpha$ -helix or a  $\beta$ -sheet, it does not have the hydrogen bond to stabilize it (Yu et al. 2007). Because of this lack of a hydrogen bond, proline will give an  $\alpha$ -helix a slight bend in the structure (Yu et al. 2007; Zhou et al. 2006). Proline at site 106 is in an  $\alpha$ -helix in the EPSP synthase so any amino acid substitution at this site will change the conformation of the  $\alpha$ -helix (Yu et al. 2007; Zhou et al. 2006). This will change the structure and function of the EPSP synthase and would therefore affect the binding of glyphosate (Ng et al. 2003; Yu et al. 2007; Zhou et al. 2006).

Because glyphosate is a competitive inhibitor of PEP, it was hypothesized that plants would not develop target site resistance because it may have a potentially deleterious effect on PEP binding resulting in a fitness cost (Bradshaw et al. 1997). The same changes to the target site that make it resistant to glyphosate binding may affect

PEP binding also because these two sites either overlap or are extremely close together (Bradshaw et al. 1997). An elevated  $K_{m(app)}(PEP)$  (apparent affinity of PEP) does not necessarily mean that it will be detrimental or lethal to the plant, however, it will have more of an impact under low PEP concentration (Bradshaw et al. 1997). This will then negatively affect the flux through the shikimate pathway (Bradshaw et al. 1997). It was shown through a cloned petunia (*Petunia × atkinsiana* D. Don ex Loudon [*axillaris × integrifolia*]) enzyme that conveyed resistance that a substitution of Gly-101 with Ala and also a Pro-106 with Ser resulted in a reduced affinity for PEP (Padgett et al. 1991). However, results from Baerson et al. (2002) with goosegrass showed that the  $K_{m(app)}(PEP)$  was 7.0  $\mu$ M for the resistant EPSPS for Pro-106 with Ser which was similar (less than two-fold difference) to the sensitive EPSPS which was 3.8  $\mu$ M. In comparison this same substitution in the petunia had a  $K_{m(app)}(PEP)$  of 44  $\mu$ M (Padgett et al. 1991). Therefore this Pro-106 with Ser substitution in goosegrass did not result in a fitness penalty in PEP binding which was predicted previously to happen with a resistant EPSPS (Bradshaw et al. 1997).

### ***Increased EPSP synthase production***

Another mechanism that can confer resistance is an increase in EPSP synthase production. This can occur through gene amplification or EPSPS overexpression through an increased rate of transcription (Pline-Srnic 2006). Both avenues of increased EPSP synthase production have been determined in tissue culture selection for glyphosate resistance in plants (reviewed by Pline-Srnic 2006). Gene amplification has been determined in GR Palmer Amaranth (*Amaranthus palmeri* S. Wats.) and in Italian ryegrass (*Lolium multiflorum* Lam.) (Gaines et al. 2010, 2011; Salas et al. 2012). Gene

amplification and EPSPS overexpression through an increased rate of transcription results in an increase in EPSPS production that can confer resistance to glyphosate.

EPSPS overexpression has only been determined in cultured cells of *Corydalis sempervirens* Pers. (Pline-Scrnici 2006; Smart et al. 1985). These cultured cells showed a 40-fold increase in EPSPS activity, however there was an accumulation of shikimic acid, the dephosphorylated substrate of the enzyme when exposed to glyphosate (Amrhein et al. 1983; Smart et al. 1985). Overproduction of the EPSP synthase was determined through gel electrophoresis and an immunoassay (Smart et al. 1985). An increased rate of EPSP synthase protein synthesis was determined through *in vivo* pulse-labeling (Smart et al. 1985). There was also no increase in the copy number of the EPSPS gene (Smart et al. 1985). The EPSP synthase from these adapted cultured cells showed identical physical, kinetic and immunological properties as the EPSP synthase from unadapted glyphosate sensitive cultured cells (Smart et al. 1985). Therefore these cultured cells were sensitive but the amplitude of EPSPS resulted in the cultured cells of *Corydalis sempervirens* Pers. conferring resistance (Amrhein et al. 1983; Smart et al. 1985).

Gene amplification as a mechanism of resistance in tissue culture has been documented in wild carrot, petunia, tobacco (*Nicotiana tabacum* L.), soybean and alfalfa (Dyer et al. 1988; Nafziger et al. 1984; Steinrücken et al. 1986; Suh et al. 1993; Widholm et al. 2001). Wild carrot was the first plant developed through tissue culture to possess gene amplification as its mechanism of resistance to glyphosate (Nafziger et al. 1984; Pline-Scrnici 2006). The EPSPS synthase activity was 12-fold higher than the original cells (Nafziger et al. 1984). Suh et al. (1993) found there was a 25-fold increase in the EPSPS gene copy number and an inverted repeat. Steinrücken et al. (1986) reported that petunia

cell culture had a 10- to 20-fold increase in the copies of the EPSPS and had a 20-fold increase in the EPSPS activity. Suspension cultures of alfalfa, soybean and tobacco conferred at least 100-fold level of resistance over original cells (Widholm et al. 2001). The EPSPS activity was 62, 21 and 800 fold higher than the original cells in alfalfa, soybean and tobacco, respectively (Widholm et al. 2001). The estimated increase of the gene copy number from Southern hybridization was 6, 9 and 48 for the suspension cultures of alfalfa, soybean and tobacco, respectively (Widholm et al. 2001). With the wild carrot and petunia tissue culture examples it was demonstrated that the EPSPS was sensitive (Nafziger et al. 1984; Steinrücken et al. 1986). Like EPSPS overexpression, gene amplification results in an increase of sensitive EPSPS production but it is a result of an increase of EPSPS gene copy number and not through an increased rate of transcription.

The first report of a glyphosate resistance due to gene amplification in naturally occurring plant populations was found in Palmer amaranth (Gaines et al. 2010). This mechanism conferred six- to eight-fold resistance, did not show differences in absorption or translocation and no shikimate accumulation was detected in the resistant leaf tissue (Culpepper et al. 2006). The EPSPS enzyme activity of GR Palmer amaranth, however, was inhibited by glyphosate when tested in an EPSPS activity assay, indicating that it is sensitive (Gaines et al. 2010). Genomes of these resistant Palmer amaranth plants contained five- to more than 160-fold more copies of the EPSPS gene than a susceptible plant (Gaines et al. 2010). Through quantitative RT-PCR on cDNA and immunoblot analysis, it was shown that EPSPS expression and EPSPS protein levels were positively correlated with the genomic copy number (Gaines et al. 2010). It was later found that the

glyphosate resistance level, overall, correlates with an increase in EPSPS genomic copy number, expression, protein level and enzymatic activity (Gaines et al. 2011). It was also suggested that with this population, 30 and 50 EPSPS genomic copies are needed to survive 0.5-1.0 kg a.e. ha<sup>-1</sup> of glyphosate (Gaines et al. 2011).

Italian ryegrass in Arkansas was found to have EPSPS gene amplification as its mechanism of resistance (Salas et al. 2012). The resistance index of the Italian ryegrass was seven- to thirteen-fold, the EPSPS activity was six-fold higher than the susceptible plant and was equally as sensitive to glyphosate (Salas et al. 2012). The Italian ryegrass was found to have up to 25 more copies of the EPSPS gene (Salas et al. 2012). There was a positive correlation with the level of resistance, an increase of EPSPS enzyme activity and the gene copy number (Salas et al. 2012). Both examples of gene amplification demonstrated a positive correlation with the gene copy number and resistance factor.

### ***Reduced Spray Retention and Absorption***

Reduced spray retention and absorption have been reported to play a role in resistance but have never been the only mechanism of glyphosate resistance. This mechanism has been reported in Italian ryegrass, Johnsongrass (*Sorghum halepense* (L.) Pers.) and sourgrass (*Digitaria insularis* (L.) Mez ex Ekman). These mechanisms were first reported in Italian ryegrass (Michitte et al. 2007). The contact angle was higher in resistance plants and spray retention and abaxial leaf surface was 35% and 40% lower, respectively, than a susceptible population (Michitte et al. 2007). It was observed that the leaf cuticle was thicker and that there were wrinkles present on the leaf surface in comparison to the susceptible plant (Michitte et al. 2004). In resistant plants the composition of the epicuticular wax was slightly different than susceptible plants with 5% more polar

compounds (alcohols and aldehydes) present (Guimarães et al. 2009). Altered translocation to the tip of the treated leaf also contributed to glyphosate resistance (Michitte et al. 2007). With Italian ryegrass decreased absorption was caused by differences in contact angle, cuticle thickness, presence of wrinkles and epicuticular wax composition.

Another case of reduced absorption is in resistant perennial Johnsongrass which shows a unique phenotypic response to glyphosate (Vila-Aiub et al. 2011). The resistant plant resprouts and tillers from the shoot meristem while the rest of the plant is severely damaged (Vila-Aiub et al. 2007). Absorption was 10-20% lower in the adaxial and 20-25% in the abaxial leaf surfaces (Vila-Aiub et al. 2011). Like the Italian ryegrass glyphosate translocation also played a role in the mechanism of resistance along with reduced absorption (Vila-Aiub et al. 2011).

The most recent report of reduced absorption of glyphosate is in sourgrass (*Digitaria insularis*) (de Carvalho et al. 2012). It absorbed at least 12% less glyphosate than the susceptible biotype at 12 hours after treatment, however there was no difference in absorption at 72 hours (de Carvalho et al. 2012). Along with reduced absorption, reduced translocation, metabolism and gene mutation contributed to resistance to glyphosate (de Carvalho et al. 2012).

Reduced absorption was also reported in legume species that showed an innate resistance to glyphosate. This innate resistance showed a seven- to fourteen-fold resistance when compared to susceptible *Amaranthus* species (Cruz-Hipolito et al. 2009, 2011; Rojano-Delgado et al. 2012). The legume species studied were *Canavalia ensiformis* (L.) DC., *Clitoria ternatea* L., *Neonotonia wightii* (Wight & Arn.) Lackey var.

*wightii* and *Mucuna pruriens* var. *utilis* (Cruz-Hipolito et al. 2009, 2011; Rojano-Delgado et al. 2012). These species possessed a thick waxy cuticle that reduced absorption by 15 to 44% in comparison to susceptible *Amaranthus* species (Cruz-Hipolito et al. 2009, 2011; Rojano-Delgado et al. 2012). Reduced translocation also contributed to glyphosate as well as enhanced metabolism in *M. pruriens* (Cruz-Hipolito et al. 2009, 2011; Rojano-Delgado et al. 2012). It was observed in *M. pruriens* that the epicuticular wax had non uniform thickness in comparison to *Amaranthus hybridus* L. (Rojano-Delgado et al. 2012). Through scanning electron microscopy it was observed that along with thicker epicuticular wax, there were small crystalline platelets in *N. wightii* and *C. ternatea* (Cruz-Hipolito et al. 2011). Therefore differences in epicuticular wax can contribute to glyphosate resistance.

### ***Reduced Translocation***

Reduced translocation can be a contributing or a sole mechanism of resistance to glyphosate. Reduced translocation is the sole mechanism of resistance to glyphosate in rigid ryegrass (Lorraine-Colwill et al. 2003; Wakelin et al. 2004). It was found that glyphosate accumulated in the leaf tips of resistant plants (Lorraine-Colwill et al. 2003). This conferred a level of resistance of ten-fold (Powles et al. 1998). It was theorized by Lorraine-Colwill et al. (2003) that there may be presence of a cellular glyphosate pump which would retain glyphosate in the apoplastic space and prevent it from entering neighbouring cells to pass into phloem tissue. Reduced translocation can confer a high level of glyphosate resistance.

There are populations of rigid ryegrass that also have a target site mutation in EPSPS along with the reduced glyphosate translocation mechanism in South Australia



(Bostamam et al. 2012). Populations that possess both of these mechanisms possessed 5.6-fold to greater than 10-fold resistance whereas the populations that contained just the target site mutation conferred two-fold resistance and just the reduced translocation mechanism conferred four-fold resistance (Bostamam et al. 2012). Therefore having both mechanisms of resistance present in the population of rigid ryegrass contributed to a greater level of resistance than if each mechanism of resistance was present singularly.

Reduced translocation was also found to be the mechanism of resistance in Canada fleabane (Feng et al. 2004). When plants were sprayed or applied with a single drop of  $^{14}\text{C}$ -glyphosate, less glyphosate was translocated to the roots (Feng et al. 2004). With droplet application of  $^{14}\text{C}$ -glyphosate it was also demonstrated that less glyphosate was being translocated out of the treated leaf in comparison to a susceptible plant (Feng et al. 2004). Autoradiography of these droplet treated leaves revealed that glyphosate remained localized and phloem loading and export were delayed in comparison to susceptible leaves (Feng et al. 2004). This delay in phloem loading and export in Canada fleabane was causing less glyphosate translocated out of the treated leaf and to the roots.

Feng et al. (2004) found that there was a lower shikimate to glyphosate ratio in GR Canada fleabane tissue relative to susceptible tissue. This would suggest that glyphosate is less able to inhibit the EPSPS (Feng et al. 2004). However, there was shikimate accumulation in the resistant tissue which would indicate a sensitive EPSPS (Feng et al. 2004; Mueller et al. 2003). A possible explanation of this would be that glyphosate could be partially excluded from the plastids in GR plants which would result in less-efficient EPSPS inhibition (Feng et al. 2004). The mechanism that is causing decreased phloem loading and export may be also partially excluding the glyphosate from

the plastids.

### ***Rapid Vacuolar Sequestration***

Vacuolar sequestration and a concomitant decrease in translocation in GR Canada fleabane and ryegrass prevents glyphosate from reaching the target site (Ge et al. 2010, 2012). Through  $^{31}\text{P}$  NMR experiments it was found that GR Canada fleabane had greater than 85% glyphosate fractional occupancy in the vacuole of the source tissue compared to approximately 15% in susceptible Canada fleabane (Ge et al. 2010). Vacuoles in the sink tissue of the GR Canada fleabane contained the majority of the glyphosate compared to the cytoplasm whereas all of the glyphosate in the sink tissue of the susceptible Canada fleabane was found in the cytoplasm (Ge et al. 2010). Uptake into the vacuole was also more rapid in the GR Canada fleabane than in the susceptible Canada fleabane (Ge et al. 2010). This provides an explanation for reduced translocation in GR Canada fleabane (Feng et al. 2004; Ge et al. 2010; Koger and Reddy 2005).

