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# The influence of ambient environment on the growth and fitness of glyphosate-resistant and -susceptible giant ragweed (Ambrosia trifida L.) biotypes

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

# THE INFLUENCE OF ENVIRONMENT AND SPRAY DOSE ON THE GROWTH AND FITNESS OF GLYPHOSATE-RESISTANT AND -SUSCEPTIBLE GIANT RAGWEED (*AMBROSIA TRIFIDA* L.) BIOTYPES

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## ABSTRACT

Glyphosate-resistant giant ragweed can be found in agricultural fields throughout the mid-western United States and southwestern Ontario. Environmental factors can influence growth and herbicide efficacy in $C_3$ plant species. I measured the aboveground injury to resistant and susceptible seedlings for 28 d following glyphosate treatment to test the hypothesis that young leaf stages would be more susceptible to glyphosate under warm, dry, low-$CO_2$ conditions. Glyphosate-resistance was not affected by environmental factors, leaf stage, or glyphosate dose, but plants grown at the highest temperature after spray had the least glyphosate injury. Resistant alleles may be associated with fitness penalties because they are rare in populations without herbicide selection pressures; however, in a greenhouse trial, resistant biotypes recovered from glyphosate injury and produced seeds. My results suggest that potentially stressful growth conditions and treatment at young growth stages will not improve the control of resistant giant ragweed biotypes.



**Key Words**: giant ragweed, glyphosate, resistance, leaf stage, fitness, $CO_2$, temperature, drought, seed germination, herbicide injury

## CO-AUTHORSHIP STATEMENT

Drs. Robert Nurse and Hugh Henry will be co-authors on any published manuscript(s) arising from the content of this thesis. Dr. Jeff Stachler will be an additional co-author on any manuscript(s) that contain results for the Ohio biotype.

## DEDICATION

I dedicate this thesis to my super granny Sarah Thompson, who was a fierce competitor and the “strongest” and most determined woman I know.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| ABA | abscisic acid |
| ae | acid equivalent |
| ALS | acetolactate synthase |
| ATP | adenosine triphosphate |
| DAHP | 3-deoxy-D-*arabino*-heptulosonate 7-phosphate |
| DAP | days after planting |
| DAT | days after treatment |
| DHQ | 3-dehydroquinate dehydratase |
| EPSP | 5-enolpyruvylshikimate-3-phosphate |
| EPSPS | 5-enolpyruvylshikimate-3-phosphate synthase |
| $F'_m$ | maximum fluorescence in the light |
| $F_m$ | maximum fluorescence |
| $F_o$ | zero fluorescence |
| MCPA | 2-methyl-4-chlorophenoxyacetic acid |
| NPQ | non-photochemical quenching |
| $P_i$ | inorganic phosphate |
| PEP | phosphoenolpyruvate |
| PSII | photosystem II |
| $PQH_2$ | reduced plastoquinone |
| RuBP | ribulose-1,5-bisphosphate |
| TZ | tetrazolium |
| ybp | years before present |

# CHAPTER 1

# General Introduction

## 1.0 Weed management

Growers aim to manage weeds in agro-ecosystems to minimize crop yield losses that occur as a result of competition with weeds. A weed is defined as a vascular plant that grows in an area where it is not wanted (Rao 2000). In Ontario, crop losses that result from competition with weeds can exceed $159 million (Swanton et al. 1993). In general, crop species are most vulnerable to competition with weeds during early growth stages, but the specific weed-free requirement varies between fields and crops (Hall et al. 1992). Weed exclusion strategies such as hand removal (in practice since 12000 years before present) require large investments in labour, while animal (1000 ybp) or mechanically powered (90 ybp) ploughs cause soil compaction and deplete organic matter (Hay 1974). Although these techniques are fairly effective and currently incorporated into weed management programs, they tend to be more costly than chemical weed control.

### 1.0.1 Herbicide development

In 1947, a significant breakthrough improved the efficiency with which growers were able to control weeds. After three decades of research and development, two synthetic auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA), were registered and released onto the market to control dicot plants (Peterson 1967; Rao 2000; Troyer 2001). The success of these herbicides encouraged the development of additional herbicide modes of action, and by 1962 there were approximately 6000 formulations of 100 different herbicides on the market (Peterson 1967). These herbicides were either specific or broad spectrum and were developed to target multiple plant pathways and structures. In 1974, the compound glyphosate [N-(phosphonomethyl) glycine] was combined with an isopropylamine salt and registered for use in several crops. Glyphosate is applied post-emergence and has broad spectrum control over both $C_3$ and $C_4$ monocot and dicot annual and perennial species. Glyphosate

is more desirable than many existing herbicides because it has a low environmental impact; it targets a pathway found only in plants and microbes, binds tightly to the soil, and is unlikely to run off into ground water (Kovach et al. 1992; WHO 1994; Williams et al. 2000; Geisy et al. 2000; Duke and Powles 2008). Because of such properties, and the development of glyphosate-tolerant crops, glyphosate has become the most widely used herbicide globally (Baylis 2000; Duke and Powles 2008).

### 1.0.2 Glyphosate mode of action

Glyphosate must be applied post-emergence because its short soil residual time makes root uptake negligible (Geisy et al. 2000). After making contact with foliage, glyphosate is absorbed through the mesophyll cells and makes its way into the phloem tissues through passive and active transport (Gougler and Geiger 1981; Shaner 2009). Once inside the phloem, it follows sucrose movement to the metabolic sinks where it specifically targets the shikimic acid pathway (Gougler and Geiger 1984; McAllister and Haderlie 1985; Shaner 2009). The first step of the shikimic acid pathway involves the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate from the pentose phosphate cycle to produce 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) (Herrmann and Weaver 1999; Figure 1.1). DAHP is converted to shikimate in a series of three reactions catalyzed by 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, and shikimate dedydrogenase. Shikimate is subsequently converted into shikimate-3-phosphate by shikimate kinase, which requires an input of adenosine triphosphate (ATP). The enzyme catalyzing the reaction between shikimate-3-phosphate and 5-enolpyruvylshikimate-3-phosphate (EPSP) is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). When glyphosate enters the chloroplasts it competes with PEP for the binding site on EPSPS (Steinrücken and Amrhein 1980; Rubin et al. 1982), halting the pathway and causing an accumulation of shikimate. This observation suggests there is no inhibitory feedback in plants preventing the diversion of PEP and erythrose-4-phosphate into the shikimic acid pathway (Amrhein et al. 1980; Jensen 1986; Geiger et al. 1986).

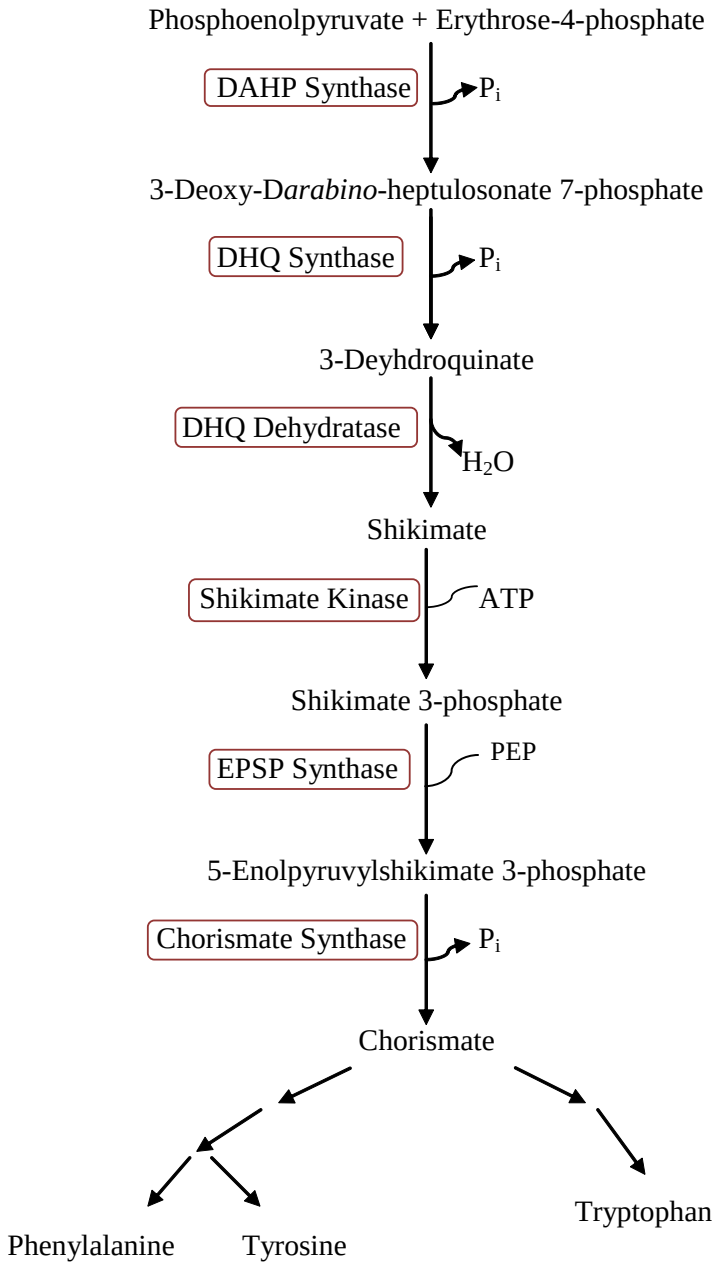


**Figure 1.1**Schematic of the shikimate pathway.

The final reaction of the shikimic acid pathway is catalyzed by chorismate synthase, which converts EPSP into chorismate. Chorismate is used to produce the aromatic amino acids tryptophan, tyrosine, and phenylalanine as well as many other aromatic secondary metabolites (Jensen 1986; Herrmann 1995a; Herrmann 1995b; Schmid and Amrhein 1995). Glyphosate therefore inhibits the production of chorismate and ceases the production of the three aromatic amino acids and many secondary metabolites including anthocyanins, flavanoids, and phytohormones (Herrmann and Weaver 1999). In susceptible plants, glyphosate application ultimately leads to plant death.

### 1.0.3 Glyphosate indirectly affects carbon metabolism

EPSPS is the only glyphosate-sensitive enzyme in the chloroplastic shikimic acid pathway, so any upstream reactions continue to take place in the presence of glyphosate. Therefore, PEP, erythrose-4-phosphate, and ATP are still drawn into the pathway, which alters the carbon and energy balance between the chloroplast and cytosol and indirectly disrupts carbon metabolism (Jensen 1986). Erythrose-4-phosphate is an important intermediate in the regeneration of ribulose-1,5-bisphosphate (RuBP), so diverting erythrose-4-phosphate to the shikimic acid pathway reduces the amount available for RuBP regeneration. Therefore, approximately 4 hours after treatment, the amount of available RuBP in the Calvin cycle decreases, and after 8 hours RuBP levels drop to about 20% of those of the control plants (Geiger et al. 1987; Servaites et al. 1987). This undoubtedly lowers the rate of carbon assimilation so, in the same time frame, the rate of starch accumulation and the net carbon exchange are substantially reduced (Servaites et al. 1987). The lower levels of RuBP cause an over-reduction of the photosynthetic electron transport chain and result in photoinhibition of photosystem II (PSII) (Coruzzi and Last 2000). Because some enzymes of the Calvin cycle require an input of ATP, the cycle is also indirectly inhibited by glyphosate (Malkin and Niyogi 2000).

### 1.0.4 Measuring damage to carbon metabolism

The negative feedback initiated by glyphosate can be measured using chlorophyll fluorescence. When the amount of light energy absorbed by the chlorophyll is greater than the amount required to drive photochemistry, the excess energy is dissipated as heat

or re-emitted as light. This re-emitted light is called chlorophyll fluorescence and is correlated with changes in photosynthetic efficiency (Kautsky et al. 1960). The mechanics of fluorescence measurement are described in Maxwell and Johnson (2000) as follows. After dark-adapting the leaf, the reaction centres of PSII are in the 'open' state, which means they are ready to accept an electron. The fluorescence measurement taken in the dark is termed zero fluorescence ($F_o$). When the dark-adapted leaf is exposed to a quick flash of saturating light, the reaction centres of PSII become saturated with electrons (i.e. they enter the 'closed' state), and the maximum fluorescence ($F_m$) is determined. In the 'closed' state, plastoquinone, the PSII electron acceptor, is fully reduced and cannot accept any additional electrons. After approximately 15-20 minutes in actinic light, the conversion between 'open' and 'closed' PSII reaction centres reaches a steady-state, so any additional saturating light pulses will provide a measure of the maximum fluorescence value in the light ($F'_m$). The diffusion of reduced plastoquinone ($PQH_2$) through the thylakoid membrane to deliver electrons to the cytochrome $b_6f$ complex is the rate limiting step of photosynthesis; therefore, any damage to the rate at which electrons are accepted and used by downstream processes can result in a build-up of electrons in the PSII reaction centres, and decreases photosynthetic efficiency. To protect the reaction centres from photoinhibition, the non-photochemical quenching (NPQ) processes increase to dissipate the incoming light energy as heat or re-emit the energy as light (Müller et al. 2001). Any changes in photosynthetic efficiency can be detected by measuring chlorophyll fluorescence.

Chlorophyll fluorescence has been used effectively to identify biotypes of pigweed (*Amaranthus retroflexus* and *Amaranthus powellii*), common bean (*Phaseolus vulgaris*), and wild turnip rape (*Brassica campestris*) that are resistant to triazines. When compared to corresponding control leaf disks soaked in a phosphate buffer, susceptible biotypes had higher (108-138%) leaf fluorescence after 24 hours of soaking in $10^{-4}$ M atrazine solution; in contrast, treated resistant biotypes, had leaf fluorescence values similar to the control (Ali and Souza Machado 1981). Studies with glyphosate have reported variable chlorophyll fluorescence values, which indicate that the response of chlorophyll fluorescence is dose- and species-dependent. Christensen et al. (2003) observed sugar beet leaves increase chlorophyll fluorescence as much as 4 times in response to

glyphosate (1000 g ae/ha) as soon as 4 h after treatment. In contrast, Olesen and Cedergreen (2010), found no consistent dose-response changes to chlorophyll fluorescence in barley, so they suggested that changes to $CO_2$ assimilation may be a more sensitive measure of glyphosate damage in plants.

## 1.1 Herbicide resistance

For a weed biotype to be classified as resistant it must meet several criteria (Heap 2005). First, the biotype must fulfill the Weed Science Society of America (WSSA) and International Survey of Herbicide-Resistant Weeds definition of a resistant plant – specifically, the plant must survive and reproduce following treatment with a herbicide dose that would be lethal to the wild-type. Secondly, the biotype must have been a problem to control in the field following the recommended application guidelines. Thirdly, the resistance must be heritable and must be naturally occurring, not the result of artificial or deliberate selection.

### 1.1.1 Incidence of resistance

The first weeds to become tolerant to herbicide application were reported in 1954 (Abel 1954); however, it was not until 1957 that a biotype of wild carrot (*Daucus carota*) in Ontario (Switzer 1957) and spreading dayflower (*Comellina diffusa*) in Hawaii (Hilton 1957) were reported resistant to the synthetic auxin 2,4-D. Resistance was reported again in 1968 when common groundsel (*Senecio vulgaris*) growing in a nursery was confirmed to be resistant to simazine and atrazine after yearly application of both herbicides for 10 consecutive years (Ryan 1970). These finding emphasized the importance of having more than one herbicide mode of action for a specific crop and rotating herbicide treatments whenever possible (Ryan 1970). As of 2012, 379 resistant biotypes of 205 weed species have been reported globally (Heap 2012).

The low incidence of resistance alleles in natural populations without the selection pressure imposed by herbicide application suggests that resistance to herbicides may confer a fitness penalty (Jasieniuk et al. 1996; Purrington 2000; Preston and Powles 2002). Fitness is a measure of survival, competitive ability, and reproductive success,

which collectively describe the evolutionary success of an individual in terms of its contribution of genes to the gene pool (Warwick and Black 1994). Any trait impeding an individual's contribution to the gene pool is referred to as a fitness penalty. Herbicide resistance is a trait that can benefit plant growth in the presence of the herbicide yet interfere with plant growth in the absence of the herbicide. Therefore, when conducting fitness studies, biotypes of similar genetic background or multiple biotypes with the same mechanism of resistance should be used to reduce the likelihood that additional loci are causing any observed fitness penalties (Bergelson and Purrington 1996; Jasieniuk et al. 1996; Cousens et al. 1997).

Target site mutations resulting in herbicide resistance can interfere with enzyme or substrate binding, which in turn can influence plant function and metabolism (Powles and Preston 2006; Vila-Aiub et al. 2009; Powles and Yu 2010). Resistant plants can either produce new enzymes or increase the production of enzymes that confer herbicide resistance (Werck-Reichhart et al. 2000); however, the additional energy required to produce these enzymes can take away from the energy allocated to growth and reproduction in the absence of the herbicide application (Vila-Aiub et al. 2009). In addition, there may be pleiotropic effects of resistance because resistant plants may become less attractive to pollinators and may be more susceptible to disease (Salzmann et al. 2008). However, studies comparing the growth, competitive ability, and reproductive output of resistant and susceptible biotypes have also found minor or no fitness costs to resistance (Pedersen et al. 2007; Vila-Aiub et al. 2009; Shrestha et al. 2010; Table 1.1).

