

GLYPHOSATE RESISTANCE: POLLEN MOVEMENT WITHIN A COMMON
RAGWEED (*Ambrosia artemisiifolia* L.) POPULATION AND HERBICIDE RELEASE
FROM COMMON WATERHEMP (*Amaranthus rudis* SAUER) PLANTS

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CHAPTER I

Literature Review

Research Justification

Numerous advances in agriculture over the past century have occurred. Improvements in equipment, crop production practices, and technological advancements in crop genetics have all contributed to the productivity and profitability of the American farmer. One of the most recent advancements was the introduction of glyphosate-resistant crops. In 1996, glyphosate-resistant soybeans (*Glycine max* (L.) Merr.) were introduced in the U.S. By 2000, production of glyphosate-resistant soybeans made up 62% of the soybean area in Missouri and 54% in the U.S (USDA ASB, 2009). In 2009, glyphosate-resistant soybeans made up 89% of the soybean area planted in Missouri and 91% of the total soybean area in the United States (USDA ASB, 2009). Since the release of glyphosate-resistant soybean, other glyphosate-resistant agronomic crops were introduced: canola in 1996; cotton in 1997; and corn in 1998 (Duke, 2005). The rapid acceptance of this technology has contributed to the increase in glyphosate usage (Shaner, 2000).

Widespread adoption of glyphosate for weed control has impacted the use of other herbicide modes of action. Since 1996, the number of active ingredients used on at least 10% of the treated soybean hectares declined from 11 in 1995 to one (glyphosate) in 2002 (Young, 2006). Also, the average number of active ingredients applied to a treated area of soybean declined from 2.5 in 1994 to 1.6 in 2002 (Young, 2006). Glyphosate-

resistant crops provided a more cost effective option for growers, allowing them to spray weeds on an “as-needed” basis; therefore, reducing the need for preemergence herbicides at the time (Pline-Srnic, 2006). Lack of diversity in weed management practices through intense selection pressure (one mode of action) has been shown to lead to herbicide resistance (Gressel and Segel, 1978). Johnson et al. (2009) conclude that the evolution of glyphosate-resistant weeds could eventually deplete weed control options.

Repetitive and dedicated use of glyphosate within years and over years has resulted in selection for resistance in several weed species including rigid ryegrass (*Lolium rigidum* Gaud.), goosegrass (*Eleusine indica* (L.) Gaertn.), horseweed (*Conyza canadensis* L.), waterhemp (*Amaranthus rudis* Sauer), Palmer amaranth (*Amaranthus palmeri* (S.) Wats.), Italian ryegrass (*Lolium multiflorum* Lam.), giant ragweed (*Ambrosia trifida* L.), johnsongrass (*Sorghum halepense* (L.) Pers), hairy fleabane (*Conyza bonariensis*), kochia (*Kochia scoparia*), annual bluegrass (*Poa annua*), and common ragweed (*Ambrosia artemisiifolia* L.) (Koger et al. 2004; VanGessel, 2001; Lee and Ngim, 2000; Pratley et al. 1999; Heap, 2010; Leer, 2006).

Common ragweed (*Ambrosia artemisiifolia* L.) was declared glyphosate-resistant in 2004. Initially found in 2002 in a field near Millersburg, Missouri, common ragweed plants survived 2 postemergence (POST) applications of glyphosate (0.84 kg ae/ha) in one season. Greenhouse experiments have shown the lethal rate of glyphosate was 8 times (8x) greater than the labeled rate (1x = 0.84 kg ae/ha) on the resistant biotype compared with the susceptible biotype; the lethal rate on the susceptible biotype was 0.25x of the labeled rate (Pollard, 2007). The dose for growth inhibition of 50% (I_{50}) of the resistant biotype was 9.6 times greater than that compared to the susceptible biotype.

One plant survived a 12x rate and was able to produce viable seeds (Pollard, 2007). The field in which the resistant biotype was identified has been in a soybean/soybean/wheat followed by soybean rotation for 25 years, and has been planted with glyphosate-resistant soybean from 1996 to 2009.