Similar results were found in ryegrass, however, sensitive ryegrass showed no measurable sequestration (Ge et al. 2012). In the stronger resistant lines of ryegrass, a greater fraction of glyphosate was sequestered in the vacuole compared to moderately resistant ryegrass (Ge et al. 2012). Sequestration into the vacuoles was also more rapid in the stronger resistant lines compared to the moderately resistant ryegrass (Ge et al. 2012). Due to this correlation of resistance level and vacuolar sequestration, it is suggested that vacuolar sequestration plays a major role in the mechanism of resistance (Ge et al. 2012).

More evidence that supports vacuolar sequestration being the primary mechanism, is that it is found that vacuolar sequestration in Canada fleabane is significantly suppressed when cold acclimated and treated with glyphosate at low temperatures

(~12°C) (Ge et al. 2011). This suppression was reversible if plants were exposed to warmer conditions (Ge et al. 2011). The effect of suppression of vacuolar sequestration in low temperatures was able to make a glyphosate resistant Canada fleabane susceptible (Ge et al. 2011).

It is strongly suggested by Ge et al. (2010, 2012) that there is presence of a tonoplast membrane pump being over expressed or upregulated because vacuolar sequestration of resistant Canada fleabane and ryegrass occurred on a time scale of hours. What has been observed with glyphosate loading in resistant Canada fleabane and ryegrass is similar to reports of an ATP-binding cassette (ABC) transporter system, being upregulated or overexpressed, used to detoxify xenobiotics into a vacuole by active transport (Ge et al. 2010; reviewed by Yuan et al. 2007). Because of these similarities, a presence of a tonoplast membrane pump is strongly suggested for causing rapid vacuolar sequestration.

### ***Metabolism***

Metabolism is not a usually suspected mechanism of glyphosate resistance. For a long time many scientists have considered soil microbes to be the only organisms that significantly degraded glyphosate (Duke 2011). Until 2011, only limited metabolism has been reported and proven not to significantly reduce phytotoxicity of glyphosate or play a role in the resistance mechanism (de Carvalho et al. 2012; Duke 2011; Duke et al. 2003; Putnam 1976; Sandberg et al. 1980; Simarmata et al. 2003; Wyrill and Burnside 1976). There are two pathways of glyphosate metabolism that occur in soil (Duke 2011). One pathway is from a direct C-P lyase enzyme that results in sarcosine and inorganic phosphate (Duke 2011). Another pathway is a *gox* enzyme that degrades glyphosate to

AMPA and glyoxylate (Duke 2011). This pathway occurs in transgenic GR canola with an inserted *goxv247* gene conferring resistance with an inserted *cp4 epsps* gene for target site resistance (Green 2009). Both of these glyphosate metabolism pathways do not naturally occur in plants, which is why it is usually not suspected to be a mechanism of resistance to glyphosate.

There has been metabolism reported in sourgrass, but it is not the sole mechanism of resistance to glyphosate (de Carvalho et al. 2012). This weed had multiple mechanisms along with metabolism including absorption, translocation and gene mutation mechanisms (de Carvalho et al. 2012). Degradation of glyphosate in susceptible biotypes of sourgrass was much slower than in the resistant biotypes (de Carvalho et al. 2012). In the resistant plants 25 to 59% of glyphosate, in relation to its metabolites, was detected up to 48 hours after treatment (HAT) and <10% was detected at 168 HAT (de Carvalho et al. 2012). Whereas with the susceptible plants >90% of glyphosate was detected at 48 HAT and 80% at 168 HAT (de Carvalho et al. 2012). Up to 96 HAT, 37-64% of AMPA and 15-32% of glyoxylate and low levels of sarcosine were detected in the resistant biotypes, and no metabolites found in the susceptible biotypes (de Carvalho et al. 2012). There was detection of AMPA and glyoxylate at 168 HAT in the susceptible biotypes but the degradation of glyphosate into its metabolites was much more rapid in the resistant biotypes (de Carvalho et al. 2012). The difference at 168 HAT between susceptible and resistant sourgrass populations is a stark contrast, but the metabolism of glyphosate may not be rapid enough to allow the resistant population to survive if there were no other mechanisms of resistant present.

## **Glyphosate resistance in giant ragweed in Ontario**

### ***Occurrence***

In 2008, a giant ragweed population near Windsor, Ontario, Canada was suspected to be resistant to glyphosate herbicide when it was not controlled with glyphosate at the manufacturers recommended field rate (Sikkema et al. 2009). In initial growth room studies, this population was able to survive rates up to two times the field rate, while rates down to one quarter of the field rate were lethal to populations from other locations (Sikkema et al. 2009). In field trials, giant ragweed plants were able to survive rates up to 10800 g a.e. ha<sup>-1</sup> (Sikkema et al. 2009). As of 2012, GR giant ragweed has been confirmed at 82 sites in the Ontario counties of Essex, Kent, Lambton, Lennox-Addington and Middlesex (Follings et al. 2013b; Vink et al. 2012d). Five of these sites from Essex, Kent and Lambton contain populations resistant to both glyphosate and cloransulam-methyl (Follings et al. 2013b). These resistant giant ragweed populations are widespread in Southwestern Ontario and able to survive high rates of glyphosate.

### ***Control***

There are a limited number of herbicides which provided acceptable control of GR giant ragweed in soybean. The most effective herbicides are 2,4-D ester and amitrole applied preplant (PP) to emerged GR giant ragweed prior to seeding of soybean. In dicamba-tolerant soybeans, GR giant ragweed is controlled with a sequential application of dicamba applied PP followed by POST (100%) (Follings et al. 2013a; Vink et al. 2012 a,b,c). There are a limited number of herbicides for the control of GR giant ragweed, this problem is exacerbated when there is multiple resistance to both glyphosate and cloransulam-methyl (Follings et al. 2013b).

### ***Herbicide Symptomology***

The first confirmed glyphosate resistant population from Windsor, and many other Ontario populations with confirmed resistance, exhibit an unusual symptomology to glyphosate. Within 24 hours of application, the mature leaves rapidly dehydrate curling upwards, eventually becoming necrotic while the young developing leaves and apical meristems escape injury. These unaffected meristematic areas will continue to grow allowing plant survival.

While surveying the occurrence of glyphosate resistance in giant ragweed in Ontario, Vink (2012) observed giant ragweed surviving the resistance screening dose of 1800 g a.e. ha<sup>-1</sup> that did not have the rapid necrosis symptomology previously described. Instead, this population from Leamington, Ontario had similar symptomology to susceptible plants treated with glyphosate in which the plants began to yellow at the meristematic tissue. Instead of wilting and dying like susceptible plants, these plants remained stunted and began to regrow after one to three weeks. Therefore, it is probable that there are two distinct mechanisms of resistance that confer resistance to glyphosate in the resistant populations of giant ragweed in Ontario.

### **Objectives and Hypothesis**

The mechanism of glyphosate resistance in giant ragweed in Ontario has yet to be determined. Because there are two glyphosate resistance phenotypes defined by different symptomologies, there may be two different mechanisms of resistance to glyphosate occurring in giant ragweed in Ontario.

The overall aim of this thesis is to identify the mechanisms of resistance to

glyphosate in giant ragweed in Ontario. In order to reach this aim, three specific objectives were set:

- (i) Determine the level of resistance of the two resistant populations compared to susceptible populations;
- (ii) Determine if shikimate can accumulate in leaf discs of resistant and susceptible populations and if there is an effect from the presence or absence of light on shikimate accumulation;
- (iii) Determine if the mechanism of resistance is through reduced absorption and translocation.

The null hypotheses of this research are:

- (i) There will be no difference in biomass accumulation and survival between resistant and susceptible giant ragweed populations.
- (ii) There will be no difference in accumulation of shikimate in leaf tissue of susceptible and resistant populations that has been incubated in glyphosate.
- (iii) There will be no effect of the presence of light on the accumulation of shikimate in leaf tissue that has been incubated in glyphosate.
- (iv) There will be no difference in absorption of glyphosate into plants of susceptible and resistant giant ragweed populations.
- (v) There will be no difference of translocation of glyphosate within the plants of susceptible and resistant giant ragweed populations.

## **MATERIALS AND METHODS**

### **Plant material and growth conditions**

Two resistant giant ragweed populations were compared to two susceptible populations in these experiments. Each population came from a different location in Ontario, Canada.

The resistant giant ragweed populations were collected from agricultural fields near Windsor (R1) and Leamington (R2) and were numbered 006 and 054, respectively. R1 seeds were collected in the fall of 2008, while the R2 population was collected in the fall of 2009 (Vink et al. 2012). Both fields had Roundup Ready soybean grown frequently.

The susceptible populations were numbers 003 from Cambridge (S1) and 005 from Windsor (S2). S1 was collected from a river bank in Cambridge, while S2 was collected from an agricultural field near Windsor. Populations R1 and R2 were previously screened for resistance (Vink et al. 2012). For the R1 population, 20 surviving plants were grown in a greenhouse in isolation under an 18 H light phase at 25°C and a 6 H dark phase at 18°C. Plants were watered as needed and a solution of fertilizer (20-20-20 N:P:K) was added weekly at 1.5g L<sup>-1</sup>. The R2 population's seeds were collected from the field site in the Leamington area. Populations were grown to maturity to produce more seed for experimentation. Seeds from R and S populations were stored in a refrigerator between two and five °C until two to three months prior to use for experimentation.

Seeds were sterilized before planting to prevent mold development while breaking dormancy. Working under a flow hood, batches of seed were put into sterile 50 mL centrifuge tubes. Thirty mL of ethanol (95%, v/v) was added to tubes and placed on a rotator for 4.5 to 5 minutes. Ethanol was then strained from seeds and then 10 to 15 mL



of a sodium hypochlorite (30% w/v) was added to the tubes containing the seeds. The tubes were then put on a rotator for 20 minutes. The sodium hypochlorite solution was strained from the seeds and 30 ml of deionized water was added and mixed by inverting the tube multiple times. Water was strained and fresh deionized water was added and mixed again. This step was repeated three times. This procedure sterilized seeds to prevent mold growth while seeds were kept in cool moist soil to break the dormancy.

Giant ragweed seed dormancy was broken using methodology similar to that of Stachler (2008). Greenhouse transplant trays (18-cell) were filled approximately half way with a moist potting media (Pro-Mix PGX<sup>1</sup>). Batches of 100 to 150 seeds of each population were placed into individual cells and then covered with two to three cm of soil. Soil was watered until it reached maximum water holding capacity. Trays were then placed into individual lidded plastic containers and stored in a refrigerator at three to six °C for two to three months. Seedlings were transplanted when they were at the radicle to cotyledon emergence stage. This method was found to be the most effective and consistent way to break the dormancy of a large amount of giant ragweed seed.

### **Dose Response**

The impact of glyphosate on biomass and survival was determined in a series of dose response experiments. Seedlings from all four populations were transplanted into a potting media (Pro-Mix PGX) in 11.4 x 11.4 cm pots for the first two runs of the experiment and 15.2 cm diameter round pots for the remaining runs. Plants from each population were arranged in a completely randomized design with 5 replications until time of spray. Plants were grown in a growth room under a regime of a 16 h light phase at 25°C and an 8 h dark phase at 20°C with humidity at 75%. Photosynthetically active

radiation was  $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Plants were watered with fertilizer (20-20-20 N:P:K) at  $0.3 \text{ g L}^{-1}$  as needed. Glyphosate was applied to plants at the two- to three-node stage, above the cotyledons. The time between transplanting and spray application ranged between 12 to 14 days. Due to variable height at the time of spraying, plants were allocated into blocks based on size. Susceptible plants were sprayed with a commercial formulation of glyphosate<sup>2</sup> at doses ranging from 7 to  $1800 \text{ g a.e. ha}^{-1}$ . Resistant plants were sprayed with doses ranging from 56 to  $14400 \text{ g a.e. ha}^{-1}$ . These rates were chosen based on the field rate  $900 \text{ g a.e. ha}^{-1}$  and a previous preliminary dose response performed by Dr. François Tardif. Glyphosate was applied with a laboratory chamber track sprayer with a single 8002 even flat fan nozzle (TeeJet, Spraying Systems Co., Wheaton, IL) positioned 50 cm above leaves calibrated to deliver a spray volume of  $210 \text{ L ha}^{-1}$  at 276 kPa. After application, plants were returned to the growth room and placed according to a completely randomized block design. Plants were rotated two plant spaces diagonally every 4 to 5 days to minimize environmental effects. At 23 days after treatment, survival was determined and all above ground plant material was cut at soil level, placed in individual paper bags and dried at  $70^{\circ}\text{C}$  for 48 h; prior to measurement of dry weights. This experiment had a wide range of doses and multiple repetitions to ensure the least amount of error when predicting the parameters of the dose response curve.

Non-linear regressions were performed on percent survival and above ground biomass (measured as percentage of the untreated) as functions of herbicide dose, using the PROC NLIN procedure in SAS (Seefeldt 1995). A sigmoidal log-logistic curve model was chosen as it most precisely fits the dose response behaviour (Bowley 2008; Seefeldt 1995). The dose response equation used is as follows:

$$Y = C + (D - C) / [1 + (\text{rate} / I_{50})^b]$$

where Y is the percent survival or percent above ground biomass, C is the lower limit, D is the upper limit, *b* is the slope of the curve at the inflexion point, and *I*<sub>50</sub> is the rate giving 50 percent of the response between the upper and lower limits. Separate upper limits were used when analyzing above ground biomass in PROC NLIN because they predicted the data significantly better than using the same upper limit using the lack-of-fit *F*-test. Resistance Index (RI) values were calculated by dividing the LD50 or G50 values of R by that of S.