### 1.1.2 Glyphosate-resistant weeds

When glyphosate first came onto the market, it was thought that it would be extremely unlikely for plants to evolve resistance because glyphosate acts on an essential pathway and alterations to this pathway would be detrimental to plant growth (Bradshaw et al. 1997). However, recent over-dependence on glyphosate, such as multiple in-season applications in glyphosate-tolerant crops and application at the wrong weed growth stage, has created a strong selection pressure for resistance. In particular, glyphosate usage increased after the introduction of glyphosate-tolerant soybean (1996), canola (1996), cotton (1997), and corn (1998). The selection pressure for the evolution of the resistant

trait has resulted in unequal control of weeds in a single population; susceptible plants succumb to glyphosate injury while the resistant plants survive and reproduce leading to an increase in the number of resistant plants in the following growing season. Therefore, ineffective chemical weed control can lead to a shift towards the resistant biotype within a population (Weller et al. 2010; Lingenfelter 2011). Because of the delay between the introduction of glyphosate and the evolution of glyphosate-resistant weeds, it is thought that the frequency of resistant alleles in populations is quite low and the appearance of glyphosate-resistant weeds results from the strong selection pressure imposed by repeated glyphosate application. The first reported glyphosate-resistant weed was a biotype of rigid ryegrass (*Lolium rigidum*) discovered in a crop grown in Australia (Powles et al. 1998; Pratley et al. 1999). To date, 22 weed species globally have biotypes confirmed to have evolved resistance to glyphosate (Heap 2012).

**Table 1.1** Fitness cost of glyphosate-resistance in weeds.

| Species | Herbicide Resistance | Fitness | Reference |
|---|---|---|---|
| *Lolium rigidum* | glyphosate | Seed weight of resistant plants greater, but fewer seeds at low levels of competition | Pedersen et al. 2007 |
| *Lolium rigidum* | glyphosate | After 3 growing seasons proportion of resistant plants declined in the absence of glyphosate selection pressure | Preston et al. 2009 |
| *Conyza canadensis* | glyphosate<br>glyphosate + ALS | Resistant plants had similar seed # and shoot mass as susceptible plants | Davis et al. 2009 |
| *Conyza canadensis* | glyphosate | Resistant plants were more competitive than susceptible plants when grown at high densities and low soil moisture | Shrestha et al. 2010 |
| *Ambrosia trifida* | glyphosate | Resistant plants flowered earlier, but produced 25% less seed than susceptible plants | Brabham et al. 2011 |

### 1.1.3 Mechanisms of glyphosate resistance

The two known strategies for glyphosate-resistance in weeds are target site mutations and non-target site alterations. Some glyphosate-resistant weeds prevent glyphosate from binding to the target site EPSPS by replacing a proline at site 106 with a serine, alanine, or threonine (Baerson et al. 2002; Wakelin and Preston 2006a; Powles and Preston 2006). This target site mutation causes nearby amino acids to extend into the glyphosate binding site, which overlaps with the binding site for PEP (Healy-Fried et al. 2007; Preston et al. 2009). More recently a target site mutation resulting from the over-expression of the EPSPS enzyme was demonstrated in a biotype of palmer amaranth (*Amaranthus palmeri*) found in Georgia (Gaines et al. 2010).

Glyphosate-resistant weeds with a non-target site mutation can alter the translocation mechanism of the herbicide to divert delivery to the susceptible, actively growing tissues (Shaner 2009). This type of resistance is caused by a nuclear encoded gene with partial or complete dominance (Lorraine-Colwill et al. 2001; Wakelin and Preston 2006b; Preston et al. 2009). Resistant rigid ryegrass plants with the altered translocation accumulated about 50% of glyphosate in the leaf tips, compared to susceptible plants, which accumulated the majority of glyphosate in the roots and shoot meristem (Lorraine-Colwill et al. 2002). Feng et al. (2004) observed reduced translocation from the leaves to the root tissues in resistant horseweed (*Conyza canadensis*) biotypes, compared to the susceptible biotypes. Glyphosate-resistant horseweed biotypes are able to trap the majority of glyphosate in the vacuoles of mature leaves within 24 h of application (Ge et al. 2010). It is thought that a glyphosate transporter on the tonoplast is either only present or is up-regulated in resistant biotypes; however, the mechanism and the transporter are still undescribed (Yuan et al. 2007; Shaner 2009).

A rapid necrosis response has been reported in some glyphosate-resistant giant ragweed biotypes with the altered translocation mechanism of resistance. The rapid necrosis response in plants has been commonly used to describe a plant's response to pathogen invasion, where infected cells die to prevent the spread of the pathogen (Stakman 1915). Similarly, the treated leaves of glyphosate-resistant giant ragweed become necrotic and drop within hours to days of glyphosate application, supposedly to prevent glyphosate

from escaping the mature leaf tissues (Brabham et al. 2011). However, glyphosate that escapes the initial sequestration can travel to the sink tissues, where it can enter the chloroplast and bind to the target site on EPSPS. Plant tissues have varying degrees of sensitivity to glyphosate. In velvetleaf (*Abutilon theophrasti*), Feng et al. (2003) showed that the mature segments of the stem had a significantly higher threshold for glyphosate compared to the young meristem and root tissues. Therefore, at sub-lethal doses, glyphosate primarily disrupts the sensitive apical meristems, freeing the plant from apical dominance and allowing the lateral meristems to begin producing buds (Thomas et al. 2005).

### 1.2 Giant Ragweed

Giant ragweed (*Ambrosia trifida* L.) is a broadleaved $C_3$ weed that is native to North America. It can be found throughout the United States and Canada, with some exceptions including the Canadian territories and Newfoundland, Alaska, and Hawaii. It moved into Canada after the retreat of the last glacial ice (Bassett and Crompton 1982). It grows on disturbed soils of roadside ditches, waste sites, riverbanks, and meadows, and over the past 30 years giant ragweed has moved into disturbed agricultural fields. The cotyledons are spatulate while leaves after the second true leaf pair display a characteristic 3 or 5 deep-lobed phenotype (Bassett and Crompton 1982).

Giant ragweed seedlings begin to emerge in March or April, before many other weed species, giving them time to establish a canopy and become the dominant species (Bassett and Crompton 1982). In agricultural soils, a recent shift in the biology of giant ragweed has extended the emergence well into June or July, which is after corn and soybean seeding (Johnson et al. 2006). A prolonged emergence pattern has the potential to create a large economic burden on growers because they must invest in management programs that have long soil residues or multiple post-emergent applications to prevent significant yield losses. Giant ragweed prevents other annual weeds and crops from growing in close proximity because it is a strong competitor for light, nutrients, and water (Abul-Fatih and Bazzaz 1979a). It can grow to 5 m tall, but its height is largely dependent on the density of neighbouring species (Abul-Fatih et al. 1979). For instance, in agricultural fields it usually grows between 0.30 - 1.52 m taller than the crop with which it is competing

(Johnson et al. 2006). Even at low densities (1.4 plants/$m^2$), giant ragweed emerging at the same time as corn can reduce grain yields by up to 60% (Harrison et al. 2001). In Ohio, season-long interference from 1 giant ragweed plant/$m^2$ in soybean decreased yield by up to 75% (Webster et al. 1994). Furthermore, if giant ragweed reaches reproductive maturity, one plant is capable of producing ≤ 5100 seeds (when grown with soybean), which contribute to the seed bank (Abul-Fatih and Bazzaz 1979b; Baysinger and Sims 1991; Johnson et al. 2006). However, the high seed number is probably an adaptation to low seed viability and high predation rates (Abul-Fatih and Bazzaz 1979b; Stoller and Wax 1974; Harrison et al. 2001). Without the yearly addition of fresh seeds, the seed bank would be depleted by ≥ 90% after four growing seasons (Harrison et al. 2007). In addition to seeds lost from the seed bank due to fungal growth and deep germination with no emergence, about 10-20% of the total seed production is made up of seedless fruits (Stoller and Wax 1973; Amatangelo 1974; Harrison et al. 2001; Schutte et al. 2006).

### 1.2.1 Reproduction

A single giant ragweed plant produces multiple male racemes with clusters of female flowers at the base of each spike. Depending on its position in the stand, a single giant ragweed plant may invest more heavily in female or male reproductive structures; plants higher in the canopy may distribute resources more evenly compared to plants lower in the canopy, which may invest more resources in female structures (Abul-Fatih et al. 1979). The flowering date, which is largely controlled by photoperiod, can occur anytime between July and October (Bassett and Crompton 1982; Johnson et al. 2006). A single plant is capable of producing over a billion pollen grains during its life, which makes it a huge contributor to hay fever (Bassett et al. 1978). Even though the plants are monoecious, they are self-incompatible so they rely on wind to transfer pollen grains between plants in a single stand. Because cross pollination is required for successful reproduction, the genetic diversity within a biotype is quite large (Johnson et al. 2006).

After pollination occurs, the seeds mature on the parent plant before dropping in the late-summer or early-fall. The seeds are covered by hard involucres with crown-like spikes around the top, which protect the embryo and regulate dormancy (Schutte et al. 2004). Giant ragweed seeds have a combination of non-deep physiological dormancy and coat

imposed dormancy, so the seeds require a period of cold stratification before germination can occur (Ballard et al. 1996). This is achieved by overwintering in the soil seed bank, where the cool moist soil conditions weaken the hard involucres. During this period, there is also an increase in giberellic acid biosynthesis and ABA degradation, which releases the seed from non-deep physiological dormancy and promotes germination under appropriate light and water conditions (Ali-Rachedi et al. 2004; Finch-Savage and Leubner-Metzger 2006).

### 1.2.2 Giant ragweed response to glyphosate

Glyphosate-susceptible giant ragweed plants treated with glyphosate begin to show herbicide injury symptoms about 1 week after treatment. The leaves become chlorotic, and by 21 days after treatment the aboveground plant tissues are usually completely necrotic (Singh and Shaner 1998; Hoss et al. 2003). On the other hand, glyphosate-resistant giant ragweed biotypes respond to glyphosate treatment in one of two ways. Some resistant giant ragweed biotypes show a quick response to glyphosate treatment, which was described in section 1.1.3 as a rapid necrosis response. The response involves the leaves becoming chlorotic then necrotic within hours to days after glyphosate treatment. This response suggests that the biotype has an altered translocation mechanism, with the glyphosate becoming trapped in the mature leaves, to prevent glyphosate from disrupting the meristem tissues. The biotypes with the rapid necrosis response were found to have a resistance index of about 6.5 (Green et al. 2011), which also hints that the biotypes have a reduced translocation mechanism of resistance (Shaner 2010). Other resistant giant ragweed biotypes show minimal visible injury and continue to grow as if not treated with glyphosate. This response suggests a target site mutation at the EPSPS enzyme. In this case, a mutation on the EPSPS enzyme prevents glyphosate from binding or causes an overproduction of the enzyme which allows the shikimic acid pathway to produce chorismate after treatment with glyphosate. A resistant index of about 4 has been found for the no-symptom biotypes (Green et al. 2011), which suggests that the resistant mechanism is caused by a target site mutation (Shaner 2010). Because the exact mechanisms causing glyphosate resistance in giant ragweed have not been described, the suggested mechanisms are based on similar responses described in other

weed species.

### 1.3 Environmental change and weed growth

Human activities have led to rising levels of atmospheric $CO_2$, contributing to the increasing temperature of Earth's surface. Land use changes, such as clearing land for agriculture and forestry, coupled with the burning of fossil fuels for energy, industry, transportation, and recreation are largely responsible for the rising levels of atmospheric $CO_2$ (IPCC 2001). Current atmospheric $CO_2$ levels are approximately 390 ppm (NOAA 2011), but the level is predicted to rise to 700 ppm by 2100 (IPCC 2001). By 2100, the surface temperature of Earth is expected to increase by 1.1-6.4$^{o}$ C, with the largest changes expected at high latitudes (IPCC 2007). In addition, Atmosphere-Ocean general circulation models predict the time between rainfall events to increase leading to extended periods of drought (IPCC 2007). Overall, these changes have been shown to influence the growth and competitive ability of $C_3$ weeds by altering processes such as carbon fixation and disrupting herbicide efficacy (Patterson 1995).

### 1.4 Thesis objectives and hypotheses

The first objective of my research project was to determine the influence of the ambient environment on the growth and herbicide efficacy of glyphosate-resistant and -susceptible giant ragweed biotypes. This objective was tested using 6 climate and $CO_2$ controlled greenhouses in the Biotron facility. Seedlings were grown to one of 4 leaf stages and treated with one of 3 doses of glyphosate to determine if there were any environment, growth stage, and dose response interactions. I hypothesized that glyphosate-resistant biotypes would be controlled with glyphosate treatments at very early growth stages, especially when grown under elevated $CO_2$ concentrations, elevated temperatures, and drought conditions. I predicted that there would be no major phenotypic differences between resistant and susceptible biotypes grown in the same greenhouse; however, resistant plants grown in the warm environments would initially have greater aboveground injury compared to the resistant plants grown in the cooler environments.

The second objective of my research project was to determine if the glyphosate-resistance

trait imparts a fitness penalty. This objective was tested by conducting a greenhouse experiment where plants were treated with glyphosate at the cotyledon, 2-leaf, 4-leaf, and 8-leaf stages and subsequently grown to seed. I hypothesized that glyphosate-resistant biotypes would have a fitness penalty limiting the seed output in the absence of glyphosate treatment because the resistant trait would only benefit the plants if it helps them survive glyphosate treatments. I predicted that there would be an early season growth advantage for susceptible plants and glyphosate-resistant plants would flower earlier and produce less aboveground biomass compared to the susceptible controls. I also predicted that in the absence of glyphosate, resistant biotypes would produce less seed when compared to the susceptible biotypes.

### 1.5 Thesis format

My thesis has been written in an integrated-article format and contains two manuscripts. Chapter 1 describes the background information and rationale for my objectives and hypotheses. Chapters 2 and 3 contain manuscripts corresponding to my first and second objectives, respectively. In chapter 4, I conclude with a general discussion and suggestions for future experiments.

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# CHAPTER 2

## The influence of ambient environment and glyphosate treatment on the growth and recovery of glyphosate-resistant and susceptible giant ragweed (*Ambrosia trifida* L.) biotypes

## 2.1 Introduction

Giant ragweed (*Ambrosia trifida* L.) biotypes found in agricultural fields in the midwestern United States and southwestern Ontario, have recently been confirmed to be resistant to the broad spectrum herbicide glyphosate. The discovery of glyphosate-resistant weeds in glyphosate-tolerant cropping systems threatens the utility of these systems and increases the importance of early season weed control. Early season weed control is one the main principles in integrated weed management (Swanton and Weise 1991); however, it is difficult to control species such as giant ragweed because it emerges in multiple waves throughout the growing season (Johnson et al. 2006). Giant ragweed plants that escape early season weed control quickly establish a canopy (Abul-Fatih and Bazzaz 1979; Bassett and Crompton 1982) and cause large yield reductions to soybean and corn crops (Webster et al. 1994; Harrison et al. 2001).

Currently, there are two known responses to glyphosate that are displayed by resistant giant ragweed biotypes (personal communication F. Tardif, University of Guelph). The first example is a rapid necrosis response where the mature leaves become chlorotic and necrotic within hours of glyphosate application (Brabham et al. 2011). However, within a week these plants recover from the glyphosate injury by initiating growth from their apical and lateral meristems. The second example involves biotypes that show no symptoms of glyphosate injury besides halting their growth for the first week after treatment. After one week, the resistant biotypes continue to grow while the susceptible biotypes become completely necrotic within 21-28 d after treatment. The specific mechanisms of resistance have not been identified so it is unclear if they will be affected by the growth environment.

Glyphosate-resistant giant ragweed biotypes have been difficult to control in the field, but control at young leaf stages has been achieved in greenhouse trials. For example, Norsworthy et al. (2011) controlled 57 and 60% of two glyphosate-resistant giant ragweed biotypes from Arkansas treated (840 g ae/ha of glyphosate) at the 2-leaf stage and Norsworthy et al. (2010) controlled 72% of a glyphosate-resistant giant ragweed biotype from Tennessee treated (870 g ae/ha of glyphosate) at the 2-leaf stage. Therefore, applying the maximum glyphosate dose for a single application (1800 g ae/ha) should achieve complete control of resistant biotypes at the cotyledon and 2-leaf stages.

In the coming decades, climatic factors, such as elevated temperatures and water stress, are likely to interact with rising levels of atmospheric $CO_2$ to affect the growth and competitiveness of $C_3$ plant species such as giant ragweed. Higher temperatures increase the solubility of $CO_2$, promoting the rubisco oxygenation reaction resulting in an increase in photorespiration (Jordan and Ogren 1984). However, Long (1991) showed that as $CO_2$ concentrations increased from 350 to 650 ppm the light saturated photosynthetic rate of a range of $C_3$ species increased by 14% (at 10˚C), 54% (at 20˚C), and 73% (at 30˚C). Therefore, the interaction between elevated $CO_2$ and temperature could stimulate a net increase in $C_3$ photosynthesis (Patterson 1995). In contrast, water stress can decrease the photosynthetic rate of $C_3$ plants by decreasing stomatal aperture. But, the interaction between elevated $CO_2$ and temperature can improve water use efficiency (Wray and Strain 1986) and plants can adjust leaf osmotic potential using the accumulated photosynthetic products, resulting in less drought stress (Polley et al. 1993; Polley 2002).

Elevated $CO_2$, temperature, and drought, directly and indirectly affect herbicide efficacy (Patterson 1995). For example, plants grown under elevated $CO_2$ can have increased concentrations of starch in their leaves (DeLucia et al. 1985; Sage et al. 1989; Arp 1991; Patterson 1995) and fewer stomata per unit area (Ziska and Bunce 2006), which decrease herbicide absorption and overall efficacy. The absorption of foliar applied herbicides is reduced if a plant is grown under a prolonged water stress because the leaves can develop a thick cuticle to prevent water loss (Ziska and Bunce 2006). In addition, herbicide absorption and translocation either increases or decreases under elevated temperatures (Reddy 2000). Variation among studies is related to the relative humidity of the post

spray environment. In a low humidity environment, foliar applied herbicides have a higher rate of evaporation, reducing the droplet size and overall absorption into the leaf tissues (Ziska and Dukes 2011). Alternatively, high humidity environments promote a longer leaf retention time by increasing absorption and translocation because the stomata can remain open (Jordan 1977; McWhorter et al. 1980).