Common waterhemp is a summer annual, broadleaf weed which, in recent years, has become one of the most difficult weeds to control in U.S. agricultural fields. A survey conducted among growers in Illinois in 2002 revealed the *Amaranthus* spp. were the main weed species encountered in corn and soybean fields. Common waterhemp was specifically mentioned in 75% of the surveys (Hager and Sprague, 2002). Common waterhemp has many characteristics which make it one of the more difficult weeds to control. A prolific seed producer, mature waterhemp plants have been shown to produce up to 288,000 seeds per plant (Sellers et al. 2003). The weed exhibits a broad time for emergence; initially in late April and continuing into mid-July (Steckel et al. 2001). Waterhemp can emerge after soil-applied herbicides and post-applied residual herbicides have lost effectiveness in the soil (Legleiter et al. 2009).

Although there is concern over an increase in the number of glyphosate-resistant weed species, localized spread of resistant populations can lead to contamination of broad areas. A larger-seeded species such as common ragweed may move by equipment and animals, but there is the potential that pollen can also vector resistance. It is important to identify the frequency and distance that glyphosate-resistance could be spread by pollen.

Common Ragweed

Common ragweed is a native summer annual weed which is distributed throughout the continental United States in cultivated fields, pastures and wastelands (Uva et al. 1997). Plants grow to a height of 1.5 meters at maturity (Gebben, 1965). Seed germinates at an optimum temperature around 31 C with radicle and shoot elongation occurring at 29 and 31 C, respectively, between the months of April and June (Shrestha et al. 1999). Small plants may produce more than 3,000 seeds and larger plants may produce up to 62,000 seeds (Dickerson & Sweet, 1971). Plants produce two spatulate-shaped cotyledons; true leaves are finely pinnatifid and form round or slightly pointed lobes. Leaves are initially opposite in arrangement and become alternate after the fourth node. Common ragweed is a monoecious plant with the male flowers on racemes at the top of the plant and the female flowers borne in the axils of the upper leaves. Flowers are present from late summer to autumn and produce large amounts of pollen (Uva et al. 1997).

Common ragweed can become a dominant weed species due to its rapid growth and prolific seed production. Studies conducted in North Carolina revealed, a canopy of common ragweed intercepted 24, 38, and 45% of the photosynthetically active radiation by 8, 10, and 12 weeks after soybean (*Glycine max* [L.] Merr.) emergence, respectively (Coble et al. 1981). Coble et al. (1981) also concluded one common ragweed plant per 10 meters of row reduced soybean yield an average of 33 kg/ha (Coble et al. 1981). Cowbrough et al. (2003) investigated the impact of common ragweed in soybeans by investigating the break-even yield loss (BEYL) levels. The BEYL considers herbicide costs, expected crop yield and commodity price as well as variables such as seed

moisture content and the level of undesirable plant material. Common ragweed populations were classified as aggregate or uniform populations. Aggregated populations were set up in experimental units to have common ragweed distributed over 66.6% of the plot area. In the plot area, 33% of the unit contained a 2x population of common ragweed, 33% contained a 1x population and 33% of the unit was weed free. Uniform populations contained common ragweed in 100% of the plot area. In 1999, the BEYL was 4.6%, which showed economic threshold values of 0.17 and 0.31 plants/m² for the uniform and aggregate populations in 1999, respectively. In 2000, the BEYL values were 0.49 and 0.50 plants/m² for the uniform and aggregate populations, respectively. Although results varied between years, 0.2 to 0.5 common ragweed plants/m² was deemed the threshold for economic damage (Cowbrough et al. 2003). Weaver (2001) studied the impact of common ragweed on corn and soybeans in Canada and showed common ragweed had more of an impact on soybeans than corn. The estimated maximum yield loss at a high weed density (greater than 32 plants per m²) was 65 to 70% in soybean (Weaver, 2001). Studies conducted in North Carolina in 1998 and 1999 showed that peanut (*Arachis hypogaea* L.) yields declined exponentially as common ragweed biomass increased. Peanut yield decreased 1,760 kg ha⁻¹ with each kilogram increase of weed biomass per meter of crop row (Clewis et al. 2001).