## **Determination of Shikimate Accumulation in Leaf Discs**

### ***General Procedures***

Shikimate accumulation experiments were conducted to determine whether glyphosate could inhibit the target site in isolated leaf discs of R and S populations. Accumulation of shikimate (the dephosphorylated substrate of EPSPS) in this system is indicative that glyphosate reaches EPSPS and is able to inhibit its activity. If the target sites of the R populations are resistant, shikimate will not accumulate.

Plants were grown under the same conditions as in the dose response experiment and were transplanted in 15.2 cm diameter round pots. R1, R2 and S1 giant ragweed populations at the two- to three- node stage, glyphosate resistant soybeans (cv. OAC Rockwood) and conventional soybeans (cv. OAC Lakeview) at the unifoliate to first trifoliate were used for these experiments. Soybeans were included as known target site resistant and susceptible control plants. Tissue was sampled by excising leaf discs (4-mm diam) with a cork borer from the youngest tissue at the apical meristem and fully developed leaves at the second node of the giant ragweed plants and unifoliate leaves of

the soybean plants. Shikimate accumulation, extraction and analysis were carried out according to the method of Shaner et al. (2005). Leaf discs were placed into wells of a 96-well microtitre plate<sup>3</sup>, 1 disc per well. Wells contained an assay buffer of 10 mM ammonium phosphate plus 0.1% (v/v) Tween 80 surfactant (pH 4.4). Control wells contained just the assay buffer while treatment wells contained 250  $\mu$ M or 500  $\mu$ M of glyphosate in the assay buffer. Each well contained 100  $\mu$ L of solution. Plates were covered with adhesive sealing film prior to incubation under fluorescent lights at 50  $\mu$ mol  $\text{m}^{-2} \text{s}^{-1}$  at room temperature (21 to 24°C). After incubation plates were frozen in -20°C freezer for >12 h and then thawed at room temperature for 1.5 to 2 h. Twenty-five microliters of 1.25 N HCl were pipetted into each well and incubated at 60°C for 15 min. At this point, leaf discs had turned gray in colour, which indicated complete penetration of the acid into the tissue. Twenty-five microliters were collected from each well and transferred into wells of a new microtitre plate. One hundred microliters of 0.25% (w/v) periodic acid<sup>4</sup>/0.25% (w/v) *m*-periodate<sup>5</sup> were added to each well and plate was incubated at room temperature for 90 min. One hundred microliters of 0.6 N sodium hydroxide/0.22 M sodium sulfite were then added to each well. Within 30 min of this addition, optical density at 380 nm was determined using a microtitre plate spectrophotometer<sup>6</sup> at 380. Background density was determined for plant species x leaf maturity and subtracted from each respective treatment well. Shikimate concentration of each well was determined via a shikimate standard curve developed by adding known concentrations of shikimic acid<sup>7</sup> to the wells of the microtitre plates. Shikimic acid levels were reported as micromolar concentration. Means and standard errors were calculated for each experiment, which was conducted three times.

### ***Light Effect Study***

In preliminary experiments, the rapid necrosis of the R1 population treated with glyphosate was found to be light induced (data not shown). This rapid necrosis may affect shikimate accumulation in the leaf discs excised due to rapid cell death. To account for this factor leaf discs were incubated in the light and dark.

The assay buffer for this study also contained 0.5 % (w/v) of sucrose for an external carbon source in the absence of light (Dale Shaner, USDA-ARS, personal communication). Plates were incubated for 23 hours under fluorescent lights or in a cardboard box to be in the dark. Background density for this study was determined for plant species x leaf maturity x incubated with or without light and subtracted from each respective treatment well. Leaf discs were excised from five replicates of each population for the presence or absence of light treatments.

### ***Dose Response Study***

The effect of an increasing dose of glyphosate in shikimate accumulation in leaf discs was studied. Leaf discs were placed into treatment wells containing 1.1, 2.5, 5, 10, 25, 50, 100 and 250  $\mu\text{M}$  of glyphosate. Microtiter plates were incubated under fluorescent lights for 16h. Leaf discs were excised from three replicates of each population for each length of incubation under light. This experiment was conducted three times.

### **Necrosis Pattern of Spotted Application of Glyphosate at Sublethal Dose**

To observe the pattern of necrosis in the mature leaves of R1 giant ragweed, a sublethal concentration of glyphosate was spot applied to one mature leaf on each plant. Plants were grown under the same conditions as above in 11.4 x 11.4 cm square pots. R1 and S1 giant ragweed were used for this experiment. When plants were at the two- to three-

node stage, glyphosate was applied as two, single, one microliter droplets with a microliter syringe<sup>8</sup>. Droplets were applied at the most proximal, distal or mid area of the leaf, approximately 5 to 10 mm laterally from the mid vein on a leaf at the second node. Two concentrations of glyphosate were used of 14 mM and 4 mM. They are considered sublethal to S plants given that the field rate of glyphosate, 900 g a.e. ha<sup>-1</sup>, sprayed at 210 L ha<sup>-1</sup> would have a concentration of 25 mM. On the opposite leaf, paraquat (4 mM) was spot applied as a visual check of a herbicide that demonstrates no mobility in the same location where glyphosate was spot applied. Photographs were taken at 2, 3, 6, 12, 24 and 48 hours after spot application to record an image of the pattern of necrosis from the applied drop of glyphosate and to observe the direction in which the necrosis developed relative to where the drop was applied.

### **Absorption and Translocation**

To compare the amount of glyphosate that was absorbed and translocated in the R1 and S1 giant ragweed populations, radiolabeled [<sup>14</sup>C]-glyphosate was used to quantify if there were differences. Plants were grown under the same conditions as above except for the relative humidity, which was set at 60%. R1 and S1 giant ragweed was transplanted at the cotyledon stage into 24-cell trays of baked clay medium. Plants were watered with 0.25g L<sup>-1</sup> of N and 1.25 g L<sup>-1</sup> of 20-8-20 N:P:K fertilizer as needed and were placed in trays of water after glyphosate treatment to ensure no glyphosate was washed off from watering. Cotyledons 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 were at an earlier stage of development than previous experiments, one- to two- node stage, due to the sample size capacity of the biological

oxidizer<sup>9</sup>. Plants were sprayed first with formulated glyphosate<sup>2</sup> using a laboratory chamber track sprayer at 500 g a.e. ha<sup>-1</sup>. Following this application, radiolabeled [<sup>14</sup>C]-glyphosate was spot applied to the adaxial surface linearly across, bisecting the length of one leaf with five 2-μL droplets at the first node of the giant ragweed plants; [<sup>14</sup>C]-glyphosate was applied with a 10-μL Wiretrol micropipet<sup>10</sup>. The specific activity of the [<sup>14</sup>C]-glyphosate was 2 GBq mmol<sup>-1</sup>. Prior to spot application [<sup>14</sup>C]-glyphosate was dissolved into deionized water and formulated glyphosate was added to bring the concentration of glyphosate up to 14.2 mM. Each plant had an application of 3104 Bq. Four replicates of plants were harvested at 24, 48 and 96 h after treatment.

At time of harvest, plants were dissected into the section of plant below the node of the treated leaf including the roots; the treated leaf and opposite leaf; and the section of plant above the node of the treated leaf. The treated leaf along with the opposite leaf were washed twice in two 22 mL vials containing 5 mL of aqueous 9.5% (v/v) ethanol containing 0.5% (v/v) Tween 20 to remove unabsorbed [<sup>14</sup>C]-glyphosate. After washing the leaf, 5 mL of scintillation cocktail<sup>11</sup> was added to each vial. Radioactivity of wash solution was quantified by liquid scintillation spectrometry (LSS) using a Beckman LS6K-SC scintillation counter<sup>12</sup>. Following the leaf wash each portion was wrapped in tissue paper<sup>13</sup> inserted into individual coin envelopes and dried at 60°C for 48 hours. Samples wrapped in tissue paper were then combusted to <sup>14</sup>CO<sub>2</sub> using a biological oxidizer<sup>9</sup> and was trapped in carbon-14 scintillation cocktail. Radioactivity was quantified by LSS. <sup>14</sup>CO<sub>2</sub> recovery was >88% determined by combusting known quantities of [<sup>14</sup>C]-glyphosate. The experiment was repeated twice and means and standard errors were calculated.

## Sources of Materials

<sup>1</sup> Pro-mix PGX, Premier Tech Horticulture Inc., 1, avenue Premier, Rivière-du-Loup, Quebec, G5R 6C1

<sup>2</sup>Roundup WeatherMAX 540 g a.e./L, Monsanto Canada Inc., 900 – One Research Road, Winnipeg, Manitoba, R3T 6E3

<sup>3</sup>Whatman Uniplat, Whatman plc, Springfield Mill, James Whatman Way, Maidstone, Kent, ME14 2LE, UK

<sup>4</sup>Periodic Acid, Sigma Ultra, Sigma-Aldrich Canada Ltd., Oakville, Ontario

<sup>5</sup>*m*-periodate, Sigma-Aldrich Canada Ltd., Oakville, Ontario

<sup>6</sup>Spectramax, 384 Plus Spectrophotometer, Molecular Devices, Sunnyvale, CA.

<sup>7</sup>Shikimic Acid, Sigma-Aldrich Canada Ltd., Oakville, Ontario

<sup>8</sup>10 µL Syringe, Model 801, Hamilton Co. Reno, NV

<sup>9</sup>Biological Oxidizer, Model OX-300, R.J. Harvey Instrument Coop., Hillsdale, NJ 07642

<sup>10</sup>Wiretrol micropipette, Drummond Scientific Company, 500 Parkway, Box 700, Broomall, PA 19008

<sup>11</sup> Ecolite, ICN Biomedicals Inc., 15 Morgan, Irvine, CA 92618

<sup>12</sup>Scintillation counter, Beckman Instruments Inc. 2500 Harbor Blvd., Fullerton, CA 92634

<sup>13</sup> Tissue paper, KimWipe, Kimberly Clark Inc., Rosewell, GA 30076



## RESULTS

### Whole Plant Dose Response to Glyphosate

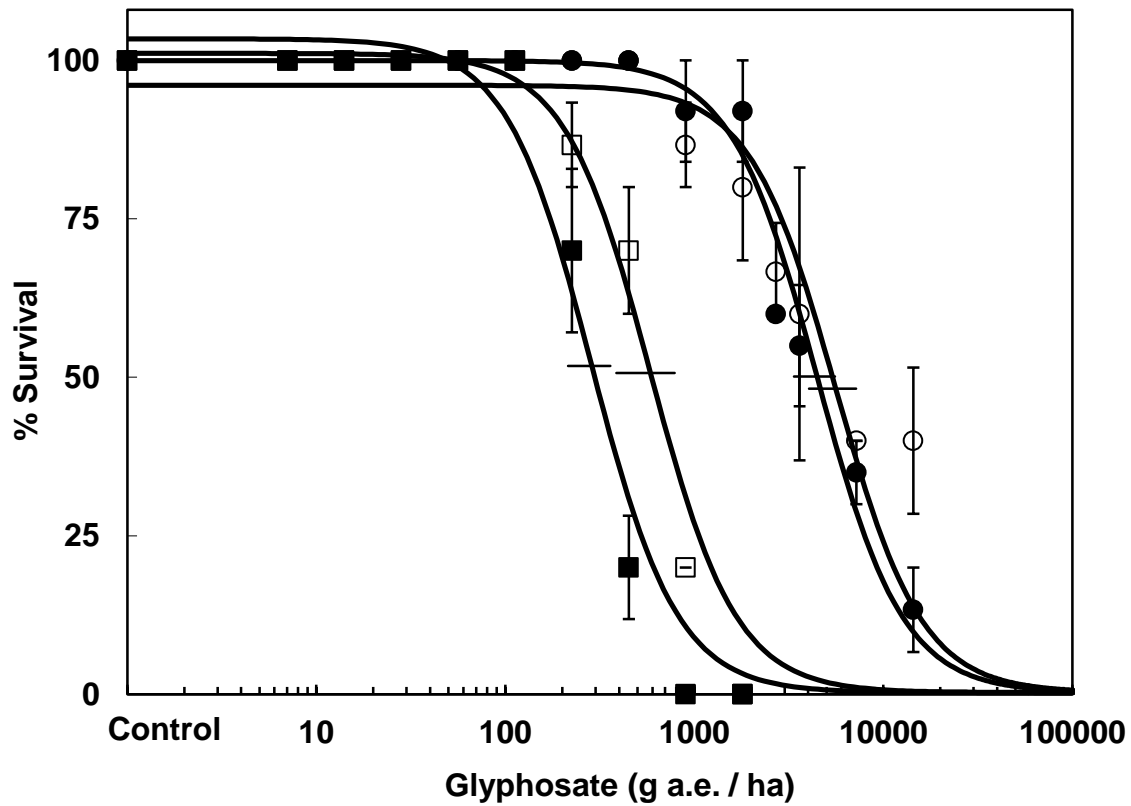
The response to glyphosate of populations R1 and R2 was clearly different from that of susceptible populations S1 and S2 based on survival and dry biomass (Figures 1 and 2).

Population R1, characterized by the rapid necrosis response, had an LD<sub>50</sub> value of 4500 g a.e. glyphosate ha<sup>-1</sup>, which was much higher than that of the two S populations.