In an environment that favours increased rates of plant development, growers have a shortened window to apply herbicides (Ziska and Dukes 2011). Increased height and leaf number can decrease herbicide efficacy even when herbicides are applied at higher doses (King and Oliver 1992) because larger weeds accumulate less herbicide per unit of plant tissue. Therefore, the objective of my research was to determine the influence of variation in $CO_2$ concentration, temperature, and moisture availability on the growth and herbicide efficacy of glyphosate-resistant and -susceptible giant ragweed biotypes. I hypothesized that the control of glyphosate-resistant biotypes would vary based on leaf stage and growth environment. I predicted that there would be no major phenotypic differences between resistant and susceptible biotypes grown in the same growth room; however, resistant plants grown in the warm environments would initially have more aboveground injury compared to the resistant plants grown in the cooler environments. Finally, I predicted that glyphosate-resistant biotypes would be controlled at the cotyledon and 2-leaf stage.

## 2.2 Methods

### 2.2.1 Seed collection

Seeds were collected from mature plants of four geographically distinct *Ambrosia trifida* biotypes (Table 2.1). All seeds were stored dry in the dark at 5˚C and a constant relative humidity (RH) of 60% until use.

### 2.2.2 Seed viability testing

I completed a tetrazolium (TZ) test (Peters 2000) on a subset of seeds to confirm the viability of each biotype. I imbibed the seeds overnight (16 h) at room temperature (ca. 21˚C) then prepared them for staining by making a longitudinal cut starting at the tip of the seed leaving the top of the cotyledons intact. I placed the prepared seeds in a 0.l % TZ solution in glass Petri dishes and incubated them at 35°C for 16 h. I cut the seed in half following the longitudinal cut and examined the staining pattern of the embryo. I considered a seed viable if the entire embryo was evenly stained red or if the radicle tip was unstained or slightly darker than the red stain. I scored a seed as nonviable if any essential embryo regions were unstained or if the seed was excessively bruised (dark red stain).

**Table 2.1** Summary of biotype information.

| Location | Coordinates | Year | Glyphosate Susceptibility[a] | Glyphosate Exposure | Expected Response[b] | Known Herbicide Resistance |
|---|---|---|---|---|---|---|
| Harrow, ON | N 42.024˚ W 82.898˚ | 2009 | S | Yes | D | – |
| Cambridge, ON | N 43.387° W 80.348° | 2008 | S | No | D | – |
| Windsor, ON | N 42.279˚ W 82.961˚ | 2009 | R | Yes | RN | glyphosate ALS |
| Pickaway, Co,Ohio | N 39.528˚ W 83.176˚ | 2007 | R | Yes | RN | glyphosate ALS |

[a] Susceptible (S) or resistant (R).
[b] Expected response to glyphosate: death (D) or rapid necrosis (RN).
Acetolactate synthase (ALS)

### 2.2.3 Seed stratification

I added sterilized field soil for the first stratification and Premier Pro-mix® Mycorise® pro for the remaining stratifications, to 4 cm depth in 28 labelled 10.2 cm diameter round plastic pots. I placed the pots on a tray and watered from the bottom until the surface soil was wet. Fifty seeds of each biotype were placed on the soil surface and the seeds were covered with an additional 5 cm of soil. I replicated this procedure seven times for a total of 350 seeds for each biotype. The pots were then watered by hand to field capacity (watered gently until water started draining from pots with subsequent waiting for the water to stop draining) and incubated at 1˚C. After 6-8 weeks of stratification, I randomly selected 2 pots of each biotype from the incubator and 5 seeds of each biotype were removed and placed into a Petri dish. I placed the Petri dishes in an incubator set to an alternating day/night temperature of 30/20˚C with a 14 h day (Andersen 1968). If < 40% of seeds for each biotype germinated after 7 days, the pots were returned to the cold stratification process. However, if > 40% of seeds of a biotype germinated, the pots from that biotype were considered to have completed stratification. I sifted the seeds from the soil, rinsed them with water, and allowed them to dry on a paper towel at room temperature (ca. 21˚C) for 3 h. I stored the stratified seeds in envelopes (1 envelope/pot) under dark and dry conditions at 1˚C. I continued the germination tests until all remaining pots met the requirements to be removed from the stratification conditions. I used the seeds within 3 months following the completion of stratification.

### 2.2.4 Experimental design

I used six independent climate and $CO_2$ controlled greenhouses located in the Biotron facility at the University of Western Ontario to create six distinct environments (Figure 2.1). The four biotypes I used in this experiment are listed in Table 2.1. I planted 144 stratified seeds of each biotype at a 2 cm depth in individually labelled 6.5 L round pots filled with Premier Pro-mix® Mycorise® pro potting media. I staggered the planting so all leaf stages were present at approximately the same time (the end of week 4). I randomized the location of pots within each greenhouse. Twelve pots of each biotype received a well-watered treatment (35 mm/week in the low temperature greenhouses;

**A**

| $CO_2$ | 390 ppm | | 550 ppm | | 700 ppm | |
|---|---|---|---|---|---|---|
| Temperature | 25/10˚C | | 25/10˚C | | 25/10˚C | |
| Precipitation | 35 mm | 17.5 mm | 35 mm | 17.5 mm | 35 mm | 17.5 mm |
| # of plants/ biotype | 12 | 12 | 12 | 12 | 12 | 12 |
| $CO_2$ | 390 ppm | | 550 ppm | | 700 ppm | |
| Temperature | 35/20˚C | | 35/20˚C | | 35/20˚C | |
| Precipitation | 70 mm | 35 mm | 70 mm | 35 mm | 70 mm | 35 mm |
| # of plants/ biotype | 12 | 12 | 12 | 12 | 12 | 12/ |

**B**

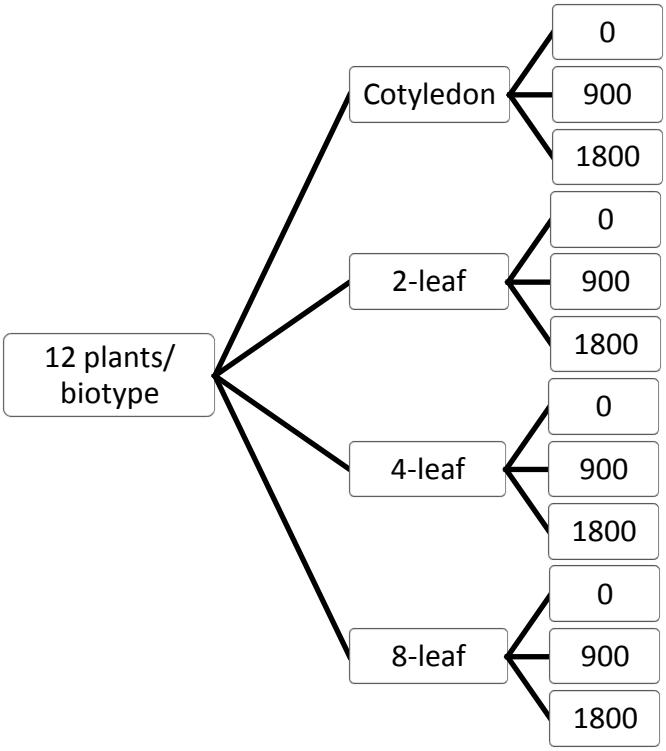


**Figure 2.1** Experimental design of the individual climate and $CO_2$ controlled greenhouses (A) located in the Biotron facility at the University of Western Ontario, London, Ontario. Each biotype was grown to (B) four leaf stages and plants were treated with glyphosate (0, 900, or 1800 g ae/ha). The experiment was repeated three times (n=3).

70 mm/week in the high temperature greenhouses) through drip tape irrigation using reverse osmosis water, while the remaining 12 were drought stressed (field capacity at seeding, then 50% of the watered treatment). In the high temperature greenhouses (35˚C), extra water loss due to increased rates of evaporation and transpiration caused the potting media to dry between watering events, so I doubled the watering treatment to 70 mm/week. The irrigation system was turned on each morning, between 07:00 and 08:00 hours, before peak day time temperatures were reached. I applied a 5g/L solution of 20-20-20, N-P-K, by hand to each pot at one week intervals after planting. The relative humidity in each biome was kept constant at 60% and a 14 hour photoperiod was maintained between 7:00 and 21:00 hours. I replicated this experiment 3 times using the same procedures.

### 2.2.5 Growth measurements

I measured height (cm) and leaf number on a weekly basis, from the day of emergence. I measured height from the soil surface to the highest growing point. I included new leaves in the leaf stage count if they had fully unfolded. I counted the axillary leaves separately once they had fully unfolded but they were not used to determine leaf stage. Subsequently, I removed subsets of 3 plants of each biotype from each greenhouse at the cotyledon, 2-leaf, 4-leaf, and 8-leaf stages and exposed the plants to glyphosate within an enclosed herbicide spray chamber. There were three treatments: 1. untreated control – reverse osmosis water; 2. glyphosate – 900 g ae/ha; 3. glyphosate – 1800 g ae/ha. In replicate one, I placed the treated plants back into the corresponding greenhouse. To test the influence of post-spray temperature on the overall herbicide injury score, I placed the treated plants in replicate two and three in a common garden in a glass growth room set to ambient environmental conditions ($CO_2$ [390 ppm] and temperature [20˚C]).

I noted visible injury symptoms at 2, 7, 14, 21, and 28 d after treatment (DAT). I scored plant injury caused by glyphosate as a percentage of aboveground tissue damage: 0% was completely healthy while 100% was completely necrotic. At 28 DAT I recorded a final height, leaf number, axillary leaf number, and axillary bud number for each plant.

### 2.2.6 Statistical analysis

I arranged this experiment as a split-split-split plot, randomized complete block design (Table 2.2). I performed a series of multiple general linear models in JMP 9.0 (SAS Institute, Inc., Cary, NC, USA) to determine the treatment effects. I used $CO_2$ concentration, temperature, precipitation, biotype, and leaf-stage as fixed factors to determine their effect on herbicide injury, height, leaf number, and lateral growth. Transformations did not improve the normality of data with the exception of height on the day of spray which was log transformed. Three-way interactions between fixed factors were analyzed for each dependent variable. The fixed factors that did not have a significant effect on the dependent variables were pooled. The post-spray temperature was used as a factor in the model. This allowed me to separate out any post-spray influence on herbicide injury and growth. I also ran a univariate repeated measures analysis to determine any differences between herbicide injury and day after treatment. Spray dose and biotype were nested within pot number and the random effects were tested. A critical $\alpha$ of 0.05 was used to assess statistical significance. A least-squares means contrast and Tukey's HSD test were used to assess significance among treatment combinations.

**Table 2.2** Statistical design of individual biomes.

| Whole Plot | Sub Plot | Sub-Sub Plot | Sub-Sub-Sub Plot[a] | | | |
|---|---|---|---|---|---|---|
| $CO_2$ and Temperature | Precipitation | Resistant (x2) | Cot | 2 | 4 | 8 |
| | | Susceptible (x2) | Cot | 2 | 4 | 8 |
| | Drought | Resistant (x2) | Cot | 2 | 4 | 8 |
| | | Susceptible (x2) | Cot | 2 | 4 | 8 |

[a] Leaf stage at which plants were treated with glyphosate: cotyledon (cot), 2-leaf (2), 4-leaf (4), or 8-leaf (8).

## 2.3 Results

### 2.3.1 Environmental effects on seedling growth

Elevated $CO_2$ and drought treatments did not affect the growth of seedlings prior to spray; however, temperature influenced the height and amount of lateral growth (Table 2.3). Seedlings grown in the cooler greenhouses (25˚C) were taller, and had more lateral leaves and buds on the day of spray (Figure 2.2 and 2.3). Harrow (susceptible) and Ohio (resistant) seedlings were taller than Cambridge (susceptible) or Windsor (resistant) seedlings ($p<0.001$; Tukey's HSD test; Figure 2.2).

**Table 2.3** Summary of p-values from three-way ANOVA for the effects of environment on growth.

| Effects | Height | Lateral Leaf Number | Lateral Bud Number |
|---|---|---|---|
| $CO_2$ | 0.145 | 0.064 | 0.337 |
| T | <0.001*** | 0.002** | <0.001*** |
| $CO_2$ *T | 0.502 | 0.198 | 0.125 |
| W | 0.915 | 0.501 | 0.742 |
| W* $CO_2$ | 0.772 | 0.484 | 0.955 |
| T*W | 0.575 | 0.139 | 0.390 |
| $CO_2$*T*W | 0.699 | 0.964 | 0.608 |

Effects: $CO_2$ level (390, 550, 700 ppm), temperature (T; 25 or 35˚C) and watering treatment (W; drought or well-watered)
Asterisks denote a significant effect (*0.05-0.01, **<0.01-0.001, ***<0.001)
Height data were log transformed

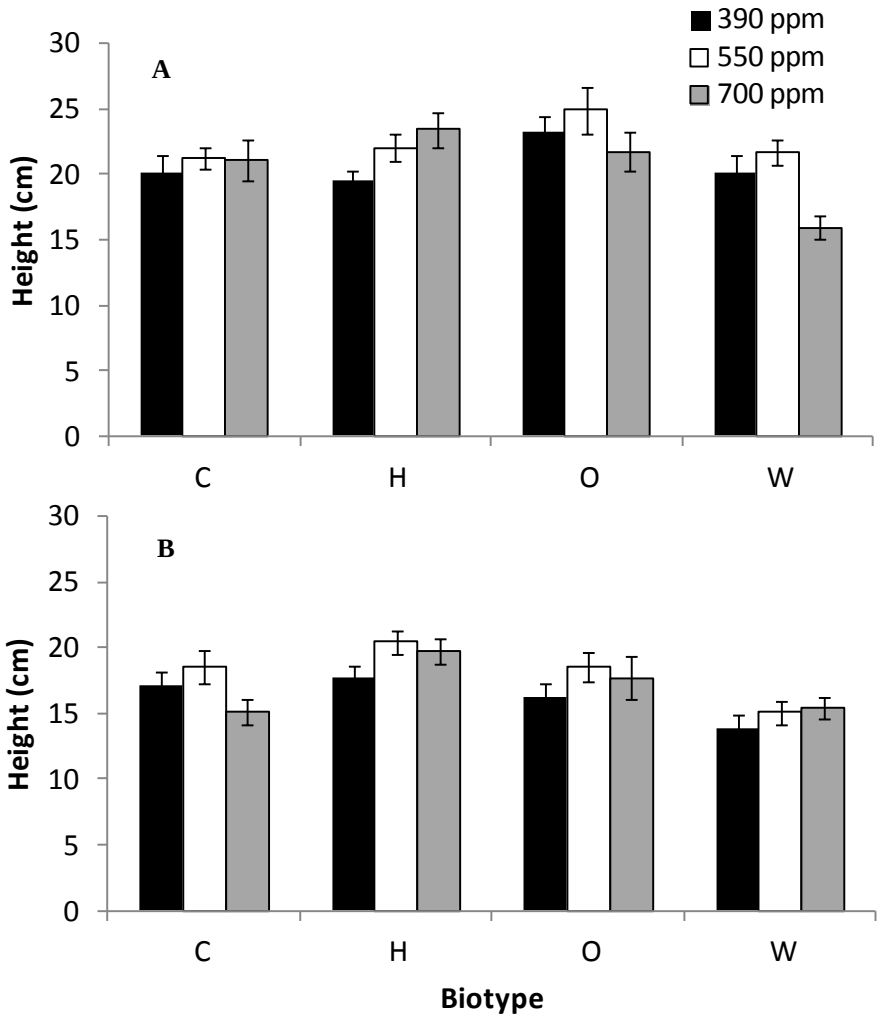


**Figure 3.2** Height of seedlings at the 8-leaf stage on the day of glyphosate treatment. Seedlings were grown in controlled growth environments with $CO_2$ levels of 390, 550, or 700 ppm and temperatures of 25/10˚C (A) or 35/20˚C (B). Mean values are plotted for each biotype: Cambridge (C), Harrow (H), Ohio (O), and Windsor (W). Cambridge and Harrow are susceptible biotypes while Ohio and Windsor are resistant biotypes. The sample size ranges from 10-18 for each treatment combination. The error bars represent standard error.

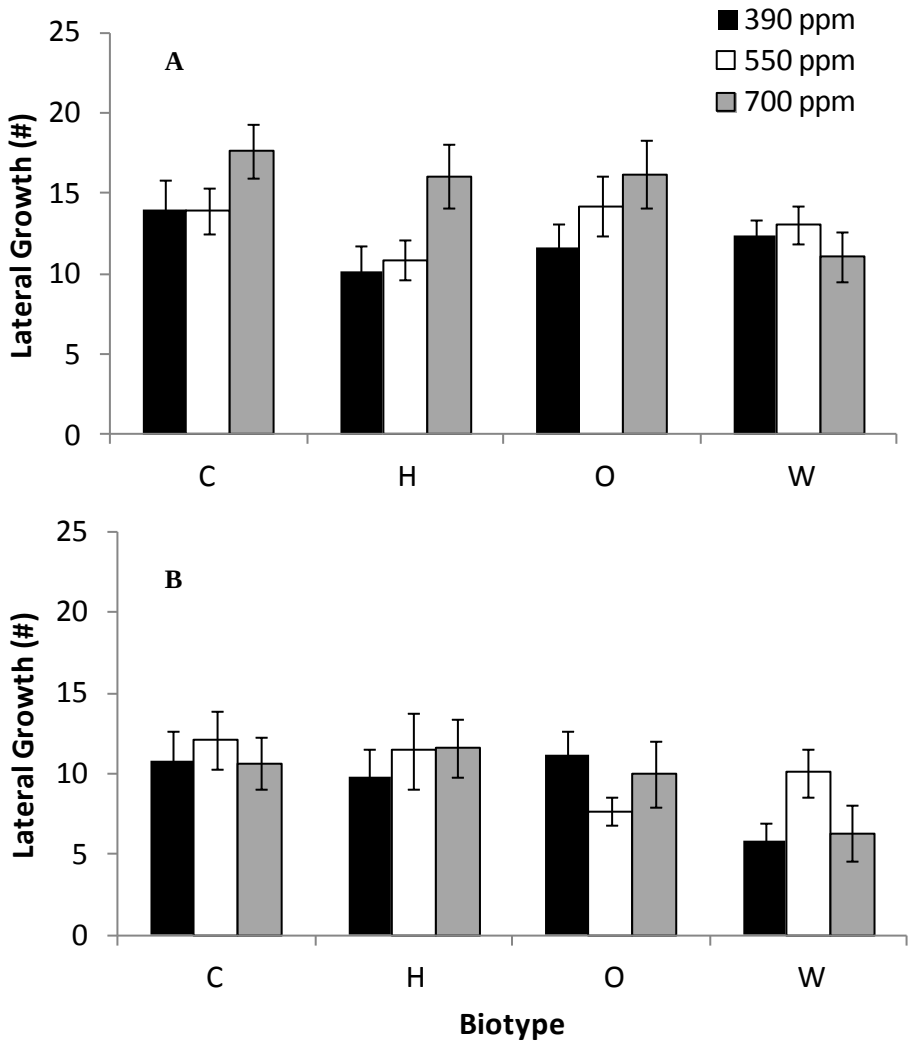


**Figure 2.3** Lateral leaves and buds (lateral growth) of seedlings at the 8-leaf stage on the day of glyphosate treatment. Seedlings were grown in controlled growth environments with $CO_2$ levels of 390, 550, or 700 ppm and temperatures of 25/10˚C (A) or 35/20˚C (B). Mean values are plotted for each biotype: Cambridge (C), Harrow (H), Ohio (O), and Windsor (W). Cambridge and Harrow are susceptible biotypes while Ohio and Windsor are resistant biotypes. The sample size ranges from 10-18 for each treatment combination. The error bars represent standard error.