The major technique for management of common ragweed is the application of herbicides. Before the introduction of glyphosate-resistant crops, control of common ragweed in soybean was often achieved with post-emergence herbicides such as lactofen, acifluorfen, bentazon, chlorimuron, imazethapyr, and cloransulam, among others. In a study by Nelson et al. (1998), weed control in soybeans with imazamox and imazethapyr

was investigated. Results illustrated that imazamox and imazethapyr reduced common ragweed dry weight by 64 and 61%, respectively by 21 days after treatment; plants survived and eventually recovered. Common ragweed mortality was 100% with the application of lactofen and tankmixes with imazamox or imazethapyr (Nelson et al. 1998). Ballard et al. (1995) identified common ragweed regrowth by 10 to 14 days after treatment with imazethapyr indicating the plants were completely controlled. Patzoldt et al. (2001) discovered a population of common ragweed in Indiana resistant to the acetolactate synthase (ALS)-inhibiting herbicide, cloransulam-methyl. In greenhouse experiments, 87% of the resistant plants tested displayed little or no injury, while 95% of the susceptible plants were deemed sensitive. Lab studies identified the I_{50} (herbicide dose to inhibit growth by 50%) for the resistant population was more than 5,000-fold higher than the susceptible population. The basis for resistance was an altered site of action (Patzoldt et al. 2001).

Soil-applied herbicides also have activity on common ragweed. Niekamp and Johnson (2001) studied weed management in no-till soybeans using sulfentrazone and flumioxazin. The study reported that flumioxazin alone resulted in 79 and 83% control of common ragweed in 1996 and 1997, respectively. Sulfentrazone alone resulted in 58 and 63% control in 1996 and 1997, respectively. Herbicide mixtures containing flumioxazin + clomazone and flumioxazin + chlorimuron + clomazone or pendimethalin resulted in 86 to 100% control of common ragweed, respectively. Sulfentrazone added to chlorimuron + clomazone or pendimethalin resulted in greater control compared to sulfentrazone + clomazone (Niekamp and Johnson, 2001). Although a number of

herbicides with residual activity suppress common ragweed, the cost of herbicides used and reduced flexibility in timing favor greater use of post-emergence herbicides.

With the introduction of glyphosate-resistant crops such as soybean, glyphosate has become one of the most effective means of controlling common ragweed. Advantages include lower costs, crop safety and flexibility in application timing. Field studies in Michigan in 1996 and 1997 showed that glyphosate provided consistent common ragweed control in wide- and narrow-soybean (Nelson and Renner, 1999). With the discovery of glyphosate-resistant common ragweed, control of common ragweed will become more difficult.

Common Waterhemp

Common waterhemp is a dioecious plant, with male and female flowers occurring on separate plants (Gleason and Cronquist, 1991). This forced hybridization results in greater genetic diversity, which enables plants to adapt to abiotic stresses such as herbicides (Hager et al. 1997). This ability to adapt has contributed to waterhemp developing resistance to ALS inhibitors (1993), photosystem II inhibitors (1994), protoporphyrinogen oxidase (PPO) inhibitors (2001), glycine herbicides (2005), and 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors (2009) (Heap, 2010).

Common waterhemp was confirmed glyphosate resistant in 2005. The population was initially located in a soybean field in northwest Missouri (Leglieter and Bradley, 2008). The infested site had been in continuous glyphosate-resistant soybean production for the previous six years, and received at least one application of glyphosate per growing season. In greenhouse experiments, the resistant biotype required 2.3 kg ae

ha⁻¹ to reduce the fresh weight biomass by 50% (GR₅₀). The resistant population displayed a level of resistance 19-fold greater for the resistant compared to a susceptible population of waterhemp (Legleiter and Bradley, 2008).

Glyphosate

Glyphosate is a broad-spectrum, non-selective herbicide, initially introduced in 1974 for control of vegetation in non-crop conditions. Glyphosate has activity on many annual and perennial weeds (Franz et al. 1997).