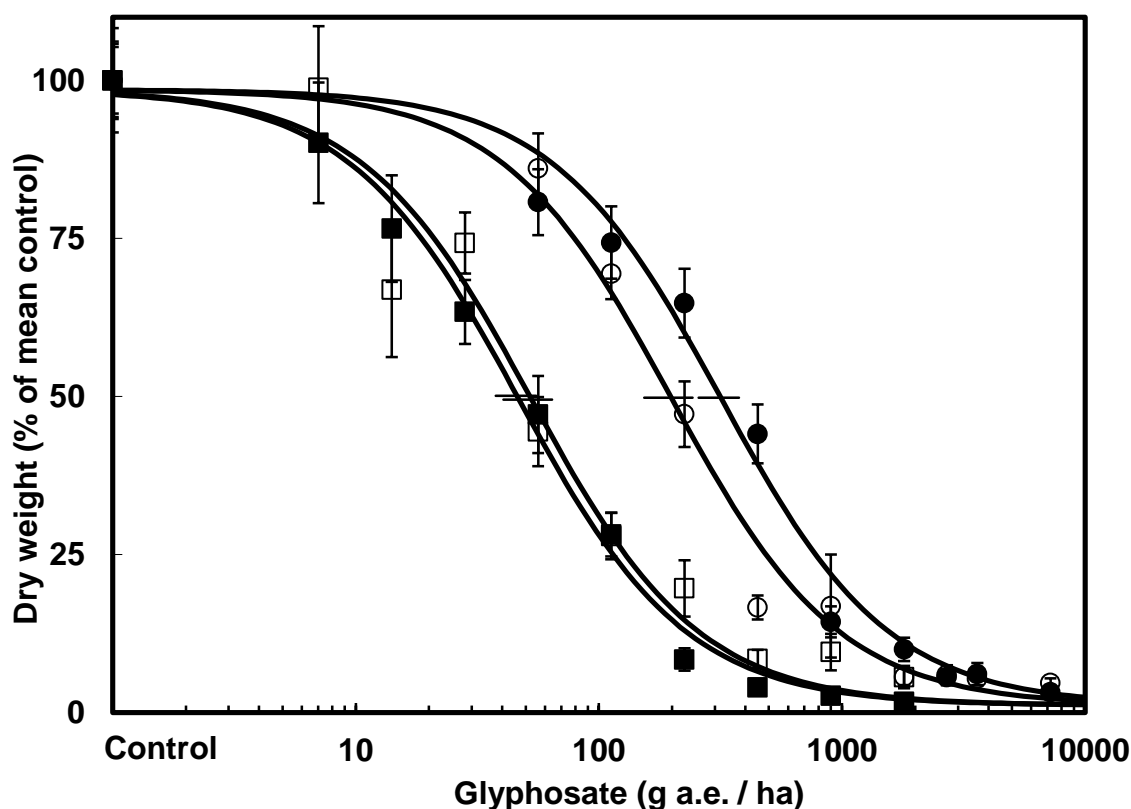
Resistance index (RI) values for R1 were 16 and 8 compared to S1 and S2, respectively (Figure 1). Population R2, which shows the slow recovery phenotype, had an LD<sub>50</sub> value of 5600 g a.e. ha<sup>-1</sup>. This was a 19-fold resistance over S1 and 9-fold over S2. Based on survival assessment, the response of R1 and R2 was similar, while S2 was slightly higher than S1 (Figure 1). Based on GR<sub>50</sub> values, population R1 had RI values of 7.0 and 6.2 compared to S1 and S2, respectively (Figure 2). Population R2 had a lower GR<sub>50</sub> value, with RI values of 4.3 and 3.8 compared to S1 and S2, respectively. The GR<sub>50</sub> values of S1 and S2 were similar.

The effect of glyphosate on biomass was observed at lower doses than the effect on survival. For the resistant populations, survival was impacted at doses of 2700 g a.e. ha<sup>-1</sup> or greater while biomass was reduced from doses as low as 56 g a.e. ha<sup>-1</sup> (Figures 1 and 2). The same response was observed for the susceptible populations with the effect on biomass occurring at lower rates than the effect on survival. This explains the higher values for LD<sub>50</sub> compared to GR<sub>50</sub> as well as the steeper slope for the survival dose response curves compared to the biomass dose response curves.

While the survival data points to similar level of resistance between the two R populations, the biomass data suggests that population R2 has a lower level of resistance



**Figure 1.** Effect of glyphosate on the survival of giant ragweed populations 23 DAT. Resistant populations R1 (●), R2 (○) and susceptible populations S1 (■) and S2 (□) were treated with glyphosate. Each point represents the average of three (R1 and S1) or five (R2 and S2) experiments with 5 replicates each. Vertical error bars represent the SEM ( $n=15$ , R1 and S1; and  $n=25$ , R2 and S2). Dose-response curves were generated by non-linear regression using a log-logistic model with the following equations: R1,  $y=100/(1+[x/4500]^{1.9})$ ; R2,  $y=96/(1+[x/5600]^{1.9})$ ; S1,  $y=103/(1+[x/290]^{1.9})$ ; S2,  $y=101/(1+[x/600]^{1.9})$ ; where  $y$  is the survival and  $x$  is the glyphosate dose. Horizontal error bars represent the 95% confidence intervals at  $LD_{50}$ .



**Figure 2.** Effect of glyphosate on the above ground dry biomass of giant ragweed populations 23 DAT. Resistant populations R1 (●), R2 (○) and susceptible populations S1 (■) and S2 (□) were treated with glyphosate. Each point represents the average of three (R1 and S1) or five (R2 and S2) experiments with 5 replicates each at 23 DAT. Vertical error bars represent the SEM (n= 15, R1 and S1; and n=25, R2 and S2). Dose–response curves were generated by non-linear regression using a log-logistic model with the following equations: R1,  $y=1+([99-1]/(1+[x/320]^{1.25}))$ ; R2,  $y=1+([99-1]/(1+[x/200]^{1.25}))$ ; S1,  $y=1+([99-1]/(1+[x/46]^{1.25}))$ ; S2,  $y=1+([99-1]/(1+[x/52]^{1.25}))$ ; where  $y$  is the survival and  $x$  is the glyphosate dose. Horizontal error bars represent the 95% confidence intervals at GR<sub>50</sub>.

compared to R1 at 23 days after treatment (DAT). The difference between the two populations could also be due to the speed at which treated plants recover from the herbicide application. At 23 DAT, there were plants in the R2 population that were only beginning to develop new axillary shoots, while most treated R1 plants had produced new growth shortly after treatment. In addition, once R2 plants started developing new shoots, there was minimal internode elongation; moreover, these new shoots appeared rigid and were slow to develop. The severity of this symptomology increased with dose (data not shown). The R1 population, however, had necrotic mature leaves but the young apical shoots developed and expanded more rapidly after treatment and overall had more vigor than the R2 population at the same dose. This explains why the survival curves were similar for R1 and R2 but the above ground biomass curves were different.

## **Shikimate Accumulation in Leaf Discs**

### ***Light Effect Study***

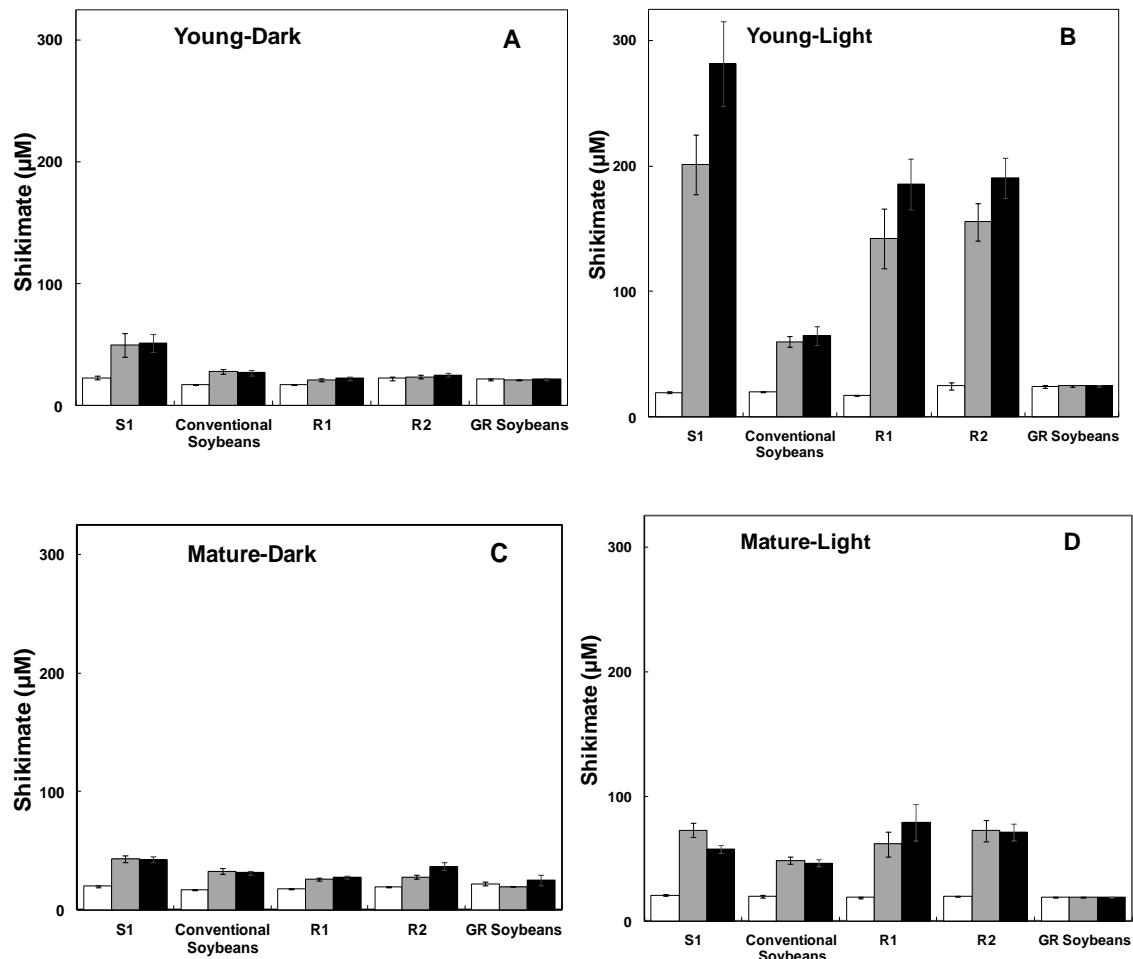
In all plants, except GR soybean, exposure to glyphosate in the light caused much higher accumulation of shikimate compared to incubation in the dark (Figure 3). There was no difference in shikimate accumulation in leaf discs from GR soybean regardless of glyphosate concentration (Figure 3). Under light, leaf discs removed from the young apical shoots and mature leaves of all of the giant ragweed populations and the conventional soybean accumulated more shikimate at 250 and 500  $\mu$ M glyphosate compared to the control (Figures 3B and 3D). This was the same in leaf discs from mature leaves incubated in the dark (Figure 3C). In leaf discs from young apical shoots incubated in the dark, there was more shikimate accumulation in the S1 and R1 giant ragweed populations and conventional soybean incubated in 250  $\mu$ M and 500  $\mu$ M

glyphosate compared to the control (Figure 3A). There was no difference in shikimate accumulation between treatments in the R2 giant ragweed populations and the GR soybean (Figure 3A).

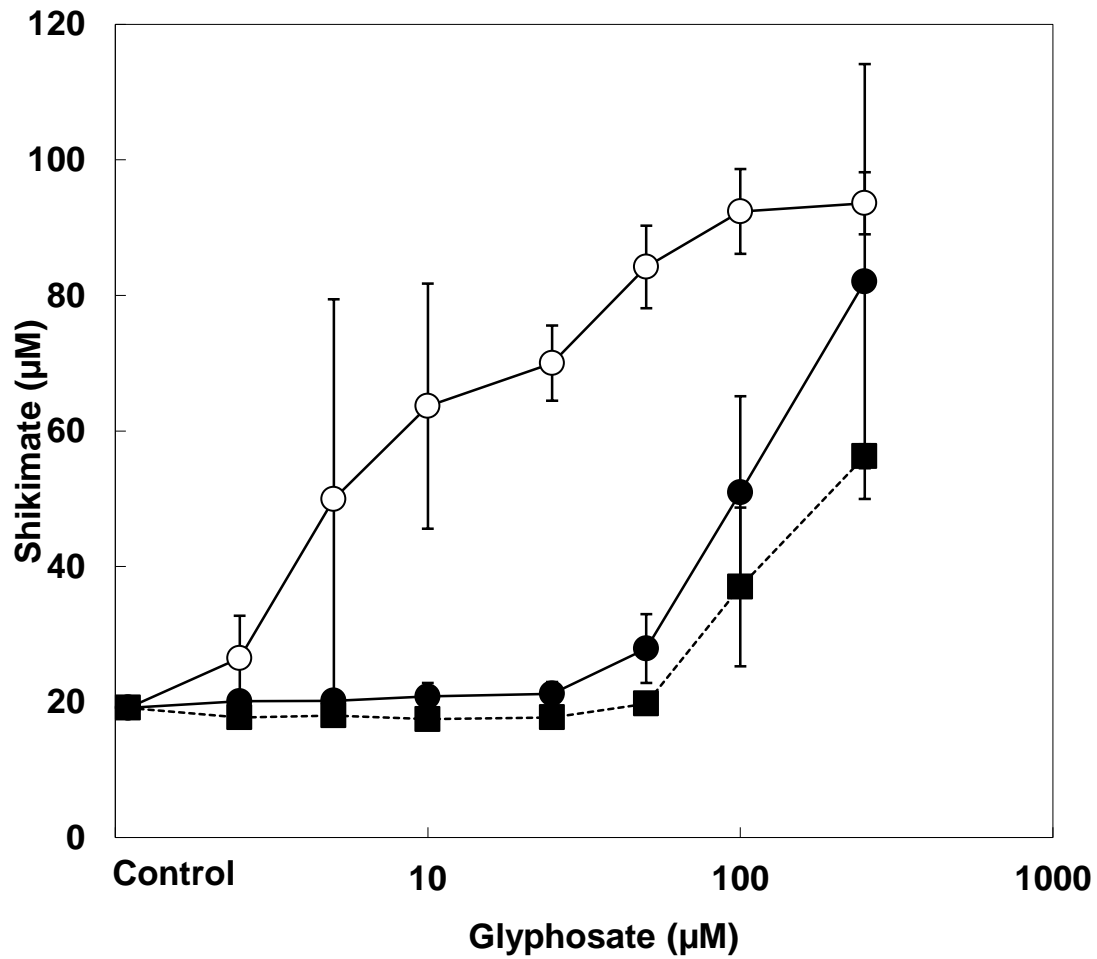
There was greater amount of shikimate accumulated in the leaf discs from the S1 population compared to the R1 and R2 populations in young apical shoots in both the light and dark incubations at 250 and 500  $\mu\text{M}$  of glyphosate (Figures 3A and 3B). In the leaf discs incubated in the light, there were similar amounts of shikimate accumulated at 250 and 500 $\mu\text{M}$  between the two R populations showing an intermediate level of shikimate accumulation in comparison to the S population and GR soybean (Figure 3B).