### 2.3.2 Glyphosate treatment and herbicide injury

Following glyphosate treatment, the temperature of the individual growth rooms or common garden influenced the herbicide injury scores ($p=0.001$). Resistant plants placed into a warm environment (35˚C) post-spray, showed less injury than plants of the same treatment placed into a cool environment (20 or 25˚C); however, susceptible biotypes had more injury 7 and 14 days after treatment if they were placed at 25 and 35˚C compared to plants at 20˚C (Tukey's HSD test; Figure 2.4). Glyphosate treatment (900 and 1800 g ae/ha) induced the rapid necrosis response in both resistant biotypes ($p=0.014$; Figure 2.4). All plants treated with glyphosate were more injured than the untreated controls ($p<0.001$). Plants grown in the warmer growth rooms or in the drought treatment prior to glyphosate treatment were not different from the untreated controls ($p=0.056$, $p=0.052$, respectively). Even though the treatments were almost significant, data had to be pooled due to the low sample size of individual treatments. Resistant plants treated at the cotyledon and 2-leaf stages showed less initial injury, but by 28 days after treatment the resistant biotypes treated with glyphosate all showed similar levels of injury ($p=0.012$). All susceptible biotypes treated with 900 or 1800 g ae/ha of glyphosate died from glyphosate injury ($p<0.001$).

Treating seedlings with glyphosate (900 and 1800 g ae/ha) reduced the height and lateral growth of susceptible and resistant biotypes compared to the untreated controls ($p<0.001$; Figure 2.5). Placing the plants at the warm temperature (35˚C) after spray increased the number of lateral leaves ($p<0.001$; Figure 2.5). Plants in the 25 and 35˚C environments had more lateral buds following glyphosate treatment ($p<0.001$; Figure 2.5).

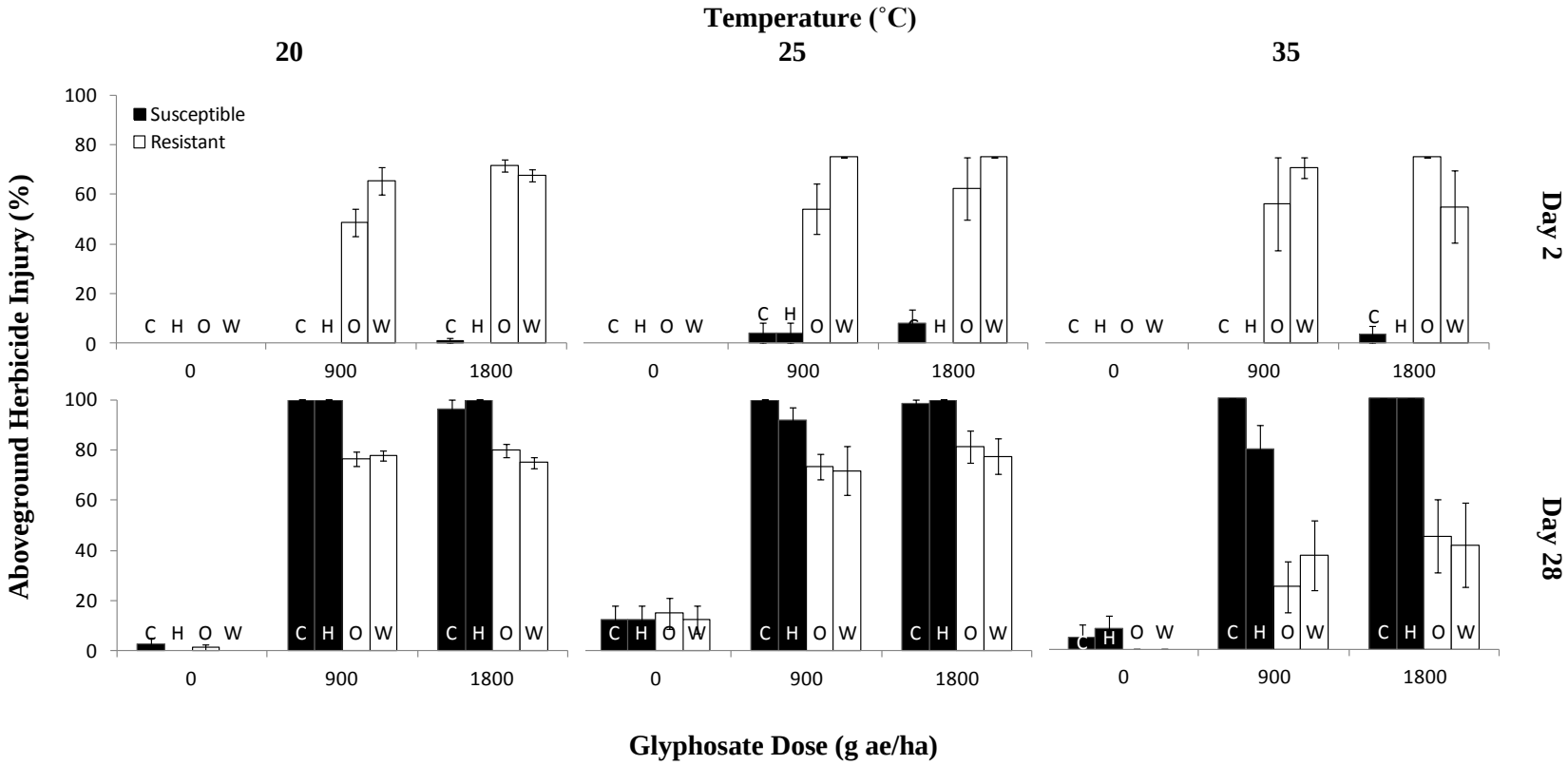


**Figure 2.4** Mean percentage of aboveground herbicide injury on seedlings treated with glyphosate at the 8-leaf stage. Seedlings were grown in controlled growth environments with $CO_2$ levels of 390, 550, or 700 ppm and temperatures of 25/10˚C or 35/20˚C. After spray the seedlings were place back into the corresponding environment or were transferred into a common garden (390 ppm, 20˚C). Cambridge (C) and Harrow (H) are susceptible biotypes while Ohio (O) and Windsor (W) are resistant biotypes that show the rapid necrosis response. The sample size ranges from 4-23 for each treatment. The error bars represent standard error.

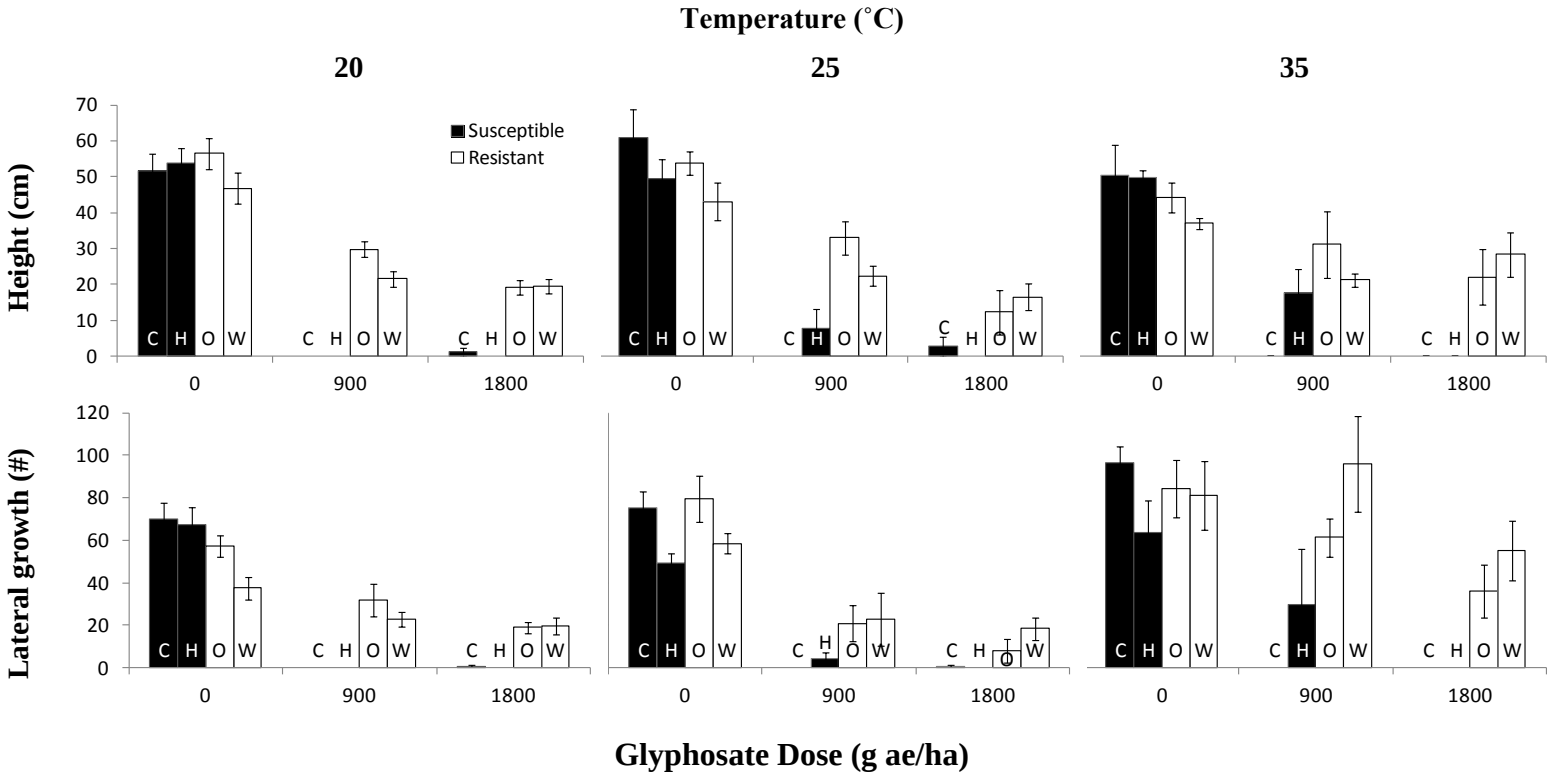


**Figure 2.5** Mean height and lateral growth (lateral leaves and buds) on seedlings treated with glyphosate at the 8-leaf stage. Seedlings were grown in controlled growth environments with $CO_2$ levels of 390, 550, or 700 ppm and temperatures of 25/10˚C or 35/20˚C. After spray the seedlings were place back into the corresponding environment or were transferred into a common garden (390 ppm, 20˚C). Cambridge (C) and Harrow (H) are susceptible biotypes while Ohio (O) and Windsor (W) are resistant and show the rapid necrosis response. The sample size ranges from 3-22 for each treatment. The error bars represent standard error.

## 2.4 Discussion

Contrary to my hypothesis, treating the resistant biotypes at the cotyledon and 2-leaf stage did not improve the overall control, regardless of the ambient growth environment. All resistant biotypes survived glyphosate treatment while the susceptible biotypes succumbed to glyphosate injury. Control of resistant weeds species such as common lambsquarters (*Chenopodium album*) and horseweed (*Conyza canadensis* and *Conyza bonariensis*) was achieved when plants were treated between the 2- and 5-leaf stage (Schuster et al. 2007; Shreshta et al. 2007; Vila-Aiub et al. 2007; Dinelli et al. 2008). Using a biotype of glyphosate-resistant giant ragweed from Tennessee, Norsworthy et al. (2010) observed 72% control of seedlings treated at the 2-leaf stage. Even though the resistant seedlings were not completely controlled by the glyphosate treatments, some seedlings produced numerous lateral buds suggesting that the apical dominance was disrupted. These buds did not appear healthy and never developed into leaves. It is possible that the seedlings with this response would never fully recover and produce seed, so monitoring the recovery past 28 days after treatment may be beneficial.

Although cooler temperatures improved plant growth prior to treatment, it did not influence glyphosate efficacy. By 2 days after treatment, biotypes displaying the rapid necrosis were showing the expected response to glyphosate. The leaves became chlorotic starting at the tips and moving towards the petiole, which caused the leaves to curl under. Seedlings that were placed back into warmer temperatures had a more rapid response to glyphosate. Higher temperatures can be correlated with higher rates of transpiration, which can increase the absorption and translocation of glyphosate (Jordon 1977; McWhorter et al. 1980). Transpiration measurements taken on a small sample of plants before glyphosate treatment confirmed that rates were the highest for biotypes grown in the warmer environments (data not shown). However, at 28 days after treatment the resistant biotypes recovering at 35˚C had a lower percentage of injury compared to plants recovering at 20 or 25˚C. Growth in warmer environments promotes lateral branching and treatment with glyphosate can initiate bud formation at the lateral meristems by relieving the apical dominance (Thomas et al. 2005; Norsworthy et al. 2010). Therefore, the glyphosate-resistant plants recovering in the warmer temperatures recovered some of the

lost biomass by 28 days after treatment by producing lateral branches.

Elevated levels of $CO_2$ can reduce the absorption and overall efficacy of glyphosate as a result of increasing starch concentrations in the leaves and decreasing stomata number per unit area (Wong 1990; Patterson 1995; Ziska et al. 1999; Ziska and Bunce 2006). The reduced efficacy could potentially have major implications for crop/weed interactions (Archambault et al. 2001); however, in the tested environments there were no noticeable differences between glyphosate injury on plants from ambient to elevated $CO_2$ environments. There were also no interactions between $CO_2$ and temperature suggesting that the interactions between environmental factors are species-specific rather than $C_3$ and $C_4$ plant specific (Archambault et al. 2001). Further studies should be conducted to determine if growth in elevated $CO_2$ actually increases the starch concentration and affects the stomatal density on giant ragweed leaves.

The drought treatment did not influence the growth of seedlings prior to glyphosate treatment. Giant ragweed prefers to grow in moist soils (Bassett and Crompton 1982; Abul-Fatih and Bazzaz 1979), but it has also been described as being slightly drought tolerant; therefore the drought treatment in my experiment may not have been severe enough to affect plant growth. More severe drought conditions were tested in pre-trials but the plants wilted almost to the point of plant death. The amount of water applied to the drought treatment increased for my experiment, to prevent water stress from being confused with glyphosate injury. The drought treatment did slightly increase glyphosate injury, but it did not affect the overall control at 28 days after treatment. This contrasts other studies that suggest drought stressed plants can be more tolerant to glyphosate because a thicker cuticle develops reducing the absorption and translocation compared to well-watered plants (Zhou et al. 2008).

## 2.5 Conclusions

In conclusion, a three-way interaction among $CO_2$, temperature, and drought treatments was not detected. The ambient environment did not alter the growth of resistant biotypes in comparison to susceptible biotypes. Additionally, glyphosate did not improve the control of resistant plants at any of the tested leaf stages including the cotyledon stage.

This has important implications for management because under growth room conditions that were potentially stressful for plant growth, the control of resistant biotypes was not improved even after treatment with glyphosate at 2x (1800 g ae/ha) the labelled rate – the maximum rate a grower can apply in one application. This was surprising because many other studies have shown that resistant biotypes could be controlled with labelled rates if they were treated at early growth stages. Relaxing the selection pressure imposed by continuous glyphosate applications may lead to a species shift back to the susceptible biotype if the resistance trait is associated with a fitness penalty. In addition, as was shown in this experiment, some resistant biotypes surviving glyphosate treatment appeared to be damaged at the apical meristem; this may prevent the plant from reaching reproductive maturity and contributing seeds to the seed bank. Experiments testing fitness penalties would help determine whether glyphosate-resistant giant ragweed biotypes are at a disadvantage at any life stage compared to the susceptible biotypes. However, without relaxing the selection pressure, the number of glyphosate resistant biotypes is expected to increase.