Glyphosate inhibits a major step in the shikimic acid pathway. This pathway links the metabolism of carbohydrates to biosynthesis of aromatic amino acids (tryptophan, phenylalanine, and tyrosine) (Franz et al. 1997). There are 7 metabolic steps involved in the shikimate pathway. It begins with the condensation of phosphoenolpyruvate (PEP) and ends with the synthesis of chorismate. The shikimate pathway is only found in microorganisms and plants (Hermann and Weaver, 1999); glyphosate is relatively non-toxic to animals. It has been estimated that 35% or more of the ultimate plant mass in dry weight is derived from the activity of the shikimate pathway (Franz et al. 1997).

Glyphosate is actively taken up by plant material and was originally determined to display no soil activity. Originally it was stated that the simplicity of the glyphosate molecule resulted in ready metabolism by soil microorganisms and the compound was tightly bound to soil particles and, therefore not leached (Franz et al. 1997). Recent research has suggested glyphosate in a readily available form can persist for at least 6 months after application in amounts which would not affect crop yield or human health

(Simonsen et al. 2008). Haney et al. (2000) does show glyphosate applied to the soil surface is quickly degraded. The addition of glyphosate also stimulated microbial activity. By measuring carbon (C) and nitrogen (N) mineralization, there was a significant relationship between C and N added by glyphosate versus C and N mineralized in the soil. The amount of C and N mineralized increased linearly with increasing rates of applied glyphosate (Haney et al. 2000)

The primary target of glyphosate in the shikimate pathway is the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS). This enzyme catalyzes the formation of EPSP from phosphoenolpyruvate (PEP) and shikimate 3-phosphate (S3P) (Hermann and Weaver, 1999). This reaction is the penultimate step in the shikimate pathway, which produces the important intermediate, chorismate (Franz et al. 1997; Hermann and Weaver, 1999). Although the exact events responsible for plant death are unknown, the disruption of EPSP synthase and the reduction of aromatic amino acid production ultimately contribute to plant death (Franz et al. 1997).

Monsanto prepared the first test for glyphosate in 1970. Preliminary greenhouse tests of the experimental herbicide were so promising that several secondary tests were bypassed so the herbicide could be put into field tests earlier than anticipated (Franz et al. 1997). The herbicide was first available for use in 1974. World-wide, glyphosate is the largest-selling herbicide on the market today (Woodburn, 2000). Much of this is due to the use of glyphosate in crop plants engineered with resistance. Glyphosate provides a more cost effective option for growers than using many other herbicides, allowing treatment of weeds on an “as-needed” basis and reducing the need for preemergence herbicides (Pline-Srnic, 2006).

The popularity of glyphosate has negative consequences. The rapid acceptance of herbicide-resistant crop technology has led to the increase in glyphosate usage (Shaner, 2000). Natural genetic mutations occur within weed populations, and the end result of intense selective pressure exerted by a lack of diversity in weed management practices (Gressel and Segel, 1978) is herbicide resistance. The repetitive use of glyphosate has selected for resistance in several weed species including: rigid ryegrass (*Lolium rigidum* Gaud.) in Australia and the United States; goosegrass (*Eleusine indica* [L.] Gaertn.) in Malaysia; and horseweed (*Conyza canadensis* L), waterhemp (*Amaranthus rudis* Sauer), Palmer amaranth (*Amaranthus palmeri*), Italian ryegrass (*Lolium multiflorum*), johnsongrass (*Sorghum halepense* [L.] Pers), hairy fleabane (*Conyza bonariensis*), kochia (*Kochia scoparia*), annual bluegrass (*Poa annua*), common ragweed (*Ambrosia artemisiifolia* L.), and giant ragweed (*Ambrosia trifida*) in the United States (Koger et al. 2004; VanGessel, 2001; Lee and Ngim, 2000; Pratley et al. 1999; Heap, 2010).