### ***Dose Response Study***

In the dose response study, the R and S populations started to accumulate shikimate at different doses of glyphosate. In the S1 population there was a relatively steady increase in shikimate accumulation as the dose of glyphosate increased with a difference, compared to the control, beginning at 10  $\mu\text{M}$  glyphosate (Figure 4). R1 and R2 populations started to accumulate shikimate at 100  $\mu\text{M}$  glyphosate with a point of inflection around 50  $\mu\text{M}$  glyphosate (Figure 4). At this point of inflection, shikimate levels increased rapidly in the R1 population almost intersecting with S1 population's shikimate levels at 250  $\mu\text{M}$  glyphosate (Figure 4). At 250  $\mu\text{M}$  glyphosate there was no difference in shikimate accumulation between S1 and R1 populations and R1 and R2 populations.



**Figure 3.** Effect of the presence of light on shikimate accumulation of excised leaf discs from glyphosate resistant giant ragweed populations R1, R2, a susceptible populations and conventional and Roundup Ready soybeans incubated in 0(□), 250 (■) and 500 μM (■) glyphosate for 24H. Figure subheadings indicate the maturity of the leaf where discs were excised from and whether they were incubated in the light or dark. Each bar represents the average of two (conventional soybean) or three (R1, R2, S1 and GR soybean) runs of the experiment with four tissue samples taken from each of five plants of each population. Vertical error bars represent the SEM (n = 40, conventional soybean; and n = 60, R1, R2 and S1).



**Figure 4.** Effect of glyphosate dose on shikimate accumulation of S1 (○), R1 (●) and R2 (■) Giant Ragweed (*L.*) populations incubated for 16 h. Each point represents the mean of three leaf discs and error bars represent the SEM (n=3). One run presented.

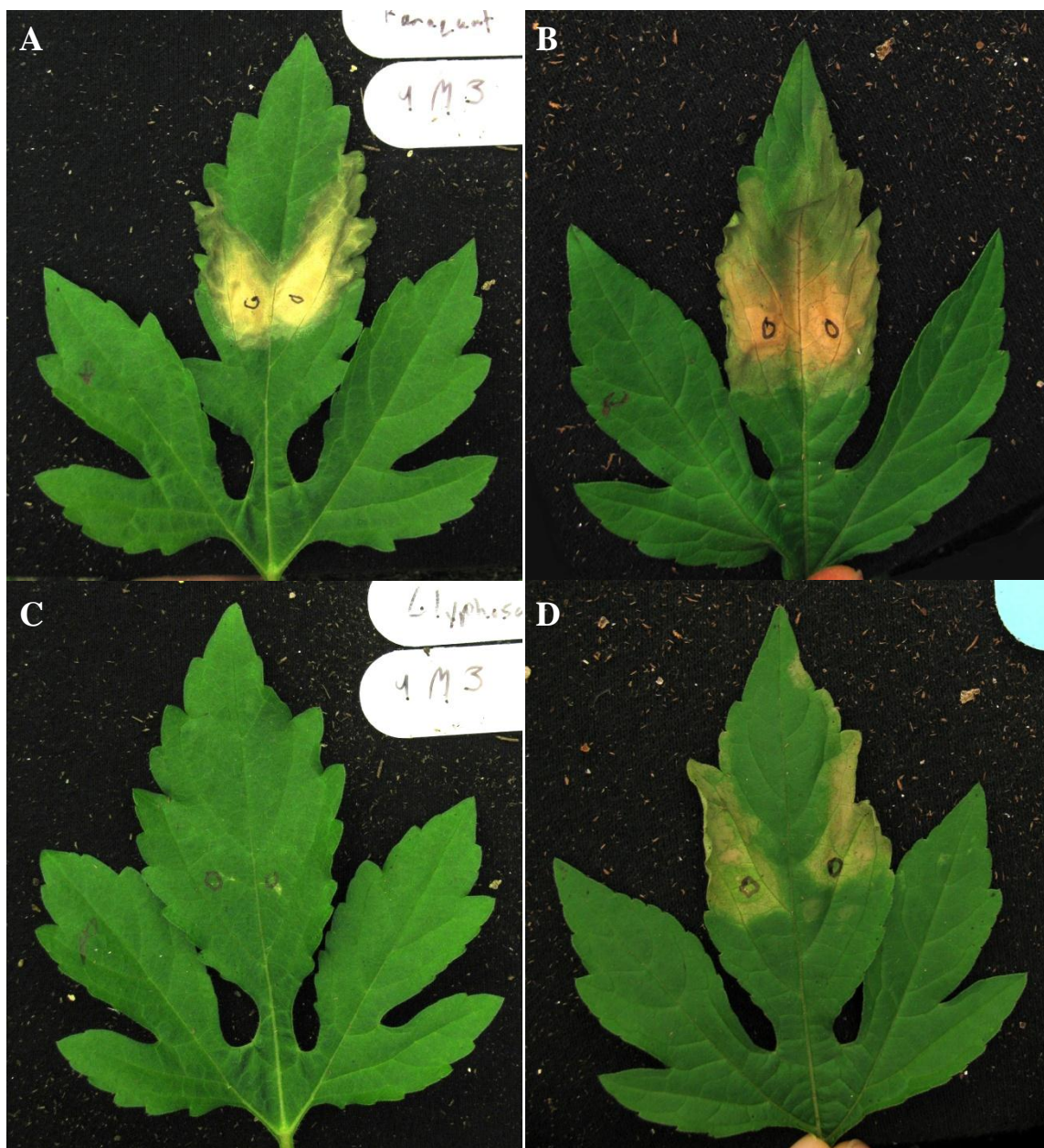
### **Necrosis Pattern of Spot Applied Sublethal Glyphosate**

After 6 hours after treatment (HAT), chlorosis started to appear on two thirds of the S1 and R1 leaves that were treated with paraquat and a fifth of the R1 leaves treated with 14 mM glyphosate. At 12 HAT, chlorotic and necrotic patches appeared on all of the S1 and R1 leaves treated with paraquat and half of the R1 leaves treated with 14 mM glyphosate and a third of the R1 leaves treated with 4 mM glyphosate. At 24 and 48 HAT 75% of the leaves treated with 14 mM glyphosate had necrotic spots. Leaves that had glyphosate spot applied to the most proximal area of the leaf did not consistently show necrosis unlike the mid and distal spot applications. No symptomology appeared on the S1 leaves that were treated with glyphosate.

The pattern of the spread of necrosis from 14 mM glyphosate on the R1 plants was similar to that of paraquat where the pattern of necrosis indicated acropetal movement of the herbicide. When glyphosate was applied in the mid area of the leaf, the majority of necrotic tissue spread in the direction away from the midvein, acropetally, towards the edge of the leaf (Figure 5). When glyphosate was applied to the base it stayed localized or spread along the edge of the base (Figure 6). When it was applied to the distal area of the leaf, the necrosis spread distally and along the edge of the leaf proximally (Figure 7).

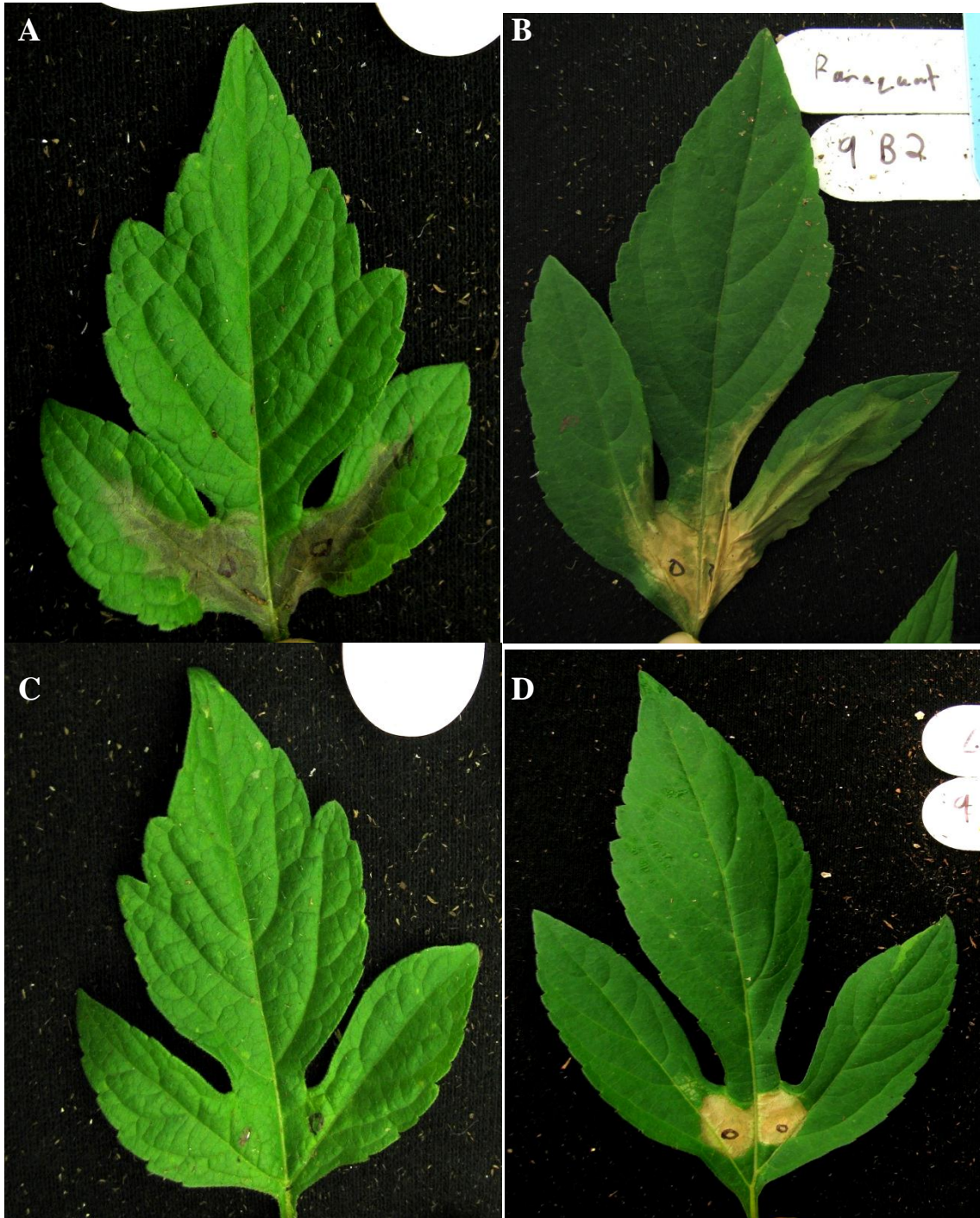
With the 4 mM glyphosate concentration on R1 the necrosis stayed localized to the spot in which it was applied if necrosis appeared. Necrosis only appeared on R1 leaves with the mid and distal area applications at this concentration in the second run (Figure 8).





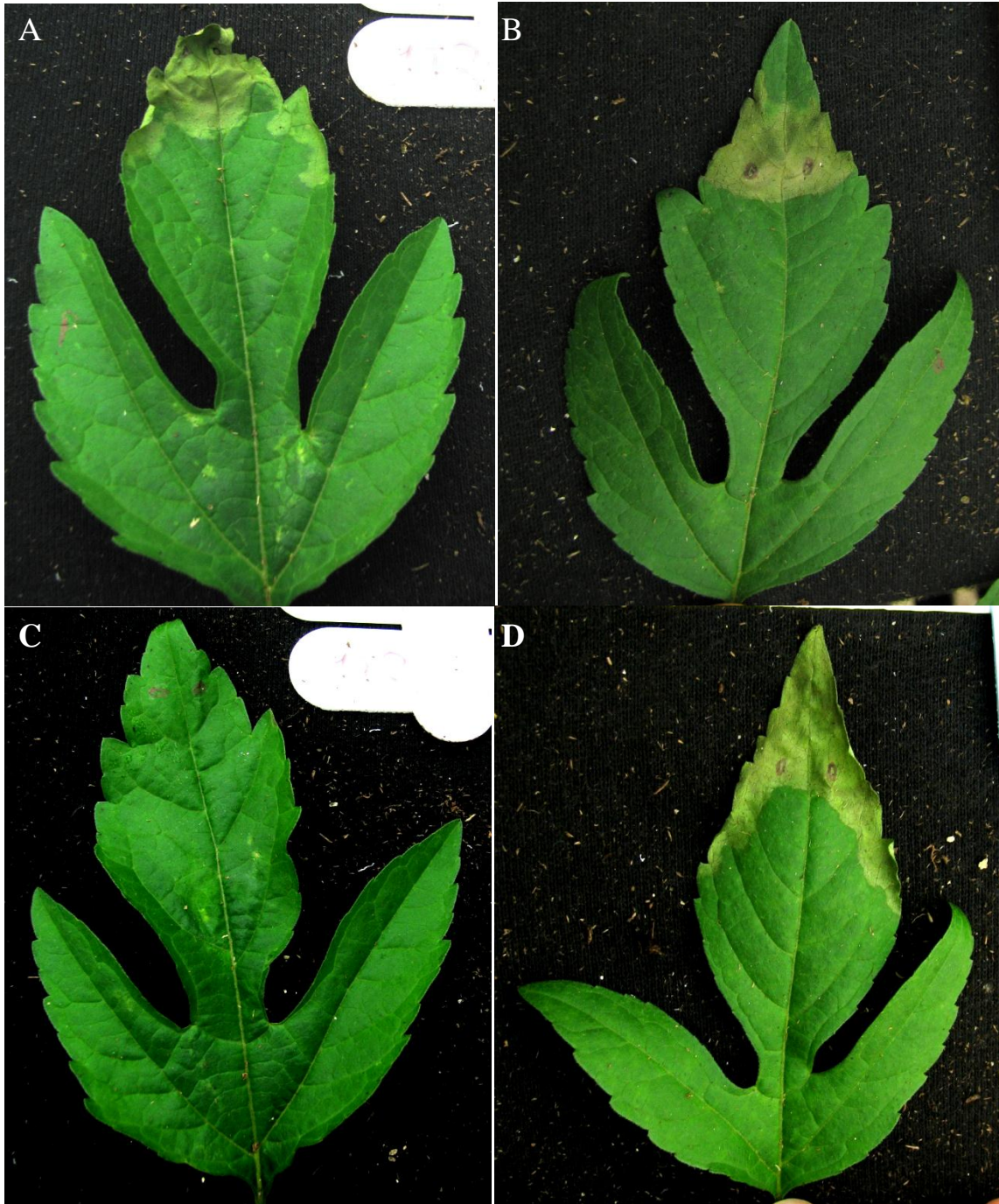
**Figure 5.** Necrosis pattern from application of two one  $\mu\text{L}$  droplets of paraquat (4 mM) (A and B) and glyphosate (14 mM) (C and D) on the mid area of the leaves at the second node of S1 (A and C) and R1 (B and D) giant ragweed plants. Photographs were taken at 24 hours after application.