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# Chapter 3

## The influence of glyphosate treatment on the growth and fitness of glyphosate-resistant and susceptible giant ragweed (*Ambrosia trifida* L.) biotypes

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### 3.1 Introduction

Giant ragweed is a summer annual plant species that is becoming an increasing concern in agricultural fields because biotypes in the mid-Western United States and southwestern Ontario have evolved resistant to the herbicide glyphosate. The primary target of glyphosate is the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme of the shikimic acid pathway (Gougler and Geiger 1984; McAllister and Haderlie 1985; Shaner 2009). Glyphosate competitively inhibits the binding of phosphoenolpyruvate (PEP) to EPSPS which ultimately halts the pathway (Steinrücken and Amrhein 1980; Rubin et al. 1982; Herrmann and Weaver 1999), preventing the production of chorismate and the three aromatic amino acids (Jensen 1986; Herrmann 1995a; Herrmann 1995b; Schmid and Amrhein 1995). Originally, it was thought that weeds would not evolve resistance to glyphosate because alterations to the EPSPS enzyme could prevent aromatic amino acid production which would be detrimental to plant growth (Bradshaw et al. 1997). However, at least 22 species of weeds have evolved resistance to glyphosate (Heap 2012).

The growth characteristics of giant ragweed make the resistant biotypes especially problematic. In biotypes with reduced translocation, glyphosate-resistance is a dominant or semi-dominant nuclear encoded gene that is transferred by both pollen and seed (Preston and Wakelin 2008; Heap 2012; Beckie 2011; Brabham et al. 2011). A single giant ragweed plant can produce over a billion pollen grains in a growing season (Bassett et al. 1978) and pollen has the potential to carry the resistant trait up to1 km from the parent plant (Raynor et al. 1970). Therefore, the wind acts as a potential dispersal vector carrying the resistant trait to nearby biotypes. In agricultural fields, giant ragweed plants emerge in two waves making it difficult to control (Johnson et al. 2006) because the plants that escape control quickly become the dominant species (Abul-Fatih and Bazzaz

1979; Bassett and Crompton 1982) and cause devastating yield reductions in soybean and corn crops (Webster et al. 1994; Harrison et al. 2001). However, Norsworthy et al. (2010) controlled 72% of resistant giant ragweed seedlings treated with glyphosate at 870 g ae/ha when they were treated at the 2-leaf stage. The maximum glyphosate rate a grower can apply in one application is 1800 g ae/ha, so one treatment at the maximum rate may improve the control of resistant giant ragweed biotypes especially at the cotyledon and 2-leaf stages.

The exact mechanisms of glyphosate resistance in giant ragweed have not been identified, but target site mutations (Wakelin and Preston 2006; Powles and Preston 2006), over-expression of the EPSPS enzyme (Gaines et al. 2010), and altered translocation possibly due to vascular sequestration (Lorraine-Colwill et al. 2002; Feng et al. 2004; Shaner 2009; Ge et al. 2010) have been described in glyphosate-resistant rigid ryegrass (*Lolium rigidum*), horseweed (*Conyza canadensis*), goosegrass (*Eleusine indica),* Italian ryegrass *(Lolium multiflorum*), and palmer amaranth (*Amaranthus palmeri*). There are two distinct phenotypes of glyphosate-resistant giant ragweed following treatment: the rapid necrosis response or the no symptom response. During the rapid necrosis response the mature leaves become chlorotic and curl under within two days of glyphosate treatment. The plant recovers from this injury by initiating growth from the apical and lateral meristems. The no symptom response biotypes grow slowly for a week following glyphosate treatment. These biotypes resume growing during the following week and overall, show minimal signs of glyphosate damage.

Glyphosate may indirectly influence carbon sink processes, which feedback and have negative influence on the photosynthetic electron transport chain (Jensen 1986; Geiger et al. 1987; Servaites et al. 1987; Coruzzi and Last 2000). Christensen et al. (2003) observed a four times increase in chlorophyll *a* fluorescence of sugar beet (*Beta vulgaris*) leaves in response to glyphosate (1000 g ae/ha) as soon as 4 h after treatment. On the other hand, Olesen and Cedergreen (2010) found no changes in fluorescence in barley (*Hordeum vulgare*), but suggested that changes to $CO_2$ assimilation and conductance may be a more sensitive measure of glyphosate damage in plants. These measures have not been used to compare biotypes with multiple strategies of glyphosate-resistance within a species but

they could provide insight into the strategies of resistance or help explain fitness differences in giant ragweed biotypes.

The low incidence of resistance alleles in natural populations without the selection pressure imposed by herbicide application suggests that resistance to herbicides may confer a fitness penalty (Jasieniuk et al. 1996; Purrington 2000; Preston and Powles 2002). A fitness penalty is defined as the disruption of survival, competitive ability, or reproductive success, and it collectively describes the evolutionary success of an individual in terms of its contribution of genes to the gene pool (Warwick and Black 1994). If the resistant biotype has no associated fitness penalty and is competitive with the susceptible it can set seed restocking the seed bank for the following growing season. For example, studies with biotypes of glyphosate-resistant horseweed from Indiana (Davis et al. 2009) and California (Shrestha et al. 2010) found no apparent fitness penalties associated with resistance, suggesting they will persist even if the selection pressure is reduced. Alternatively, those biotypes associated with a fitness penalty provide a potential target for management programs if it can be exploited to reduce the number of resistant plants (Jordon et al. 1999; Zhang et al. 1999; Pedersen et al. 2007). This is done by increasing competition, reducing seed output, and rotating herbicides (Preston and Wakelin 2008; Preston et al. 2009). For instance, glyphosate-resistant rigid ryegrass produced fewer but heavier seeds at low levels of competition (Pedersen et al. 2007) and the number of resistant plants declined when the selection pressure was relaxed (Preston et al. 2009). Furthermore, a glyphosate-resistant giant ragweed biotype from Indiana flowered earlier but produced 25% less seed than the susceptible biotype in the absence of glyphosate (Brabham et al. 2011). This last study focused on one biotype, so the potential differences between glyphosate-resistant biotypes collected in geographically separated fields were not compared. In addition, the identification of biotypes with the no symptom response to glyphosate provides a comparison between giant ragweed biotypes with different mechanisms of resistance. The individual resistance mechanisms affect the fitness differently if they involve a target site and non-target site mutation.

The objective of my research was to determine if the glyphosate-resistance

trait imparts a fitness penalty in multiple biotypes of giant ragweed with the rapid-necrosis or no symptom response to glyphosate. I hypothesized that glyphosate-resistance in giant ragweed is associated with a fitness penalty. I predicted that there would be an early season growth advantage for susceptible biotypes and glyphosate-resistant biotypes would flower earlier and produce less aboveground biomass compared to the susceptible controls. I predicted that glyphosate-resistant biotypes treated with 900 or 1800 g ae/ha would be controlled at the cotyledon and 2-leaf stage. I also predicted that the resistant biotypes would produce less seed when compared to the susceptible controls.

## 3.2 Methods

### 3.2.1 Experimental design

I established a greenhouse study at the Agriculture and Agri-Food Canada, Greenhouse and Processing Crops Research Centre in Harrow, Ontario. I designed the experiment in a randomized complete block design with four replications planted simultaneously on four different benches located within one greenhouse. The greenhouse temperature alternated between 25/20˚C day/night with a 14 h photoperiod. Seeds were collected from seven distinct *A. trifida* populations and were stored dry in the dark at 5˚C and a constant relative humidity (RH) of 60% until use. The biotypes I used in this experiment are listed in Table 3.1.

**Table 3.1** Summary of biotype information.

| Location | Coordinates | Year[a] | Glyphosate Susceptible[b] | Previous Glyphosate Exposure | Expected Response[c] | Known Resistance[d] |
|---|---|---|---|---|---|---|
| Cambridge, ON | N 43.387° W 80.348° | 2008 | S | No | D | – |
| Ridgetown, ON | N 42.460˚ W 81.892˚ | 2009 | S | No | D | – |
| Chatham, ON | N 42.440˚ W 82.305˚ | 2009 | S | No | D | – |
| Windsor, ON | N 42.279˚ W 82.961˚ | 2009 | R | Yes | RN | G ALS |
| Pickaway, Co, Ohio | N 39.528˚ W 83.176˚ | 2007 | R | Yes | RN | G ALS |
| Harrow, ON | N 42.038˚ W 82.986˚ | 2009 | R | Yes | N | G |
| Leamington, ON | N 42.099˚ W 82.644˚ | 2009 | R | Yes | N | G |

[a] Year seeds were collected in the field
[b] Susceptible (S) or resistant (R)
[c] Expected response to glyphosate: death (D), rapid necrosis (RN), or no response (N)
[d] Glyphosate (G), Acetolactate synthase (ALS)

### 3.2.2 Seed stratification and germination

I cold stratified the seeds for three months prior to the start of the experiment using the following procedure. I added Premier Pro-mix® Mycorise® pro to 4 cm depth in 28 labelled 10.2 cm diameter round plastic pots. I placed the pots on a tray and watered from the bottom until the surface was wet. I placed 50 seeds of each biotype on the surface and covered them with an additional 5 cm of potting media. I replicated this procedure 7 times for a total of 350 seeds for each biotype. The pots were then watered by hand to field capacity (watered gently until water started draining from pots with subsequent waiting for the water to stop draining) and incubated at 1˚C. After three months of stratification, I removed the seeds from the cold treatment, and prepared them for planting by rinsing them with water and drying them at room temperature.

I used a single-edged razorblade to remove the hull and seed coat from the embryo before germination. I did this by carefully cutting off the central spike and shaving the hull until it could easily be removed. Then I gently peeled the seed coat off the embryo. Excising the embryo ensured that I was germinating a viable embryo, as opposed to hollow involucres or badly bruised embryos. The excised embryos were placed in Petri dishes, moistened with 10 mL of reverse osmosis water, then incubated under alternating day/night temperatures of 30/20˚C with a 14 h photoperiod (Andersen 1968).

### 3.2.3 Experimental set-up

I planted all four replicates and leaf stages on the same day. I planted 96 germinated seeds of each resistant biotype and 24 germinated seeds of each susceptible biotype in separate pots filled with Premier Pro-mix® Mycorise® pro potting media and equally divided pots of each biotype between the 4 replicates. Six plants of each resistant biotype were grown to the cotyledon, 2-leaf, 4-leaf, and 8-leaf stage. Six plants of each susceptible biotype were grown to the 8-leaf stage and 2 plants of each susceptible biotype to each of the cotyledon, 2-leaf, and 4-leaf stage. In a previous experiment (Chapter 2) I confirmed that all susceptible biotypes exposed to glyphosate did not survive; therefore, I eliminated the herbicide treatments for the susceptible biotypes for each leaf stage from the design. I planted the seedlings that were grown to reproductive maturity in 20 L pots and plants

grown to lower leaf stages in 6.5 L pots. All of the plants could not be grown to seed, due to space limitations within the greenhouse.

### 3.2.4 Growth measurements

I monitored emergence so the morphological measurements (height, leaf number, number of axillary leaves, and number of axillay buds) could be taken weekly from the day of emergence until 28 d after treatment or plant death. When plants reached the corresponding leaf stage, I recorded height, leaf number, number of axillary leaves, and number of axillary buds on the day of herbicide application. I removed subsets of plants from each biotype and each greenhouse bench at the designated cotyledon, 2-leaf, 4-leaf, or 8-leaf stage and sprayed them with one of three treatments: 1. Untreated control – reverse osmosis water; 2. glyphosate – 900 g ae/ha; 3. glyphosate – 1800 g ae/ha. After treatment, I returned the plants to the greenhouse. I scored aboveground herbicide injury 2, 7, 14, 21, and 28 days after treatment on a scale of 0 to 100%: 0% no visible injury and 100% were completely necrotic.

The surviving 8-leaf resistant plants and the susceptible and resistant control plants were grown to reproductive maturity (64 plants/replication). At this point, I grouped similar biotypes together on the bench and hung sheer fabric (organza) screens around each biotype to prevent cross pollination. I assessed the maturity of the pollen spikes of each biotype 72 days after planting. I collected, counted and weighed all of the seeds from each plant. I also weighed three random samples of 25 seeds from each plant to calculate the average seed mass per plant. The aboveground plant material was dried at 65˚C for 1 week. I recorded the dry weight (g) of the aboveground tissue for individual plants. I stratified the seeds following the procedures outlined in section 3.2.2.

### 3.2.5 Fluorescence experiment

I conducted a second greenhouse experiment at the University of Western Ontario to quantify the herbicide damage on glyphosate-resistant and susceptible giant ragweed plants. The biotypes used for this experiment were Ridgetown, Chatham, Windsor, Harrow, and Leamington (Table 3.1). I carefully cut the involucres and seed coats off 100

seeds of each biotype following the procedures outlined in section 3.2.2. I placed the excised embryos in moistened Petri dishes and incubated them under alternating day/night temperatures of 30/20˚C with a 14 h photoperiod (Andersen 1968). I potted 12 germinated seeds of each biotype in separate 12 L pots filled with Premier Pro-mix® Mycorise® pro potting media. The 12 pots of each biotype were assigned one of three glyphosate treatments (0, 900, or 1800 g ae/h) and were divided into 4 replicates ensuring that each replicate had all three glyphosate treatments.

When the plants reached the 8-leaf stage, I measured the photosynthetic rate per unit leaf area (μmol of $CO_2/m^2/s$), stomatal conductance (mol $H_2O/m^2/s$), and chlorophyll *a* fluorescence on the middle lobe of the newest fully expanded leaf using a LI-6400 XRT Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE, USA). The leaf was dark adapted for 20 minutes prior to fluorescence measurement. I took all measurements at 390 ppm of $CO_2$ and 25˚C leaf temperature. I took the light reaction measurements at a light intensity of 1000 $\mu mol/m^2/s$ and the final recording was made 15 minutes after the fluorescence measurements. After the physiological measurements, I sprayed plants with the assigned herbicide treatment in an enclosed herbicide spray chamber. The LI-6400 XRT was used to repeat the fluorescence and photosynthetic measurements on all the 8-leaf stage plants 6-12 h, 24 h, and 1 week after glyphosate treatment.

### 3.2.7 Data analysis

I designed the experiment as a randomized complete block design with 4 blocks (benches) within a single greenhouse compartment. I ran a general linear model in JMP 4.0 (SAS Institute, Inc., Cary, NC, USA) to analyze the effects of biotype, leaf stage, and spray dose on herbicide injury, height, leaf number, lateral growth, biomass, and seed number. I analyzed two-way and three-way interactions between fixed factors for each dependent variable. The fixed factors that did not have significant effect on the dependent variables were pooled. Transforming the data did not improve the normality of the herbicide injury scores, height, or lateral leaf number; however, biomass, seed number, and seed mass were log transformed to improve normality. I also ran a univariate repeated measures analysis to determine any differences between herbicide injury and day after

treatment. Spray dose and biotype were nested with pot number and the random effects were tested. The effects were determined to be significant if $p < 0.05$. Least-squares means contrast test and Tukey's HSD test were used to compare means. I calculated the reproductive ratios for each biotype and spray dose (eqn 3.1).

reproductive ratio = (seed mass)/ (total shoot mass + seed mass)

**Eqn 3.1**

## 3.3 Results

### 3.3.1 Seedling growth

Before glyphosate treatment, biotype had no influence on the height and lateral growth, except at the 2-leaf stage (Table 3.2). By 28 days after spraying, plants treated with glyphosate (900 and 1800 g ae/ha) at the cotyledon, 2-leaf, and 4-leaf stages were shorter and had less lateral growth compared to the controls ($p<0.001$; A.1, A.2). At the 8-leaf stage, there was an interactive effect of biotype and spray dose on the height and lateral growth, as a result of the large differences in growth of the glyphosate treated susceptible plants compared to the untreated control susceptible plants ($p<0.001$; Figure 3.1, 3.2).

### 3.3.2 Herbicide injury

Biotype, spray dose, and day interacted with herbicide injury scores 2 and 7 days after glyphosate treatment ($p=0.001$). The rapid necrosis biotypes had the highest level of injury on day 2 and 7, but due to variation within the Harrow, Ridgetown, and Cambridge biotypes sprayed with 1800 g ae/ha the injury was not significantly higher (Figure 3.3). On day 7, the Cambridge and the Leamington biotypes sprayed with 1800 g ae/ha showed more injury than on day 2, while the Windsor biotype sprayed with 900 g ae/ha started to recover from injury (Figure 3.3).

**Table 3.2** Summary of p-values from a one-way ANOVA for the effects of biotype and spray dose on growth.

| Leaf Stage At Spray | Effects | Initial Height | 28-day Height | Initial Lateral Growth | 28-day Lateral Growth |
|---|---|---|---|---|---|
| 8-leaf | B | 0.240 | 0.010* | 0.086 | <0.001*** |
| | SD | 0.277 | <0.001*** | 0.743 | <0.001*** |
| | B*SD | 0.469 | <0.001*** | 0.065 | <0.001*** |
| 4-leaf | B | 0.762 | 0.263 | --- | 0.246 |
| | SD | 0.422 | <0.001*** | --- | <0.001*** |
| | B*SD | 0.532 | 0.923 | --- | 0.921 |
| 2-leaf | B | 0.001** | 0.006** | --- | 0.853 |
| | SD | 0.215 | <0.001*** | --- | <0.001*** |
| | B*SD | 0.650 | 0.352 | --- | 0.959 |
| Cotyledon | B | 0.488 | 0.001*** | --- | 0.701 |
| | SD | 0.899 | <0.001*** | --- | <0.001*** |
| | B*SD | 0.793 | 0.930 | --- | 0.659 |

Effects: Biotype (B) and spray dose (SD)
Asterisks denote a significant effect (*0.05-0.01, **<0.01-0.001, *** <0.001)

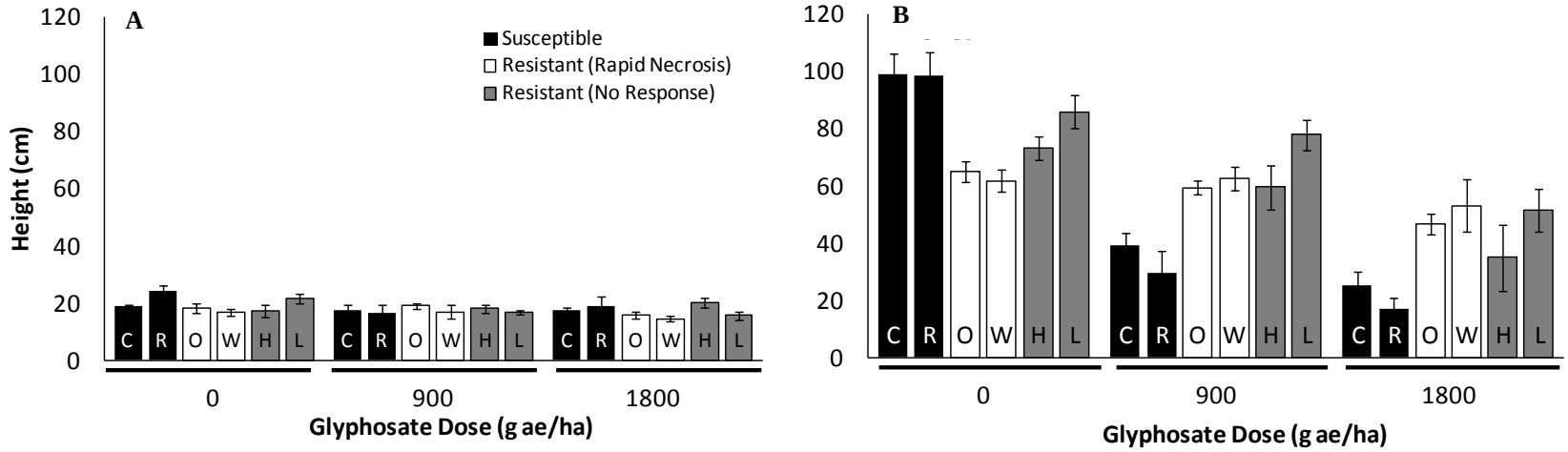


**Figure 3.1** Height (cm) of giant ragweed seedlings on the day of (A) and 28 days after (B) treatment with glyphosate. Seedlings were sprayed with glyphosate at the 8-leaf stage. The mean heights for each biotype (C= Cambridge, R= Ridgetown, O= Ohio, W= Windsor, H= Harrow, L= Leamington; table 3.1) are plotted and the error bars represent standard error (n=2-8). Associated significance values are noted in table 3.2. Figures for the cotyledon, 2-leaf, and 4-leaf stages are appended.

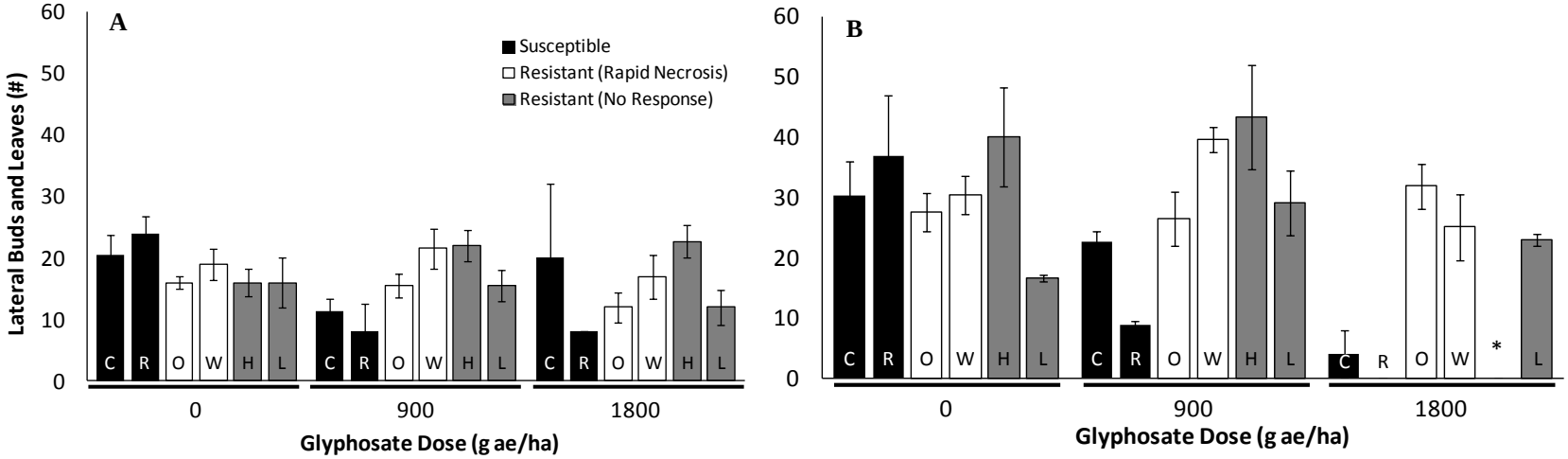


**Figure 3.2** Number of lateral leaves and buds on giant ragweed seedlings on the day of (A) and 28 days after (B) glyphosate treatment. Seedlings were sprayed with glyphosate at the 8-leaf stage. The mean number of leaves and buds for each biotype (C= Cambridge, R= Ridgetown, O= Ohio, W= Windsor, H= Harrow, L= Leamington; table 3.1) are plotted and the error bars represent standard error (n=2-8). The asterisk denotes a missing data point. Associated significance values are noted in table 3.2. Figures for seedlings sprayed at the cotyledon, 2-leaf, and 4-leaf stages are appended.

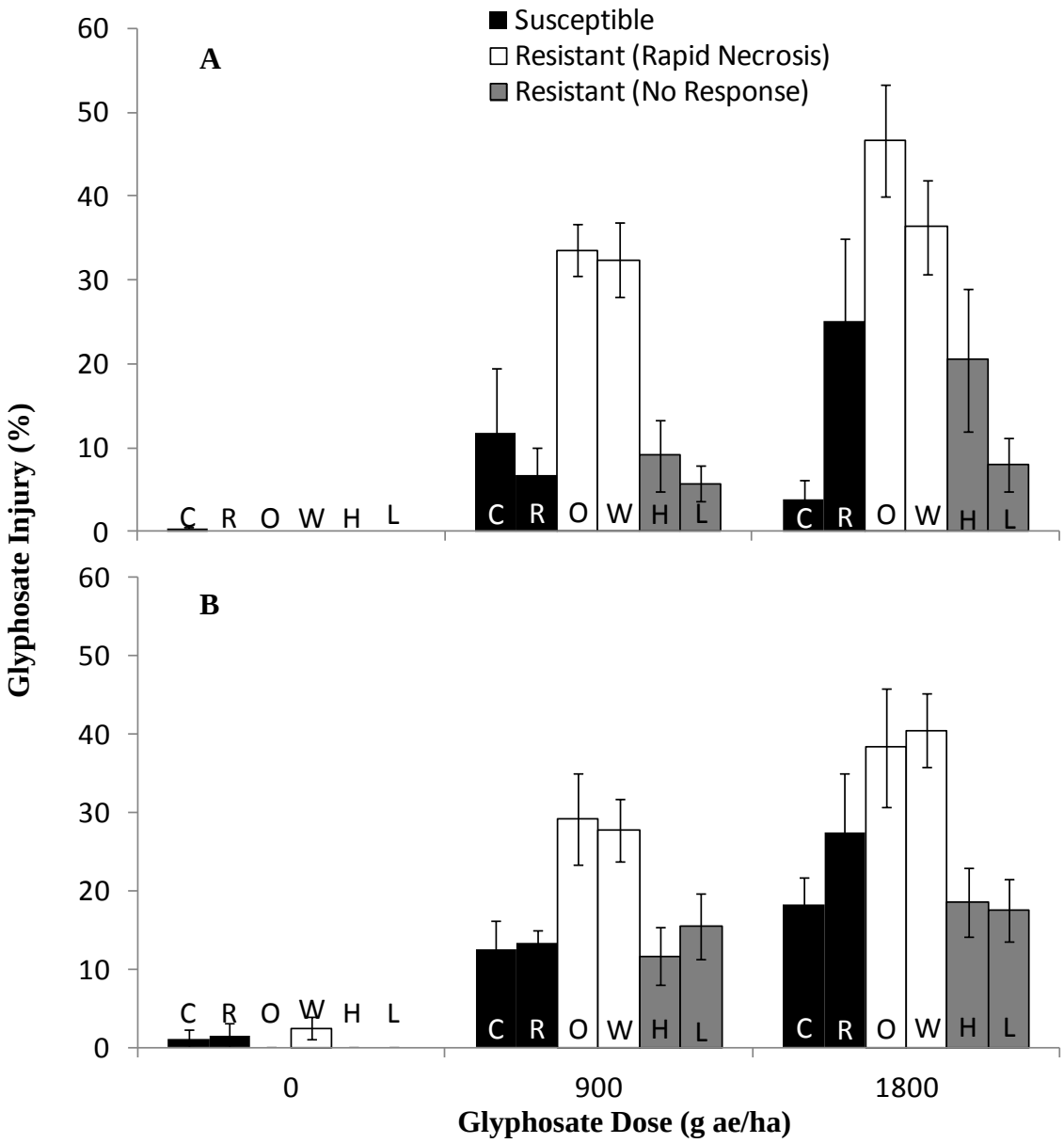


**Figure 3.3** Percentage of aboveground tissue damaged for seedlings 2 (A) and 7 (B) days after treatment with glyphosate. The mean injury scores for each biotype (C= Cambridge, R= Ridgetown, O= Ohio, W= Windsor, H= Harrow, L= Leamington; table 3.1) are plotted and the error bars represent standard error (n=2-24). One Ridgetown outlier (1800 g ae/ha) was removed because insect damage, inflated the herbicide injury scores.

### 3.3.3 Aboveground biomass and seed harvest

By 72 days after planting, about 80% of susceptible plants, 45% of rapid necrosis plants, and 40% of no response plants had produced mature pollen spikes. A mature pollen spike was recorded if it had elongated and was producing pollen. Spray dose and leaf # at the time of spray, did not affect the final biomass ($p=0.515$, $p=0.463$), seed number ($p=0.301$, $p=0.755$), or seed mass ($p=0.479$, $p=0.642$, respectively); however, biotype significantly influenced the biomass ($p=0.021$) and total seed number ($p<0.001$). The resistant biotypes with the rapid necrosis response, produced 33% less biomass than the resistant biotypes that showed no response to glyphosate ($p=0.002$; means contrast test; Figure 3.4). The susceptible and resistant rapid necrosis biotypes produced, on average, 54% and 47% respectively, fewer seeds than the no response resistant biotypes ($p=0.001$, $p<0.001$; means contrast test; Figure 3.5 A). Finally, there were differences in seed mass between biotypes ($p=0.004$). Despite producing more seeds, the resistant biotypes with no response produced seeds that were 16% lighter than the rapid necrosis biotypes and 10% lighter than the susceptible biotypes ($p<0.001$, $p=0.031$; means contrast test; Figure 3.5 B). In addition, the interaction between biotype and spray did not influence the reproductive ratios ($p<0.001$; Table 3.3).

**Table 3.3** Seed mass as a percentage of average plant mass for each biotype and spray dose.

| Biotype | Response to Glyphosate | Spray Dose (g ae/ha) | | |
|---|---|---|---|---|
| | | **0** | **900** | **1800** |
| Cambridge | Susceptible | 2.32 | 0 | 0 |
| Ridgetown | | 4.15 | 0 | 0 |
| Ohio | Rapid Necrosis | 3.08 | 3.32 | 2.45 |
| Windsor | | 2.09 | 2.17 | 2.64 |
| Harrow | No Response | 1.96 | 2.21 | 2.00 |
| Leamington | | 3.20 | 3.24 | 3.19 |

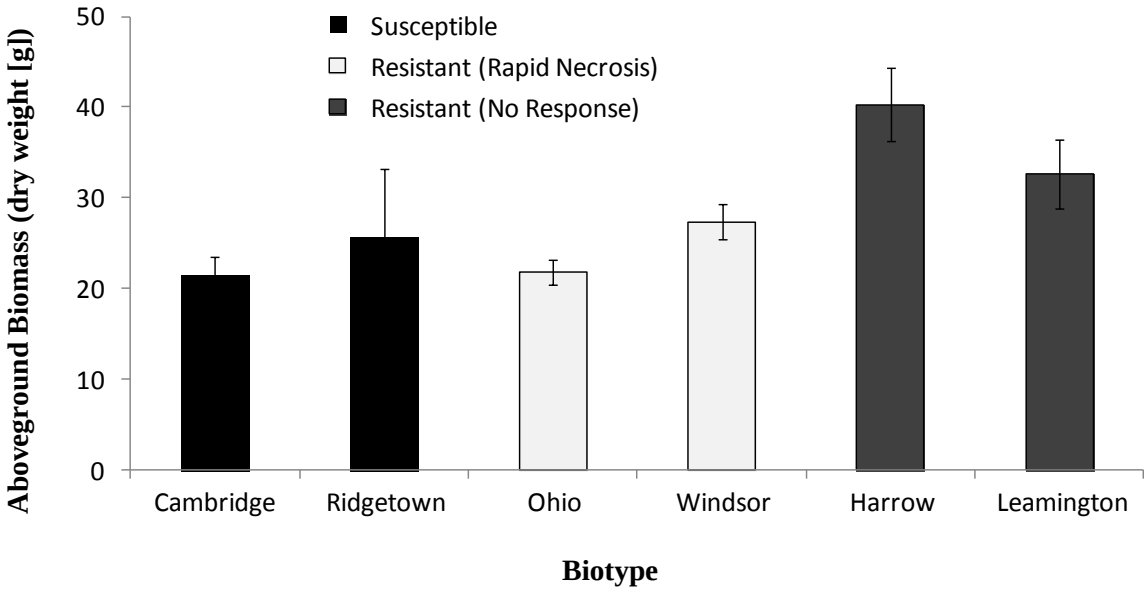


**Figure 3.4** Aboveground biomass collected on the final day of seed harvest and dried at 60˚C for 7-10 days. The mean masses for each biotype are plotted and the error bars represent standard error. Spray dose and leaf number were not significant (p=0.4229, p=0.3568 respectively), so they were pooled within a biotype. The sample size for each biotype was: Cambridge (n=7), Ridgetown (n=4), Ohio (n=18), Windsor (n=48), Harrow (n=25), Leamington (n=24).

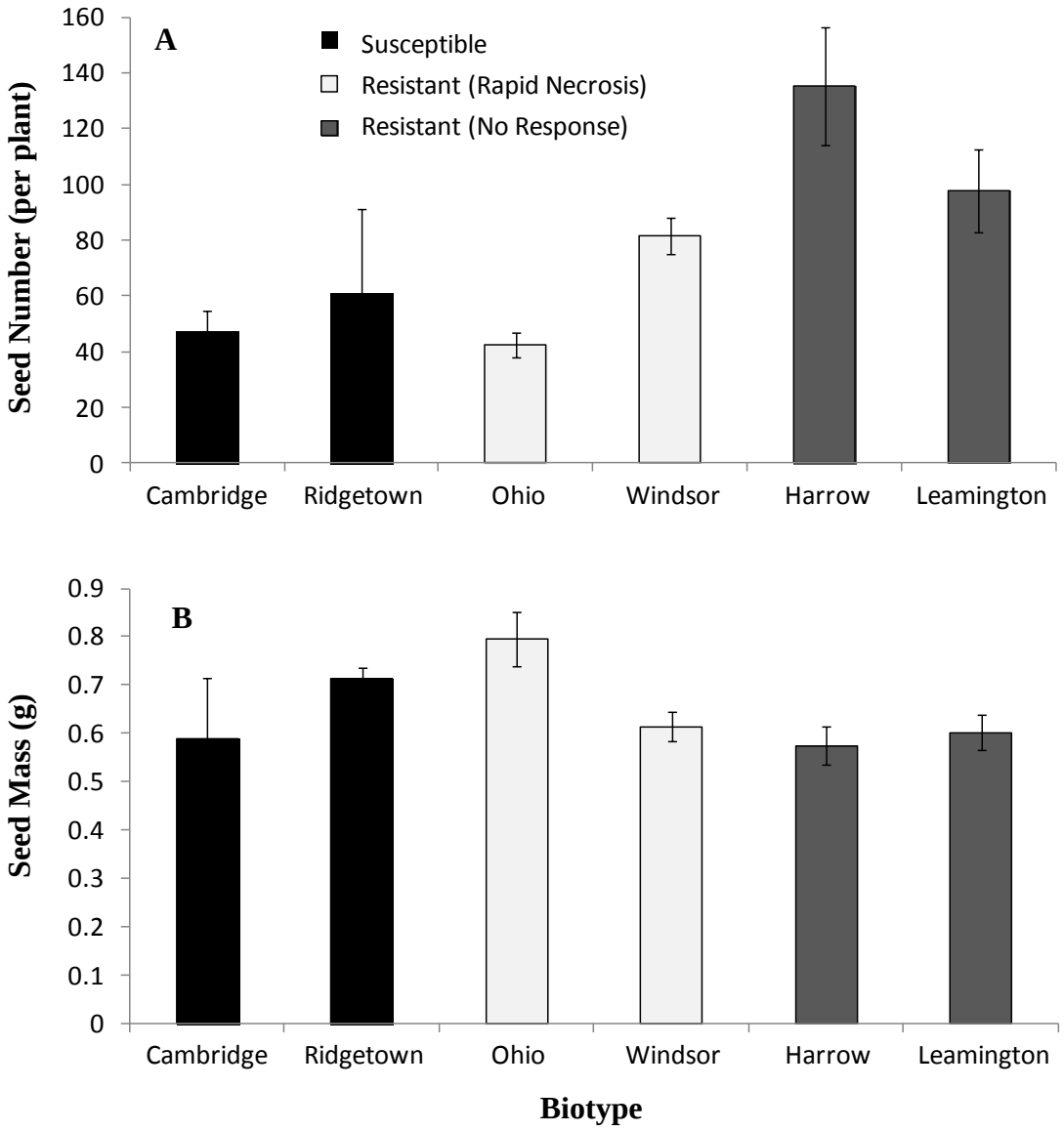


**Figure 3.5** Mean seed number per plant (A) and seed mass (B) for each biotype. The seed mass was calculated based on three random samples of 25 seeds for each individual plant. Spray dose and leaf number were pooled within a biotype. The sample size for each biotype was: Cambridge (n=6), Ridgetown (n=4), Ohio (n=18-19), Windsor (n=47-48), Harrow (n=22-24), and Leamington (n=22-24).