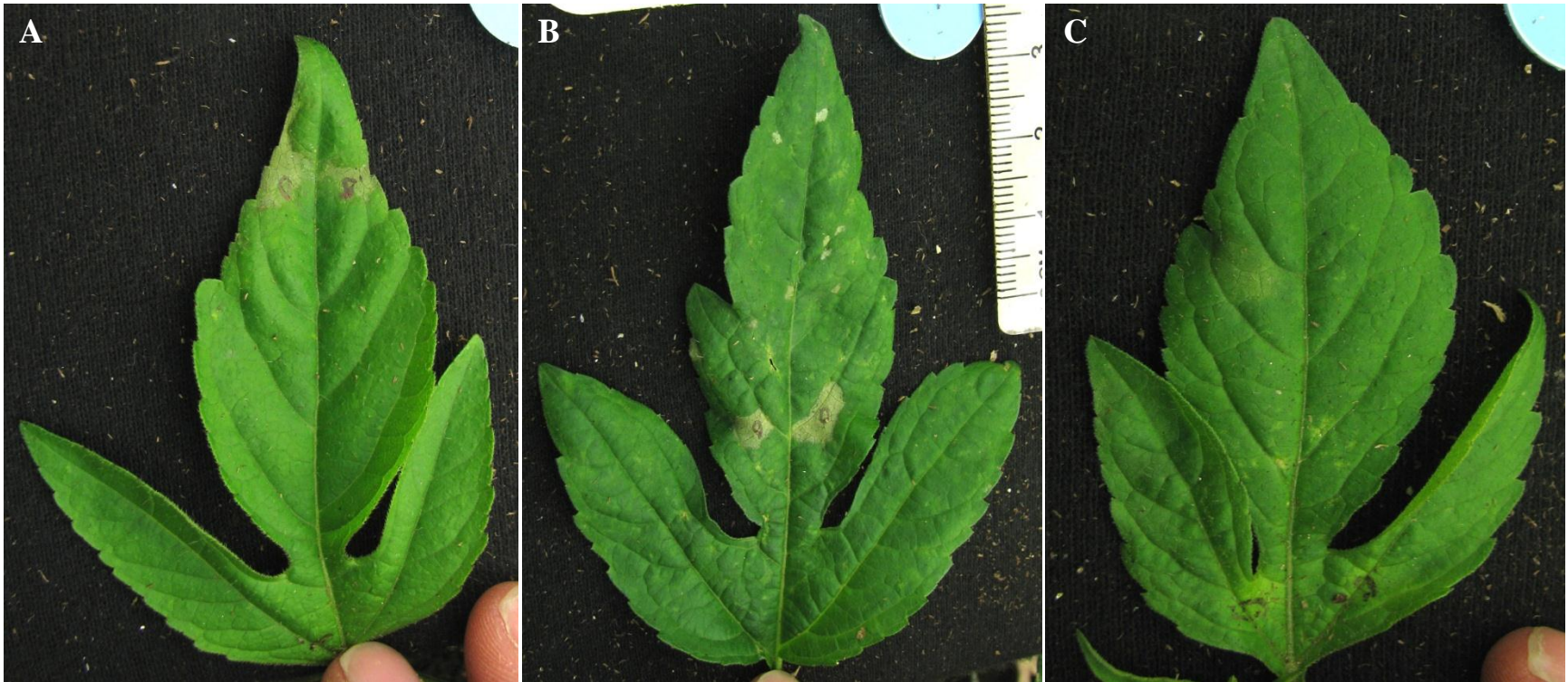


**Figure 6.** Necrosis pattern from application of two one  $\mu\text{L}$  droplets of paraquat (4 mM) (A and B) and glyphosate (14 mM) (C and D) on the proximal area of the leaves at the second node of S1 (A and C) and R1 (B and D) giant ragweed plants. Photographs were taken at 24 hours after application.





**Figure 7.** Necrosis pattern from application of two one  $\mu\text{L}$  droplets of paraquat (4 mM) (A and B) and glyphosate (14 mM) (C and D) on the distal area of the leaves at the second node of S1 (A and C) and R1 (B and D) giant ragweed plants. Photographs were taken at 24 hours after application.



**Figure 8.** Necrosis pattern from application of two one  $\mu\text{L}$  droplets of glyphosate at a 4 mM concentration on the distal (A), mid (B) and proximal (C) area of the leaves at the second node of R1 giant ragweed plants. Photographs were taken at 24 hours after application.

## Absorption and Translocation

About 50 to 65% of [ $^{14}\text{C}$ ]glyphosate was absorbed in the treated leaves of R1 and S at each sampling time. Differences between S and R1 were minimal, except at 96 HAT, where 17% more glyphosate was absorbed in R1 leaves (significant at  $p < 0.05$ ). In the S1 population, there was no increase in glyphosate absorption after 24 HAT. In contrast, in the R1 population there was an increase in glyphosate absorption to 65% at 96 HAT.

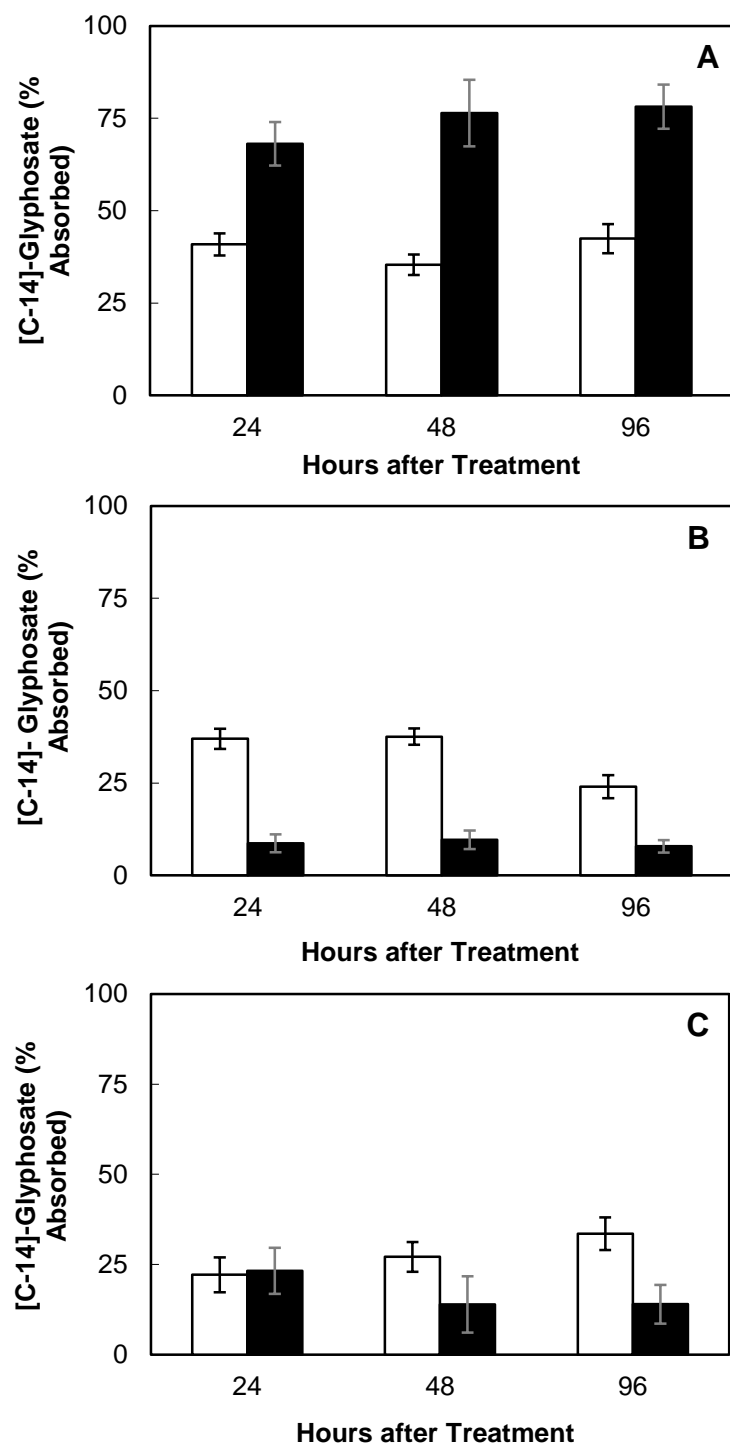
**Table 1.** Absorption of  $^{14}\text{C}$  glyphosate in treated leaves of S1 and R1 populations

| Time (h) | Absorption (%) |          |
|----------|----------------|----------|
|          | S              | R1       |
| 24       | 52 (3.2)       | 53 (4.5) |
| 48       | 51 (2.3)       | 55 (4.3) |
| 96       | 48 (3.6)       | 65 (3.9) |

Each data point is the average of 10 observations for S and 9 observations for R over two replications in time. Figures in parenthesis are standard errors of the mean.

There were differences found in the amount of glyphosate translocated in the R1 population compared to the S1 population. More [ $^{14}\text{C}$ ]glyphosate remained in the treated and opposite leaf of the R1 population than in the S1 population at 24, 48 and 96 HAT (Figure 9A). There was less [ $^{14}\text{C}$ ]glyphosate detected in the roots and stem below the treated leaf in the R1 population compared to the S1 population at every sampling time (Figure 9B). The amount of [ $^{14}\text{C}$ ]glyphosate translocated to the area of the plant above the treated leaf in the R1 population was similar to the S1 population at 24 HAT, however there was less glyphosate above the treated leaf at 48 and 96 HAT in the R1 population compared to the S1 population (Figure 9C). These results show that there was a reduction in translocation to the sink tissues above and below the treated leaf in the R1 population in comparison to the S1 population.





**Figure 9.** Distribution of [ $^{14}\text{C}$ ]glyphosate expressed as percent absorbed [ $^{14}\text{C}$ ]glyphosate in the treated leaf and the opposite leaf (A), the section of the plant below the treated leaf including the roots (B) and the section of the plant above the treated leaf (C) of R1■ and S1□ *A. trifida* over time. Each bar represents the mean of eight observation for R1 at 24 and 96 HAT; seven observations for R1 at 48 HAT; and ten observations for S1 at 24, 48 and 96 HAT. Error bars represent  $\pm$  SEM

## DISCUSSION

The objectives of this thesis were to confirm glyphosate resistance in two populations of giant ragweed and also to determine the mechanistic basis for resistance. The results of the experiments indicate that the two populations are indeed resistant to this herbicide. In addition, it does not appear that a modified target site is involved. Furthermore, resistance in the rapid necrosing population (R1) is likely due to impaired translocation.

Dose response experiments showed differences between R1 and R2 compared to S1 and S2 following glyphosate application. The resistance index (RI) values calculated with survival and biomass data were similar for R1 and R2. These RI values ranged between 3.8 and 19 which is comparable to RI values reported for other GR species. For example, RI values of 6 to 8 were documented in Palmer amaranth, 5.6 to 13 in Italian and rigid ryegrass, and 8 to 13 fold in Canada fleabane (Bostamam et al. 2012; Culpepper et al. 2006; Salas et al. 2012; VanGessel 2001). Based on this research, resistance was confirmed in two giant ragweed populations exhibiting contrasting phenotypes and their RI values were similar to other GR weeds.

Since the two different giant ragweed populations have different response to glyphosate it suggests that they have different resistance mechanisms. Although the RI values are similar for the R1 and R2 populations, this does not imply that they have the same mechanism of resistance. The various species listed above have similar RI values, but they are resistant due to different mechanisms of resistance (gene amplification, reduced translocation, target site mutation or rapid vacuolar sequestration) (Bostamam et al. 2012; Gaines et al. 2011; Ge et al. 2010; Salas et al. 2012). Therefore, it is likely that R1 and R2 also have different physiological base for resistance as indicated by their phenotypic response, and the fact they have similar RI values is coincidental.

While the LD<sub>50</sub> values were identical for R1 and R2, the GR<sub>50</sub> value for R1 was higher than that of R2 (Figures 1 and 2). This suggests that the mechanism of resistance of R1 results in more rapid recovery and growth after glyphosate application compared to R2. With the R2 population, although they survive the application of glyphosate they do not recover and resume growth as quickly as R1. It was observed that growth resumed more quickly in R1 than R2 following treatment.