### 3.3.4 Chlorophyll a fluorescence

At one week after glyphosate treatment, there was an interaction between spray dose and response to glyphosate on the photochemical efficiency (Fv/Fm) and steady state fluorescence (Fv'/Fm') ($p<0.001$, $p=0.031$, respectively). There was also an interaction between time of measurement and response to glyphosate on Fv/Fm and Fv'/Fm' ($p=0.001$, $p=0.024$, respectively). This is largely attributed to the leaves of the Windsor biotype sprayed with 1800 g ae/ha of glyphosate becoming necrotic by the 1 week measurement (Tukey's HSD test; Figure 3.6 A, B). Photochemical quenching (qP) was not influenced by biotype or spray dose, but the qP was slightly reduced by the 1 week measurement for the susceptible biotypes ($p=0.080$; Figure 3.6 C). Non-photochemical quenching declined (NPQ) at higher spray doses ($p=0.017$) and with time from glyphosate treatment ($p<0.001$; Figure 3.6 D).

### 3.3.5 Carbon fixation

The number of carbon molecules fixed per photon of light ($\varphi CO_2$) and photosynthetic rate decreased at 6 h and 1 week after glyphosate treatment ($p=0.004$, $p=0.001$, respectively; Figure 3.7A). The $\varphi CO_2$ was slightly lower when plants were treated with glyphosate at 900 and 1800 g ae/ha ($p=0.075$). Following treatment with glyphosate, the photosynthetic rate declined compared to that of the untreated controls ($p=0.001$; Figure 3.7B).

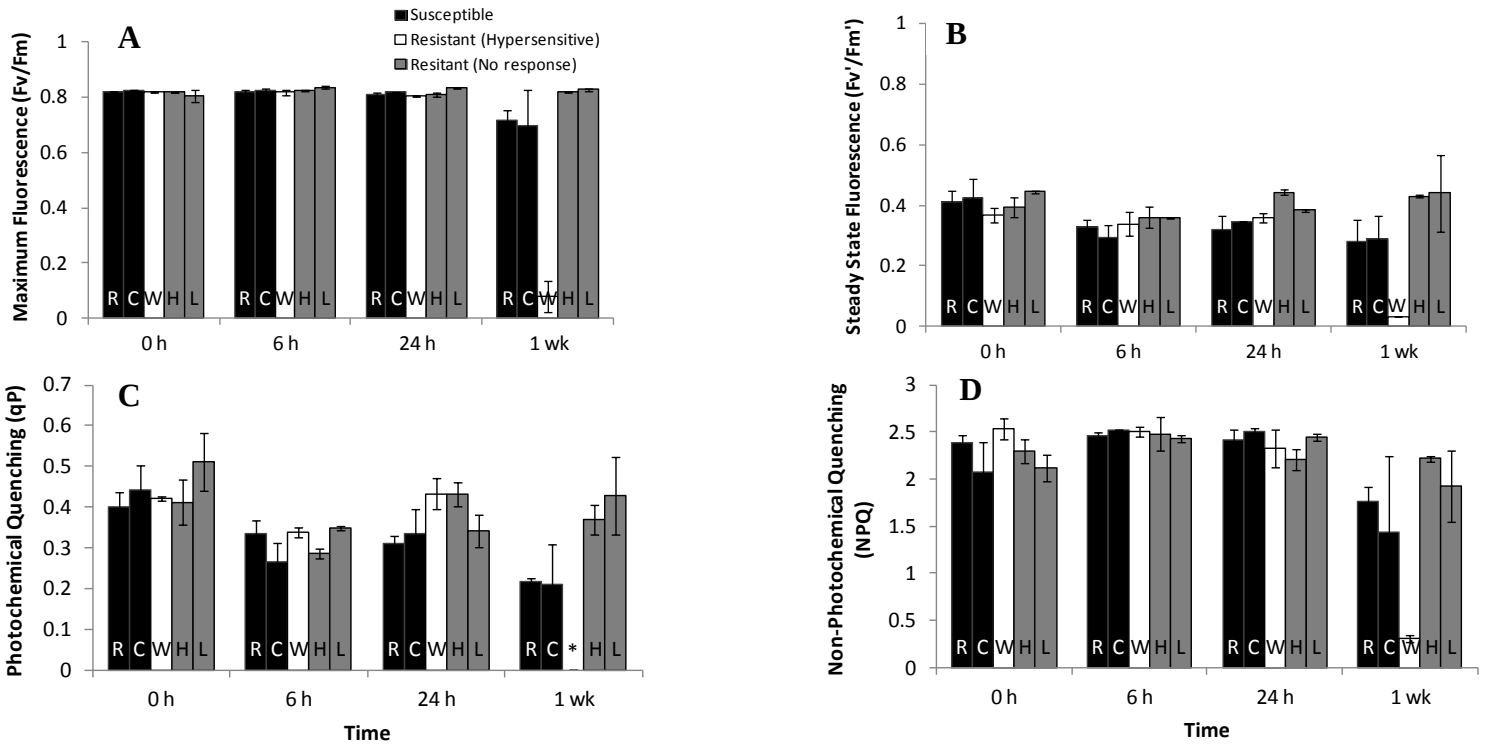


**Figure 3.6** Photochemical efficiency of the PSII reaction centre. Plants were treated with 1800 g ae/ha of glyphosate at the 8-leaf stage and measurements were taken at four time intervals from the time of glyphosate treatment. The maximum fluorescence measurements (A) were taken after a 15 minutes dark adaptation period. The steady state fluorescence (B), photochemical quenching (C), and non-photochemical quenching (D) were taken 15 minutes after the maximum fluorescence measurement. Mean values for each response are plotted and the error bars represent standard error (n=2-4). The letters on the bars identify biotypes (Table 3.1; R= Ridgetown, C=Chatham, W= Windsor, H= Harrow, L= Leamington). The asterisk denotes a missing data point.

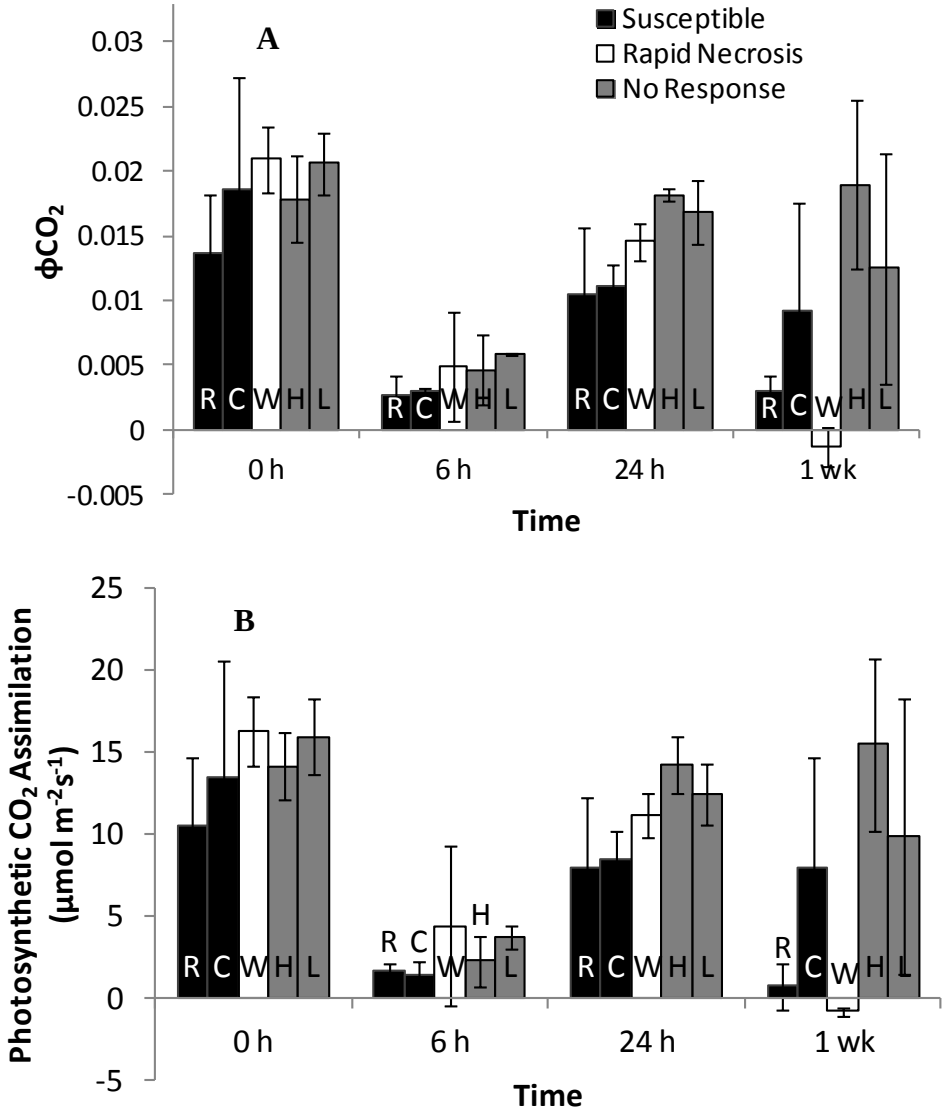


**Figure 3.7** Carbon fixation rates, measured in terms of quantum yield of $CO_2$ ($\varphi CO_2$) (A) and photosynthetic rate (B) for plants treated with 1800 g ae/ha of glyphosate at the 8-leaf stage. Measurements were taken at four time intervals from the time of glyphosate treatment. Both measurements were based on a single recording at 25˚C, 390 ppm of $CO_2$, and a 1000 µmol $m^{-2}$ $s^{-1}$ light intensity. The letters on the bars identify biotypes (R= Ridgetown, C= Chatham, W= Windsor, H= Harrow, L= Leamington; table 3.1). Mean values for each response are plotted and the error bars represent standard error (n=2-4).

## 3.4 Discussion

### 3.4.1 Seedling growth in the presence and absence of glyphosate

My experiment tested the hypothesis that glyphosate-resistant biotypes would have reduced seed output in the absence of glyphosate treatment. Contrary to my prediction, there was no growth advantage for the susceptible biotypes before glyphosate treatment suggesting that there are no observable costs for biotypes carrying the resistant trait. This contrasts the results of Brabham (2011), who found that a glyphosate-resistant giant ragweed biotype grown in the field was taller than a susceptible biotype until 43-50 days after planting (DAP) at which point, there was no difference in height. The different findings may be a result of the genetic variation between biotypes or the differences between the greenhouse and field growth environment (Warwick 1991; Jordon 1992; Warwick and Black 1994). In the absence of glyphosate, the susceptible biotypes and the Leamington (no symptom) biotype treated at the 8-leaf stage were taller than the Ohio (rapid necrosis), Windsor (rapid necrosis), and Harrow (no symptom) biotypes at 50 DAP suggesting a possible cost to resistance; however, this trend was not consistent in control plants at the 4-leaf (38 DAP), 2-leaf (35 DAP), or cotyledon (31 DAP) stages. It is possible that the growth measurements for the 4-leaf, 2-leaf, and cotyledon stage were confounded by the smaller pot size that was used to grow the susceptible control biotypes.

### 3.4.2 Glyphosate injury

Consistent with the described mechanism of resistance, the biotypes with the rapid necrosis response had the greatest percentage of injury on day 2 and 7. The leaves of these plants became chlorotic and necrotic from the leaf tip inwards and by 2 days after treatment, the leaves were curling under. The no response resistant biotypes developed chlorosis on the apical buds and petioles by 7 days after treatment showing that they did have mild symptoms of glyphosate stress.

At doses of glyphosate lethal to the susceptible biotypes, the resistant biotypes recovered and grew to a similar height and produced a similar number of lateral leaves and buds as the controls by 28 days after treatment. The death of the susceptible and recovery of the

resistant plants caused the interactive effect of biotype and spray dose on the height and lateral growth of glyphosate treatment (Figure 3.1, 3.2). The interaction between spray dose, biotype, and day on herbicide injury can be explained by the slight recovery of the rapid necrosis biotypes on day 7 as well as the large variation in percent injury of the susceptible and no response biotypes on both days. Because there was no significant difference between percent injury of 2- and 8-leaf stage plants, treating biotypes at very early leaf stages did not improve the control of resistant biotypes. In fact, control of resistant plants was not achieved at any of the tested leaf stages (data not shown). This contrasts the results of Norsworthy et al. (2010) who reported 72% control of glyphosate-resistant giant ragweed plants at the 2 node stage (2-leaf), 44% at the 4 node stage (6-leaf), and 49% at the 6 node stage (10-leaf) 28 days after treating plants with 870 g ae/ha of glyphosate. It also contrasts the findings of studies which have reported better control over glyphosate-resistant horseweed and hairy fleabane at younger growth stages (Dinelli et al. 2006; Urbano et al. 2007). The different levels of control can probably be attributed to the biotypes having different genetic backgrounds (Jasieniuk et al. 1996; Vila-Aiub et al. 2009).

### 3.4.3 Biomass and seed harvest

Contrary to my hypothesis, the resistant biotypes with the rapid necrosis response accumulated a similar amount of aboveground biomass as the susceptible controls. This suggests that recovery from the glyphosate injury is not as costly as expected. Because the aboveground biomass was similar between glyphosate treatments, resistant biotypes in the absence of glyphosate were not at a disadvantage. In fact, the no response biotypes had the most aboveground biomass at the final harvest. It is difficult to say why this was the case because the specific resistance mechanisms are unknown. However, it is possible that glyphosate-resistant plants also experience a disruption to the production of the aromatic amino acids such as tryptophan. Tryptophan is a precursor in the production of auxin, the phytohormone responsible for apical dominance (Last and Fink 1988). If the apical dominance is relieved, the resistant plants may increase axillary bud production and lateral branching by the final harvest (Cline 1991; Thomas et al. 2005). This is supported by the findings of Green et al. (2011) who found an accumulation of shikimate

in the young tissues of resistant and susceptible biotypes, minimal accumulation in the old tissues of the rapid necrosis biotype, and similar accumulation between the old tissues of susceptible and no-response resistant biotypes. Therefore, the no response biotypes may have accumulated the most aboveground biomass as a result of an increased initiation and development of lateral branches.

The reproductive ratios for the rapid necrosis biotypes suggest that these seeds were, on average, 0.24 g heavier after treatment with glyphosate (highest ratio: 900 g ae/ha [Ohio], 1800 g ae/ha [Windsor]), so these biotypes were potentially more reproductively successful following glyphosate treatment. Furthermore, the no response resistant biotypes allocated slightly more mass to seed after treatment with either dose of glyphosate, suggesting they are also more reproductively successful in the presence of glyphosate. However, when seed numbers were analyzed, no significant differences between spray doses were found. Because giant ragweed plants are known to produce seeds without embryos to deter predators, seed number is not the best indicator of reproductive output (Abul-Fatih and Bazzaz 1979; Stoller and Wax 1974; Harrison et al. 2001). In addition, seed mass may provide an indicator of the ability of a seed to germinate, because heavier seeds may have more stored resources (Primack and Kang 1989); therefore factoring in seed mass, as was done in the reproductive ratios, provides a better assessment of reproductive investment by the parent plant (Harper and Ogden 1970).

### 3.4.4 Physiological measures of herbicide injury

Approximately 6 h after spray, all glyphosate treated and untreated controls experienced a reduction in stomatal conductance, transpiration, and carbon fixation. Even though glyphosate can induce plants to close stomata (Brecke and Duke 1980) the same reduction was measured in control plants suggesting that it was caused by environmental conditions. The plants were removed from the greenhouse and isolated for 24 hours prior to glyphosate treatment to reduce the risk of insect transfer to the spray area. After spray, the plants were placed in the laboratory [ca. 20˚C] until the 6 h measurements were complete. These measurements were taken between 13:00 and 21:00 h, which is the period of the day when the guard cells begin to close (Talbott and Zeiger 1998). The 24 h

measurements taken between 07:00 and 15:00 h, the time of day when stomata are open, were more similar to the 0 h measurements. Therefore, it is very likely that the measurements were influenced by stomatal conductance, so I was not able to detect the indirect effect of glyphosate on photosynthetic electron transport or carbon fixation.

The fluorescence measurements were not influenced by glyphosate treatment. This is consistent with the study by Olesen and Cedergreen (2010) who found no specific changes in chlorophyll fluorescence of barley leaves at 24 hours after spray. Therefore, even though chlorophyll fluorescence was an effective method to determine triazine resistance in multiple species (Ali and Souza Machado 1981), it does not appear to be an effective determinant of glyphosate resistance in giant ragweed biotypes.

## 3.5 Conclusion

At 50 DAP the resistant biotypes appeared to have a fitness cost in the absence of glyphosate treatment; however, the final aboveground biomass measures suggest that the resistant plants recover and have similar or greater aboveground biomass compared to the susceptible biotypes. In addition, glyphosate treatments did not prevent the resistant biotypes from recovering and setting seed, which has important implications for the use of glyphosate in fields infested with resistant biotypes. With the continued use of glyphosate the selection pressure for the resistant traits is strong, promoting the emergence of resistant plants in future growing seasons and the evolution of new resistant biotypes. To alleviate the selection pressure, glyphosate application would have to cease, which is extremely unlikely giving the low cost and wide-usage of glyphosate in Roundup Ready cropping systems. In addition, with the production of crops with stacked herbicide tolerance to glyphosate and other herbicides, the usage of glyphosate is not expected to decline. Moreover, crops tolerant to multiple herbicides require growers to spray herbicides with different modes of action which can increase the likelihood that weeds will evolve multiple resistant traits. This challenge has caused some growers to bring back tillage and hand removal strategies (Alder 2011), in addition to chemical applications, for weed control; these methods were initially abandoned due to the increased soil erosion, increased nutrient runoff, and high production costs associated

with increased labour, respectively. Currently, the best recommendation for management of resistant weeds is to rotate crops so herbicides with multiple modes of action can be integrated into management programs (Beckie 2006) or to include herbicide tank-mixes to prevent weeds from evolving resistance (Beckie and Reboud 2009).