Herbicide symptomology exhibited by the R1 plants was dependent on glyphosate dose. When sublethal doses of glyphosate (< 900 g a.e. ha<sup>-1</sup>) were applied, it was observed that the entire mature leaf of R1 plants would not become necrotic as they would with lethal doses; instead there would be localized spots of necrosis (data not shown). In addition, no chlorosis would appear in the developing leaves. This would suggest that there is little translocation of glyphosate in the treated leaf and no (or little) translocation to the growing point at these doses. However at higher doses (> 900 g a.e. ha<sup>-1</sup>) there was often chlorosis of the young leaves suggesting that a portion of the glyphosate moved out of the treated leaves. Some glyphosate appears to be able to translocate to the growing points and this would explain increased mortality and biomass reduction at higher doses.

Shikimate accumulated in leaf discs excised from leaves of R1 and R2 populations and this was light and dose dependent (Figures 3 and 4). The leaf disc based shikimate test is an indirect determination of glyphosate resistance that may or may not indicate the presence of an altered EPSPS target site. For shikimate to accumulate under the conditions of this test, glyphosate needs to enter the leaf tissues, the cells and ultimately the chloroplasts in order to inhibit EPSPS. The fact that shikimate accumulated in R1 and R2 shows that the herbicide is able to enter the chloroplasts and inhibit EPSPS. This suggests the presence, in these plants, of



an EPSPS that can bind glyphosate. In contrast, leaf discs from GR soybean did not accumulate any shikimate (Figure 3) as a result of possessing a resistant EPSPS. Shikimate accumulated in R1 and R2 at levels less than in leaf discs from the S1 population (Figures 3 and 4). This could mean two things: the presence of an altered EPSPS, but with a reduced level of resistance compared to other R EPSPS, or the presence of a mechanism preventing glyphosate from effectively entering the cells or the chloroplasts.

A possible mechanism that could be preventing glyphosate from effectively entering the cells would be an absent or less effective phosphate transporter. This phosphate transporter is believed to actively transport glyphosate across the cell membrane at low concentrations while at high concentrations glyphosate enters the cell via passive flow (Denis and Delrot 1993; Hetherington et al. 1998; Morin et al. 1997; Shaner 2009). This absent or less effective phosphate transporter could be the explanation for intermediate levels of shikimate accumulation found in the discs from young apical tissue of the R1 and R2 populations incubated in the light (Figure 3B). Shikimate also did not accumulate in the resistant plants until 100  $\mu$ M concentration in the shikimate accumulation dose response experiment whereas the S1 population began to accumulate shikimate at 10  $\mu$ M (Figure 4). This could also explain the yellowing of the young leaves at high doses observed in the dose response experiment. With an absent or less effective phosphate transporter, glyphosate is not taken up by the cells at lower doses and therefore not loaded into the phloem, but at high concentrations, glyphosate is able to be taken-up by cells by passive flow and translocated symplastically.

The light dependence of shikimate accumulation in leaf discs has been previously documented by others (Amrhein et al. 1980; Shaner et al. 2005). With giant ragweed and the susceptible cultivar of soybean, there was always more shikimate accumulation under light than

in the dark, and this is consistent with the fact that the reaction catalyzed by EPSPS is dependent on photosynthesis. In an attempt to circumvent the lack of photosynthesis in the dark, sucrose was added to the solution as a source of carbon. However, this did not allow shikimate accumulation to occur at levels comparable to those observed under light. The only exception was with leaf discs from mature leaves from the S1 population, which accumulated shikimate when incubated with glyphosate (Figure 3A). This, however, occurred at levels that were five to seven times less than under light.

Testing shikimate accumulation under light and dark conditions was done to ascertain whether the light dependent rapid necrosing reaction associated with the R1 population could affect the ability of the assay to determine response to glyphosate. It was thought that, if necrosis occurred rapidly in leaf discs incubated with glyphosate, this would cause cell death and would therefore prevent shikimate accumulation. This could have lead to the erroneous conclusion that the EPSPS from R1 was resistant to the herbicide. The results were opposite to what was initially thought, with accumulation of shikimate higher under the light than in the dark (Figure 3). This shows that under the condition of the assay, glyphosate did not trigger the fast necrosing reaction.

In the light, leaf discs from young leaves of all giant ragweed populations accumulated more shikimate than corresponding mature leaves (Figures 3B and 3D). This is consistent with what others have reported and is likely due to the higher activity of EPSPS in developing tissues where there is a high demand for amino acids. Because of the higher level of activity in younger leaves, differences in response to glyphosate between S1 and the two R populations were observed while they were not apparent with mature leaves. Clearly, the age of the plant parts being tested could have an influence on the results and their interpretation.

It is believed that the rapid necrosing reaction prevents most of the glyphosate that is absorbed in the mature treated leaves to move to the young leaves at the apex and to other metabolic sinks. Results from radiolabeled glyphosate experiments showed that 70 to 117% more herbicide remained in the mature treated leaves of R1 compared to S1 (Figure 9). There was a corresponding decrease in the amount found in tissues above and below treated leaves. This suggests that a reduction in glyphosate translocation may contribute to resistance in the rapid necrosing population.

Work with non-labeled glyphosate, using the phenotypic reaction as a marker, shows that the damage following single droplet application was visible within 24 hours (Figures 5 to 8). In addition, while there appeared to be herbicide diffusion around the point of application, the damage was predominantly in an acropetal direction. Assuming that the damage is a result of the presence of the herbicide in the affected tissues, this suggests that glyphosate in the mature leaves, moves in the xylem. Similar pattern of damage with paraquat was observed in this study (Figure 5, 6, and 7). Paraquat tends to have localized movement in the xylem and exhibits similar acropetal distribution (Slade and Bell 1966; Smith and Davies 1965). It is possible that glyphosate, in the fast necrosing biotype, behaves in a way similar to paraquat as it moves mostly acropetally by remaining in the xylem.

There could be two mechanisms restricting translocation of glyphosate out of the mature treated leaf in rapid necrosing plants: rapid necrosis and a limited entry in the phloem. The rapid necrosing reaction, by killing cells, causes leaves to become photosynthetically non-functional. This prevents production of photosynthates and therefore their export, as well as that of glyphosate, out of these leaves. The other mechanism that would prevent glyphosate from being exported to the sinks would be due to its limited entry in phloem cells. This means that

glyphosate remains in the xylem forcing it to move according to transpiration flow in an acropetal direction. This flow of solutes in the xylem towards the tip of the leaves thus carries glyphosate further away from sinks. These two combined mechanisms would prevent most of the glyphosate from reaching metabolic sinks, hence ensuring continuous growth of the R1 plants after glyphosate application.

What was found when measuring the absorption of [ $^{14}\text{C}$ ]glyphosate was that there was similar absorption levels of [ $^{14}\text{C}$ ]glyphosate with the R1 and S1 populations at 24 and 48 hours and greater absorption with the R1 population than the S1 population at 96 hours. Thus it can be concluded that reduced absorption is not a mechanisms of resistance for these two populations.

### **Contributions of this research**

This research is the first to conclusively establish the level of resistance in two phenotypically different populations of giant ragweed and to confirm the mechanism of resistance is not due to a resistant target site. This study documented that there is reduced uptake of glyphosate at the cellular level and this may impact translocation of glyphosate in both populations. In addition, in the rapid necrosing population, there is reduced translocation from the treated leaves.

Overall this research sets the foundation for further investigation into the mechanism of resistance in giant ragweed populations of Ontario. It also helps gain a better understanding of glyphosate resistance and may assist in determining the mechanism of resistance in future glyphosate resistant weeds.

### **Limitations**

One of the main constraints in these studies was the limited sample size. Sample size was

limited because of growth room space limitations. If a greater number of plants were sampled the standard error could have been decreased.

In the shikimate assay experiments, limitations include using an *in vivo* shikimate assay versus a direct EPSPS enzyme assay. With measuring the target site's sensitivity, the most direct and accurate assay would have been to do a direct EPSPS enzyme assay, however this demands the production of shikimate-3-phosphate which requires sophisticated biochemistry equipment, which was not available (Shaner et al. 2005). This experiment was limited to the assumption that there was no limitation in cellular uptake in the resistant giant ragweed plants which was unknown at the time of the experiment. Even with the limitations of this assay there was an accumulation of shikimate which indicated that the target site is sensitive in the R1 and R2 populations.

For the radioactive absorption and translocation experiment, the limitations include the harvest times, the number of dissections and overspraying with “cold” glyphosate. Earlier harvest times of 6 and 12h would have been helpful to determine if there are differences in the rate of absorption between the R1 and S1 populations. These earlier harvest times would have been useful in documenting the translocation pattern, especially to determine if more glyphosate did translocate to the roots and then was exported back to the treated leaf through the xylem in the 0 to 24 h period. Cutting the treated leaf into three parts: area treated with radioactive glyphosate, area distal from this area and area proximal from this area, would assist in documenting glyphosate translocation in the treated leaves of the S1 and R1 populations. Overspraying with “cold” glyphosate has its limitations on translocation it has been debated that overspraying with “cold” glyphosate will cause a self limitation in translocation of the radioactive glyphosate due to toxicity of the oversprayed “cold” glyphosate (Dill et al. 2010).

Overall, additional harvest times and a greater number of dissections could contribute to a better understanding of the absorption and translocation of glyphosate in the R1 populations.

### **Future directions**

This study has provided initial insights into the mechanism of glyphosate resistance in the R1 and R2 giant ragweed populations. Future research into the mechanism can have a narrower focus because of these results. The areas that need to be studied further include gene amplification, reduced translocation, target site exclusion on a cellular level and the cause of the rapid necrosis.

In regards to translocation, the R2 population was not investigated in this study. It would be useful to determine if there is reduced translocation in this biotype. From this study's results there was found to be a difference in translocation in the R1 population; further research is needed to study translocation within the leaf by cutting the leaf into three sections: the treated area of the leaf, the section of the leaf distal to the treated area and the section of the leaf proximal to the treated area. To test out the explanation that glyphosate may be being exported out of the sink tissues into the xylem of the R1 population, [ $^{14}\text{C}$ ]glyphosate would need to be applied only to the meristematic apical shoot in an experiment similar to the one performed in this study. Autoradiography of [ $^{14}\text{C}$ ]glyphosate applied to a source and sink leaf would also be useful in documenting the translocation pattern of glyphosate visually in both R1 and R2 populations. This research would provide greater understanding of the translocation pattern of glyphosate in the R1 and R2 populations.

The mechanism causing the intermediate levels of shikimate accumulation in both the R1 and R2 giant ragweed population also needs to be studied further. A possible explanation is that

there could be a target site exclusion mechanism on the cellular level that could be caused by reduced cell uptake, chloroplast exclusion or vacuolar sequestration.

The explanation for rapid necrosis in the R1 population needs to be further researched. It is stated by Hess (2000) that “events that cause tissue damage (necrosis) are not associated with the primary target and are always due to membrane damage caused by lipid peroxidation of polyunsaturated fatty acids”. The symptomology from glyphosate application in the R1 population appears to be very similar to that of paraquat and both are light dependent. The necrosis that occurs after paraquat treatment is due to hydroxyl radicals causing membrane degradation through lipid peroxidation of polyunsaturated fatty acids (Hess 2000). Possibly the rapid necrosis observed in the R1 population after glyphosate application is due to free radical damage. This would be an exciting area for future research.

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**APPENDIX 1: Parameters of Dose Response of Resistant and Susceptible Giant Ragweed to Glyphosate applied at two- to three- node stage**

**Table 2. Parameters of Figure 1. Effect of glyphosate on the survival of giant ragweed populations.**

| Population | D             | C           | <i>b</i>       | I <sub>50</sub>   |
|------------|---------------|-------------|----------------|-------------------|
| R1         | 100 (95, 105) | 0 (-12, 12) | 1.9 (1.4, 2.4) | 4500 (3400, 5500) |
| R2         | 96 (90, 102)  | 0 (-12, 12) | 1.9 (1.4, 2.4) | 5600 (4000, 7200) |
| S1         | 103 (98, 109) | 0 (-12, 12) | 1.9 (1.4, 2.4) | 290 (210, 360)    |
| S2         | 101 (95, 107) | 0 (-12, 12) | 1.9 (1.4, 2.4) | 600 (400, 800)    |

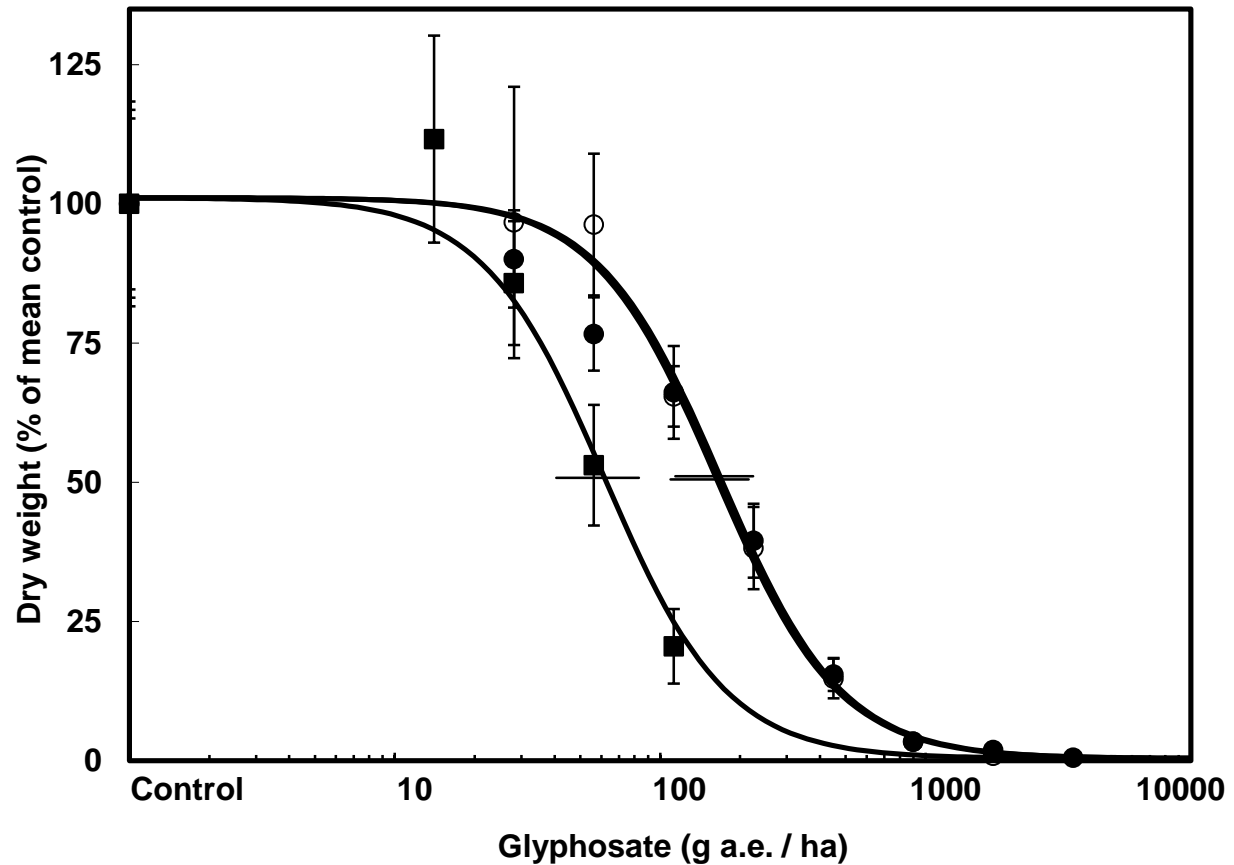
Figures in parenthesis represent the 95% confidence interval

**Table 3. Parameters of Figure 2. Effect of glyphosate on the above ground dry biomass of giant ragweed populations**

| Population | D            | C         | <i>b</i>          | I <sub>50</sub> |
|------------|--------------|-----------|-------------------|-----------------|
| R1         | 99 (94, 103) | 1 (-2, 5) | 1.25 (1.06, 1.45) | 320 (260, 380)  |
| R2         | 99 (94, 103) | 1 (-2, 5) | 1.25 (1.06, 1.45) | 200 (150, 240)  |
| S1         | 99 (94, 103) | 1 (-2, 5) | 1.25 (1.06, 1.45) | 46 (38, 55)     |
| S2         | 99 (94, 103) | 1 (-2, 5) | 1.25 (1.06, 1.45) | 52 (41, 64)     |

Figures in parenthesis represent the 95% confidence interval

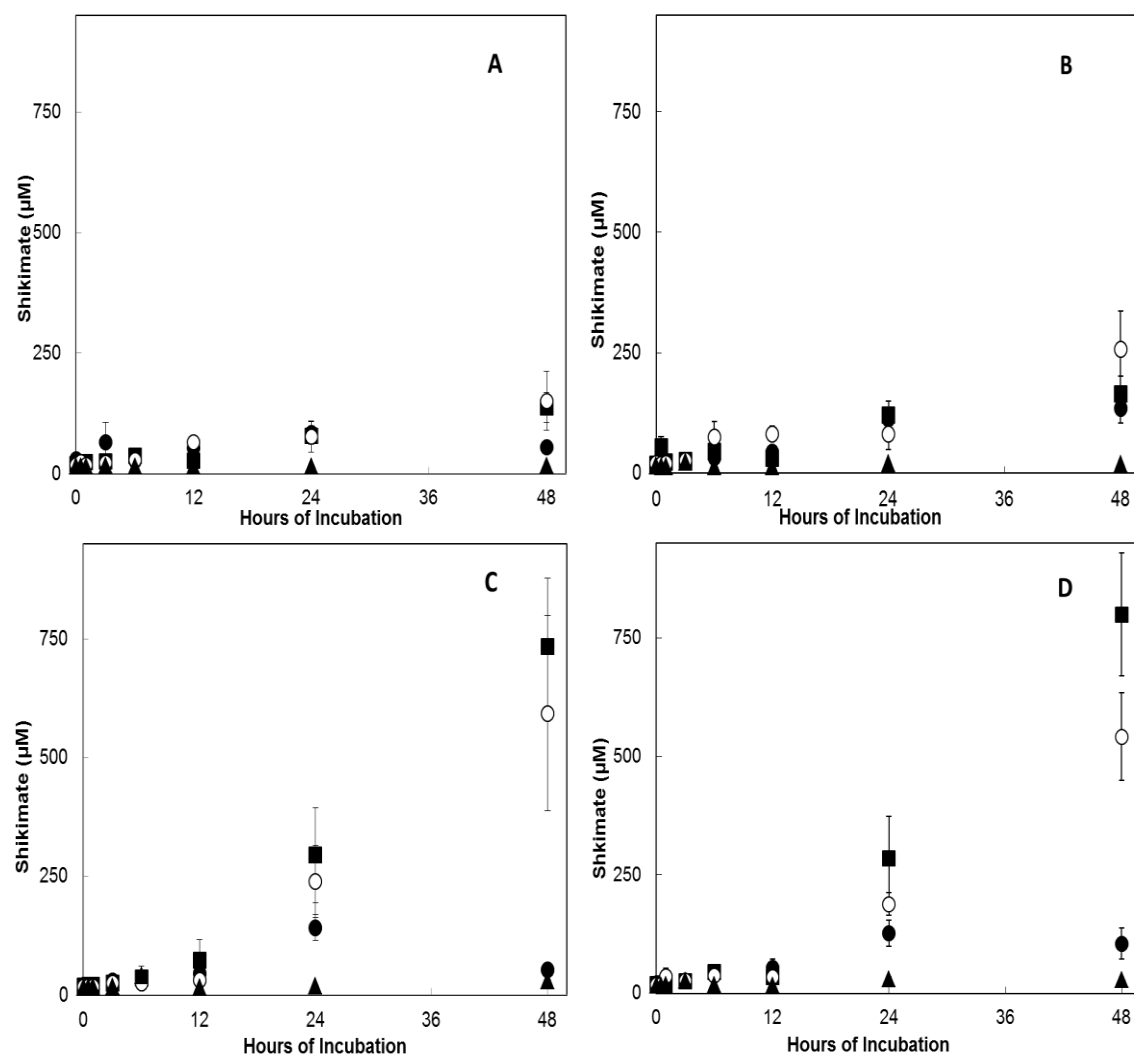
**APPENDIX 2: Dose Response of Resistant and Susceptible Giant Ragweed to Glyphosate applied at one- to two-node stage**



**Figure 10. Effect of glyphosate on the above ground dry biomass of resistant giant ragweed populations R1 (●), R2 (○) and susceptible populations S1 (■) and S2 (□) at one- to two-node stage.**

Each point represents the average of two experiments with 5 replicates each. Vertical error bars represent the SEM (n= 10) Dose–response curves were generated by non-linear regression using a log-logistic model with the following equations: R1,  $y=(101/(1+[x/160]^{1.9}))$ ; R2,  $y=(99/(1+[x/170]^{1.9}))$ ; S1,  $y=(99/(1+[x/60]^{1.9}))$ ; where  $y$  is the survival and  $x$  is the glyphosate dose. Horizontal error bars represent the 95% confidence intervals at  $GR_{50}$ .

### APPENDIX 3: Time Course Experiment of Shikimate Accumulation



**Figure 11.** Shikimate accumulation over time in excised leaf discs from glyphosate resistant (R1 (●) and R2 (○)) and susceptible (S1 (■)) *A. trifida* and glyphosate resistant soybeans (▲) incubated in glyphosate solution under continuous light. Leaf discs were excised from young apical leaves (A and B) or mature leaves (C and D). Glyphosate concentration was 250 μM (A and C) or 500 μM (B and D). Mature leaves were at the second node in *A. trifida* and the unifoliate in soybeans. Each data point represents the mean  $\pm$  the SE of 3 experiments with 5 replicates each.

## APPENDIX 4: SAS Code to Analyze Dose Response Curves

```
title 'Dose response to glyphosate using populations 003,005, 054, 006';
Data first;
input rep pop$ block rate wt;
  if rate>0 then lograte=log(rate);
else lograte=log(0.036621094/9);
cards;

;
proc print; run;
proc univariate; var wt;
run;
proc glm data=first;
class rate rep pop block;
model wt=rate rep pop block rate*rep rate*pop rate*block rep*pop rep*block
pop*block rate*pop*block/ ss4;
run;
title3'seperate slopes Cs and Ds';

proc nlin data=first;
parameters d_ResA=100 d_ResB=100 d_SusA=100 d_SusB=100 c_ResA=0 c_ResB=0
c_SusA=0 c_SusB=0
ec50_ResA=200 ec50_ResB=200 ec50_SusA=50 ec50_SusB=50 B_ResA=1.2
  B_ResB=1.2
  B_SusA=1.2
  B_SusB=1.2;

  if rate = 0 and pop='ResA' then predict=d_ResA;
if rate = 0 and pop='ResB' then predict=d_ResB;
if rate = 0 and pop='SusA' then predict=d_SusA;
if rate = 0 and pop='SusB' then predict=d_SusB;
  else if pop = 'ResA' then predict = c_ResA + (d_ResA-c_ResA)/(1+
exp(b_ResA*(lograte-log(ec50_ResA))));
  else if pop= 'ResB' then predict= c_ResB + (d_ResB-c_ResB)/(1+
exp(b_ResB*(lograte-log(ec50_ResB))));
  else if pop= 'SusA' then predict= c_SusA + (d_SusA-c_SusA)/(1+
exp(b_SusA*(lograte-log(ec50_SusA))));
  else if pop='SusB' then predict= c_SusB + (d_SusB-c_SusB)/(1+
exp(b_SusB*(lograte-log(ec50_SusB))));

model wt=predict;
run;

title3'same D';

proc nlin data=first;
parameters d=100 c_ResA=0 c_ResB=0 c_SusA=0 c_SusB=0
ec50_ResA=200 ec50_ResB=200 ec50_SusA=50 ec50_SusB=50 B_ResA=1.2
  B_ResB=1.2
  B_SusA=1.2
  B_SusB=1.2;
```

```

    if rate = 0 then predict=d;
    else if pop = 'ResA' then predict = c_ResA + (d-c_ResA)/(1+
exp(b_ResA*(lograte-log(ec50_ResA))));
    else if pop= 'ResB' then predict= c_ResB + (d-c_ResB)/(1+
exp(b_ResB*(lograte-log(ec50_ResB))));
    else if pop= 'SusA' then predict= c_SusA + (d-c_SusA)/(1+
exp(b_SusA*(lograte-log(ec50_SusA))));
    else if pop='SusB' then predict= c_SusB + (d-c_SusB)/(1+ exp(b_SusB*(lograte-
log(ec50_SusB))));

model wt=predict;
run;
title3'same C';

```

```

proc nlin data=first;
parameters d_ResA=100 d_ResB=100 d_SusA=100 d_SusB=100 c=0
ec50_ResA=200 ec50_ResB=200 ec50_SusA=50 ec50_SusB=50 B_ResA=1.2
    B_ResB=1.2
    B_SusA=1.2
    B_SusB=1.2;

```

```

    if rate = 0 and pop='ResA' then predict=d_ResA;
    if rate = 0 and pop='ResB' then predict=d_ResB;
    if rate = 0 and pop='SusA' then predict=d_SusA;
    if rate = 0 and pop='SusB' then predict=d_SusB;
    else if pop = 'ResA' then predict = c + (d_ResA-c)/(1+ exp(b_ResA*(lograte-
log(ec50_ResA))));
    else if pop= 'ResB' then predict= c + (d_ResB-c)/(1+ exp(b_ResB*(lograte-
log(ec50_ResB))));
    else if pop= 'SusA' then predict= c + (d_SusA-c)/(1+ exp(b_SusA*(lograte-
log(ec50_SusA))));
    else if pop='SusB' then predict= c + (d_SusB-c)/(1+ exp(b_SusB*(lograte-
log(ec50_SusB))));

```

```

model wt=predict;
run;

```

```

title3'same slopes';

```

```

proc nlin data=first;
parameters d_ResA=100 d_ResB=100 d_SusA=100 d_SusB=100 c_ResA=0 c_ResB=0
c_SusA=0 c_SusB=0
ec50_ResA=200 ec50_ResB=200 ec50_SusA=50 ec50_SusB=50 B=1.2
;

```

```

    if rate = 0 and pop='ResA' then predict=d_ResA;
    if rate = 0 and pop='ResB' then predict=d_ResB;
    if rate = 0 and pop='SusA' then predict=d_SusA;
    if rate = 0 and pop='SusB' then predict=d_SusB;
    else if pop = 'ResA' then predict = c_ResA + (d_ResA-c_ResA)/(1+
exp(b*(lograte-log(ec50_ResA))));
    else if pop= 'ResB' then predict= c_ResB + (d_ResB-c_ResB)/(1+
exp(b*(lograte-log(ec50_ResB))));
    else if pop= 'SusA' then predict= c_SusA + (d_SusA-c_SusA)/(1+
exp(b*(lograte-log(ec50_SusA))));
    else if pop='SusB' then predict= c_SusB + (d_SusB-c_SusB)/(1+ exp(b*(lograte-
log(ec50_SusB))));

```



```

model wt=predict;
run;
title3'same slope and C& D';
proc nlin data=first;
parameters d=100 c=0
ec50_ResA=200 ec50_ResB=200 ec50_SusA=50 ec50_SusB=50 B=1.2
;

if rate = 0 then predict=d;

else if pop ='ResA' then predict = c + (d-c)/(1+ exp(b*(lograte-
log(ec50_ResA))));
else if pop= 'ResB' then predict= c + (d-c)/(1+ exp(b*(lograte-
log(ec50_ResB))));
else if pop= 'SusA' then predict= c + (d-c)/(1+ exp(b*(lograte-
log(ec50_SusA))));
else if pop='SusB' then predict= c + (d-c)/(1+ exp(b*(lograte-
log(ec50_SusB))));

model wt=predict;
run;

```