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# Chapter 4

# Conclusions and Future Direction

## 4.1 Research findings

Overall, glyphosate-resistant biotypes tested in both the Biotron and greenhouse experiments were not controlled with glyphosate at any of the tested spray doses or leaf stages. Higher temperatures improved the initial rate of glyphosate damage as a result of increased translocation, but plants placed back into the warmer environments had less aboveground injury 28 days after treatment. The ambient $CO_2$ concentration did not influence the herbicide efficacy at any of the tested leaf stages suggesting that $CO_2$ enrichment does not reduce glyphosate uptake in giant ragweed seedlings. The changes in carbon fixation 6-12 h after glyphosate treatment were attributed to changes in stomatal conductance because the measurements were taken mid-day when stomata were likely closed; therefore, I was not able to detect any indirect effects of glyphosate on carbon metabolism. Despite not achieving complete control of the resistant biotypes, seedlings recovering from glyphosate treatment did show symptoms of glyphosate injury. For example, some seedlings lost apical dominance and produced numerous lateral buds that never developed into leaves. Originally it appeared as though the seedlings would not recover, however results from the fitness experiment suggested that plants showing symptoms of glyphosate injury can recover and produce seed.

Seed number and mass varied between resistant and susceptible biotypes and between biotypes that showed a similar response to glyphosate. The susceptible biotypes had the highest overall reproductive ratio (3.24%), while the rapid necrosis (2.75%) and no symptom (2.73%) resistant biotypes had slightly higher reproductive ratio following treatment with glyphosate at 900 g ae/ha. The resistant biotypes seem unable to match the reproductive output of the susceptible biotypes in the absence of glyphosate (Brabham et al. 2011). However, in all instances I was not able to prevent glyphosate-resistant giant ragweed biotypes from producing seeds.

As more weed species become resistant to glyphosate, weed control in Roundup Ready cropping systems becomes a challenge. As was shown in this study, labelled field rates (900 or 1800 g ae/ha) of glyphosate did not control glyphosate-resistant giant ragweed seedlings at the cotyledon and 2-leaf stages. Continued use of glyphosate promotes the survival of resistant biotypes by selectively removing susceptible plants and imposing a selection pressure for resistance (Owen 2008). Surviving resistant plants produce millions of pollen grains each day (Bassett and Crompton 1982) that can disperse up to a kilometer away from the parent plant (Raynor et al. 1970). This allows for gene flow between nearby biotypes of giant ragweed and increases the potential for resistance to spread (Jasieniuk et al. 1996) because the semi-dominant or dominant resistance trait can be carried by pollen or seed (Preston and Wakelin 2008; Heap 2012; Beckie 2011; Brabham et al. 2011).

Glyphosate is relatively inexpensive ($4-10/acre) making it unattractive for growers to abandoned Roundup Ready crops (Smith 2010) when other herbicides may cost $20-30/acre. To offer solutions to prevent or delay resistance, crops tolerant to multiple herbicides are being developed (Dill et al. 2008; Feng et al. 2010; Alder 2011); however this technology may just add additional selection pressures so weeds with multiple herbicide resistant traits evolve. In addition, the resistance genes added to crops usually include herbicides that have a longer soil residual time than glyphosate, the potential to run-off, or implications for other organisms in the environment. Tilling and manual weed pulling have been re-introduced by some growers to improve weed control (Alder 2011); however, these methods are associated with higher costs and tilling can have a greater environmental impact because it can cause nutrient run-off and soil erosion. Currently, the best solution to relax the selection pressure for resistance is to rotate crops or incorporate tank-mixes so herbicides with different modes of action can be incorporated into weed management programs (Beckie 2006; Powles 2008; Beckie and Reboud 2009; Norsworthy et al. 2010; Brabham 2011; Norsworthy et al. 2011).

### 4.2 Potential limitations

The first limitation to my study was the space constraints in the growth rooms. To accommodate the number of treatments and replications, I had to choose the pot size that

would best fit the space. Unfortunately this limited the pot size to 6 L for seedlings in the Biotron experiment and plants not grown to seed in the greenhouse experiment and 20 L for plants grown to seed. In the Biotron experiment, pot size may have also influenced the sink storage capacity of seedlings limiting the response to elevated $CO_2$ (Arp 1991). In the greenhouse experiment, smaller pots caused a noticeable reduction in the height of control plants at the 4-leaf, 2-leaf, and cotyledon stages. By the final seed harvest, the roots of plants grown to seed in the 20 L pots appeared to be pot bound, limiting the overall sink capacity; the largest pots available to fit the space were chosen so the limited sink capacity is an artifact of running greenhouse trials. The limited space also prevented me from growing all of the leaf stage and treatment combinations to seed, so I cannot draw conclusions about differences in seed production and leaf stage for each glyphosate treatment.

I experienced difficulties germinating the seeds following published germination protocols (Anderson 1968; Abul-Fatih and Bazzaz 1979). During the failed germination attempts, many seeds rotted or became moldy. I ended up cutting the seed coats off the embryos of cold stratified seeds to germinate them for the greenhouse trials. Cutting the seed coat off removed the seed coat-imposed dormancy and improved germination rates (Schutte et al. 2012). Giant ragweed seeds require a lengthy 8-12 week cold stratification period to break the physiological dormancy (Davis 1930; Ballard et al. 1996; Ali-Rachedi et al. 2004; Baskin and Baskin 2004; Finch-Savage and Leubner-Metzger 2006; Shutte et al. 2012), so it was not possible to replenish seeds lost from failed germination attempts. In addition, seeds were collected from specific biotypes in the field between 2007 and 2009, so seed stocks for each biotype were limited. Therefore, the number of seeds available for my experiment limited the number of replicates I could conduct.

Finally, variation between biotypes with the same resistance mechanisms makes it difficult to determine if other resistant biotypes respond in the same way to glyphosate treatments. Norsworthy et al. (2010) achieved 72% control of glyphosate-resistant giant ragweed seedlings from Tennessee treated at the 2-leaf stage, while Norsworthy et al. (2011) achieved 57% and 60% control of resistant seedlings from Arkansas treated at the 2-leaf stage. In my trials, I achieved 0% control of resistant biotypes by 28 days after

treatment. The major difference between studies was the particular resistant biotypes tested. Despite the differences between biotypes, all studies came to the same conclusion that management of glyphosate-resistant giant ragweed plants should include herbicides with multiple modes of action.

### 4.3 Future experiments

In future experiments, it would be beneficial to grow giant ragweed in the presence and absence of crop competition. Growing giant ragweed in competition with crops can increase seed production from < 500 seeds/plant to ≤ 5100 seeds/plant (Abul-Fatih and Bazzaz 1979; Baysinger and Sims 1991; Johnson et al. 2006; Brabham et al. 2011). Giant ragweed plants tend to grow 0.30 - 1.52 m taller than the crop they are competing with and allocate reproductive structures based on their position in the stand (Abul-Fatih et al. 1979; Johnson et al. 2006). I would expect the resource allocation to vary in competition trials and exacerbate potential fitness differences between susceptible and resistant biotypes (Pedersen et al. 2007). Therefore, I would suggest running a greenhouse fitness experiment in the presence of crop competition and I would repeat the fitness experiment under field conditions. In field trials, Brabham et al. (2011) calculated much higher reproductive ratios for glyphosate-resistant (16.2%) and susceptible (19.2%) plants in the absence of glyphosate. This is probably related to the higher number of seeds produced by giant ragweed plants in the field. Extreme caution must be taken in field trials, especially if multiple biotypes of giant ragweed are planted, to prevent the outcrossing of resistance to neighbouring giant ragweed biotypes. Long-term studies monitoring resistant giant ragweed in the absence of glyphosate could determine if it is possible for resistant biotypes to shift to susceptible biotypes in the absence of glyphosate selection pressure over multiple generations. Experiments should also continue to identify the resistance mechanisms in giant ragweed, because knowledge of the resistance mechanism may provide new target sites for controlling glyphosate-resistant biotypes.

Future studies should also describe the optimal germination and stratification conditions for seeds from multiple biotypes of giant ragweed collected in agricultural and non-agricultural fields. The published protocols were tested on non-agriculture biotypes that emerge in the early spring (Anderson 1968; Abul-Fatih and Bazzaz 1979). Because the

agricultural biotypes have a second wave of emergence in June or July, they have an extended physiological dormancy preventing germination at low temperatures (Johnson et al. 2006; Schutte et al. 2012). Therefore, they should require a longer cold stratification period and germination should occur at a higher soil temperature. In addition, the viability of seed in the soil seed bank, especially in the upper 5 cm, can be quickly depleted in the first 4 years after deposition (Stoller and Wax 1974; Harrison et al. 2003; Harrison et al. 2007). Studies characterizing the initial viability of seeds and the timing between germination and emergence could potentially identify new target sites for giant ragweed management.

Finally, studies should include light response curves to determine if the photosynthetic capacity is reduced following glyphosate treatment. Because my measurements were taken at a fixed light intensity, it is difficult to determine if I was measuring the maximum carbon fixation rates. These studies can provide more details about the indirect influences of glyphosate on the photosynthetic electron transport chain and carbon fixation. In future experiments under controlled $CO_2$, drought, and temperature treatments, measurements of leaf area and cuticle thickness should be incorporated because these variables can impact the absorption of foliar applied herbicides.

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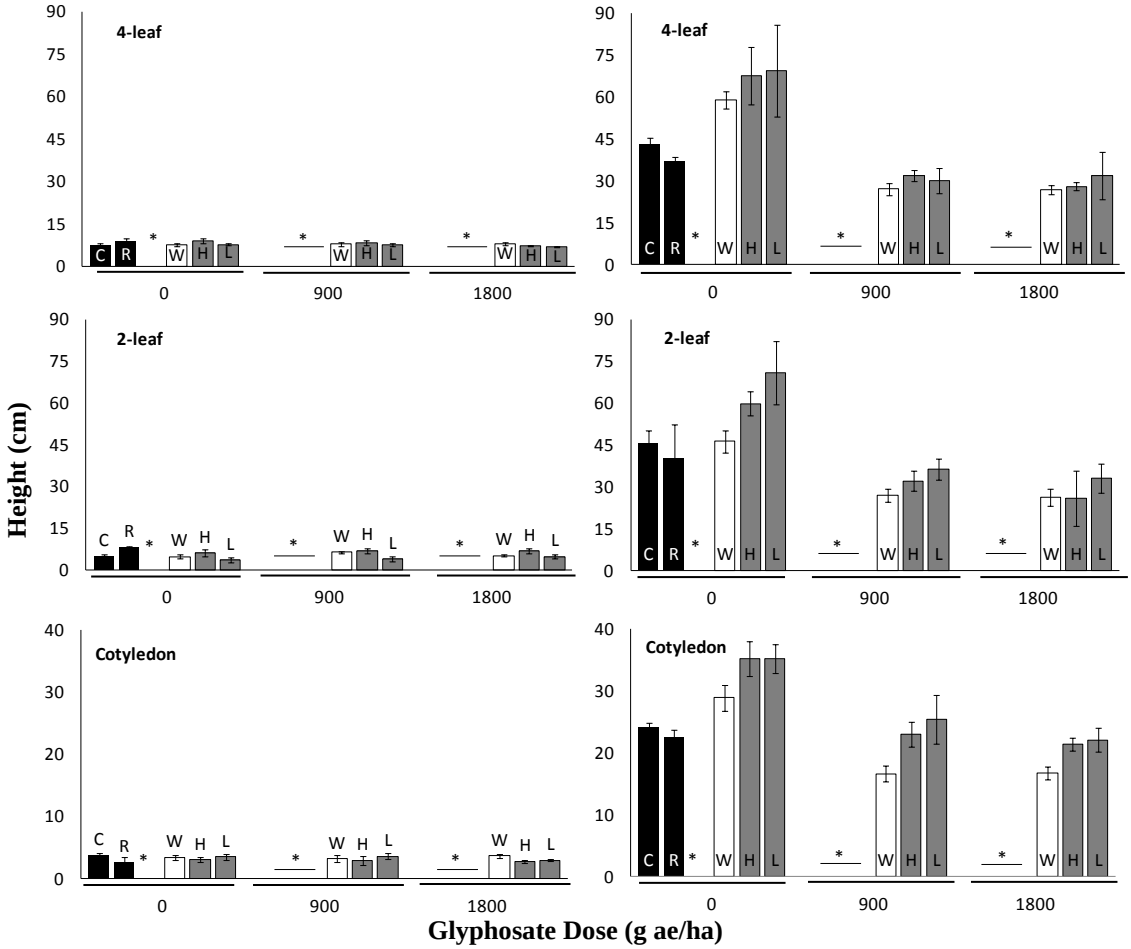
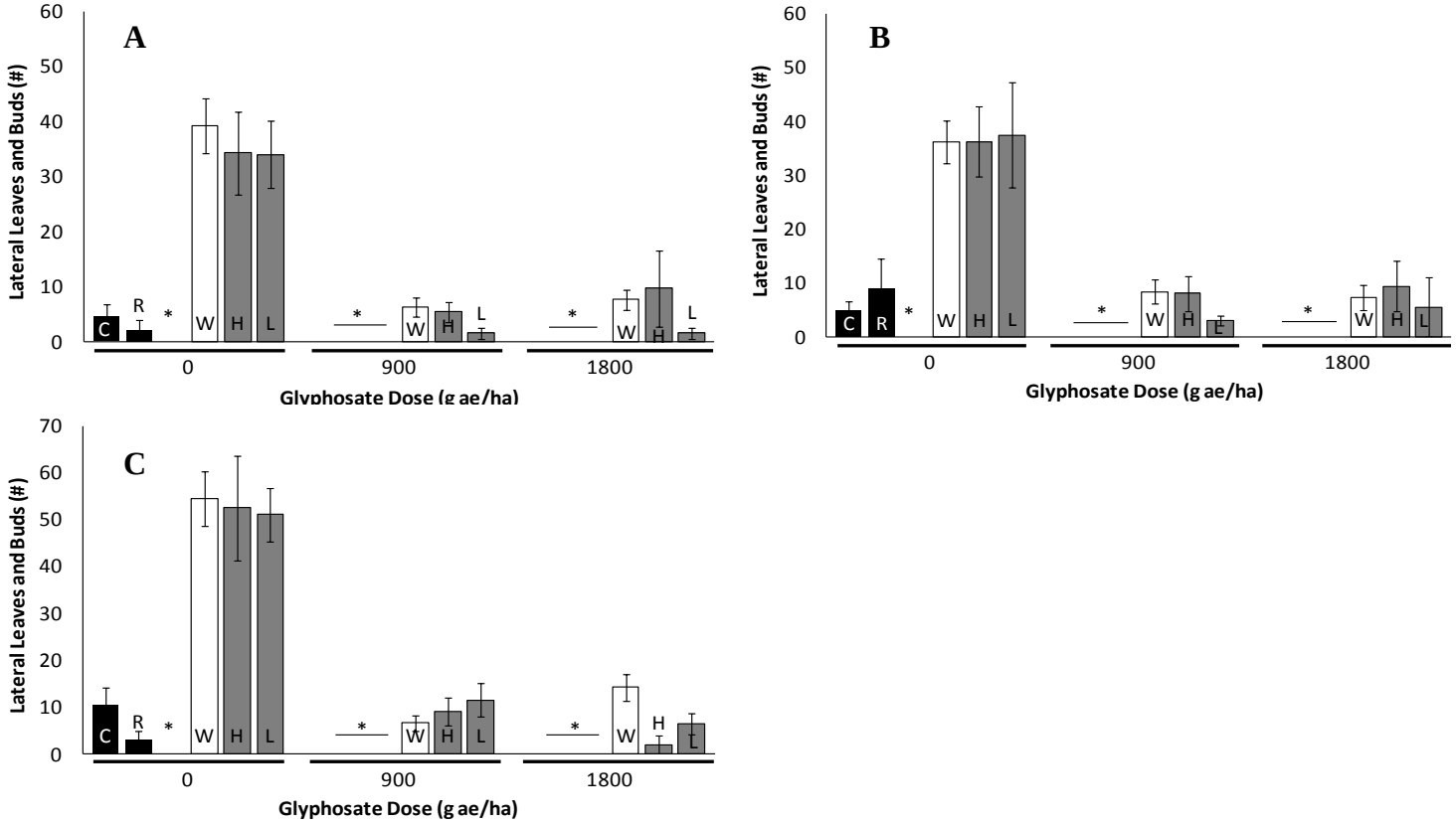
# APPENDIX




**A.1** Height (cm) of giant ragweed seedlings on the day of (left column) and 28 days after treatment (right column) with glyphosate. Seedlings were sprayed with glyphosate at the 4-leaf, 2-leaf, and cotyledon stage. The mean heights for each biotype (C= Cambridge, R= Ridgetown, O= Ohio, W= Windsor, H= Harrow, L= Leamington; table 3.1) are plotted and the error bars represent standard error (n=2-8). Associated significance values are noted in table 3.2. The asterisk denotes missing data points.




**A. 2** Lateral leaves and buds on giant ragweed seedlings 28 days after glyphosate treatment. Seedlings were sprayed with glyphosate at the 4-leaf (A), 2-leaf (B), and cotyledon (C) stage. The mean number of leaves and buds for each biotype (C= Cambridge, R= Ridgetown, O= Ohio, W=Windsor, H= Harrow, L= Leamington; table 3.1) are plotted and the error bars represent standard error (n=2-8). The asterisk denotes a missing data point. Associated significance values are noted in Table 3.2.

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