Herbicide resistance in plants can be facilitated through various mechanisms of resistance. Currently, four mechanisms have been identified which underlie glyphosate resistance. Baerson et al. (2002) revealed there was an alternate form of EPSPS which had a major contribution to the development of resistance in goosegrass. This mechanism of resistance has been identified as a cytosine to thymine nucleotide mutation in the active site of ESP synthase (Baerson et al. 2002). Wakeline et al. (2004) discovered the translocation of glyphosate was altered in rigid ryegrass. Altered translocation patterns reduced the accumulation of glyphosate in shoot meristematic zones. This prevented plant growth in susceptible plants, but had little effect on the growth of resistant plants (Wakeline et al. 2004). Ge et al. (2010) discovered a

mechanism of resistance within horseweed was vacuole sequestration of the glyphosate molecule in the plant. Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) experiments revealed significantly more glyphosate accumulation in the vacuoles of glyphosate-resistant horseweed plants than the susceptible biotype. This process removes glyphosate from the phloem, limiting translocation throughout the plant. More recently, Gaines et al. (2010) discovered the mechanism of resistance within palmer amaranth was due to an amplification of the EPSPS gene which resulted in high levels of EPSPS expression. Both resistant and susceptible populations of palmer amaranth displayed equal EPSPS enzyme inhibition by glyphosate. PCR measurements of the genome of the resistant population revealed from 5-fold to more than 160-fold more copies of the EPSPS gene than the susceptible population (Gaines et al. 2010).

The first case of glyphosate resistance occurred in 1996 with rigid ryegrass (*Lolium rigidum*) in Australia (Heap, 2010). After 15 years of successful control, glyphosate failed to control a population of rigid ryegrass (Powles et al. 1998). Four different populations of *Lolium rigidum* were evaluated for resistance in Australia. The study showed that four distinct populations were resistant, although each population had a slightly different level of resistance due to differences in the lethal dose of glyphosate for 50% of the population (LD_{50}). The LD_{50} rate ranged from 6- to 10-fold higher than the susceptible population.

Inheritance of the resistant trait in rigid ryegrass was found to be due to a single dominant allele. Eight resistant populations of rigid ryegrass were crossed with the same susceptible population, resulting in F1 progeny. Challenging F1 progeny with glyphosate revealed plants were more similar to the resistant parents than the susceptible parents

when sprayed with a range of glyphosate doses from 0 to 1,800 g ae ha⁻¹. Five of the populations of the F1 were backcrossed with susceptible plants. The progeny from the backcross segregated in a manner consistent with a single gene controlling resistance (Wakelin and Preston, 2006).

In 1997, glyphosate resistance in goosegrass was confirmed in Malaysia (Heap, 2010). Resistant biotypes were found to have an LD₅₀ 2- to 4-fold greater compared to the susceptible biotypes (Baerson et al. 2002). Another study showed the LD₅₀ values to be 8- to 12-fold greater in resistant versus susceptible plants (Lim & Ngim, 2000). Heritability studies were conducted by making reciprocal crosses between glyphosate-resistant and -susceptible biotypes of goosegrass. The progenies of self-pollinated resistant plants over two generations displayed no segregation and zero mortality after application of glyphosate at the recommended rates, demonstrating that the plants were homozygous recessive for the resistance gene. The effective dose, or dose of a substance required to produce a measurable effect on 50% of individual plants in a population (ED₅₀) of the F1 population was 665 g ha⁻¹ for the (♀Susceptible X ♂Resistant) cross and 761 g ha⁻¹ for the (♀Resistant X ♂Susceptible) cross. The ED₅₀ value for the susceptible parent population was 241 g ha⁻¹ and the value for the resistant parent was 1,531 g ha⁻¹. Therefore, the authors concluded that inheritance of the resistant gene was due to an allele(s) that is incompletely dominant. To determine the genetic control of the resistance allele(s) in goosegrass, F1 plants were self-pollinated to produce an F2 population. Three different application rates of glyphosate (540, 781, and 869 g ha⁻¹) were chosen to discriminate between susceptible, intermediate and resistant plants, respectively. The segregation pattern of the F2 revealed that 25% of the plants behaved like the susceptible

parents, 50% behaved like the F1, and 25% of the plants behaved like the resistant parents. The segregation of resistance in the F3 generation was analyzed to show whether there was a second locus involved. Fifteen intermediate and 15 resistant F3 plants were screened with an application of 868 g ha⁻¹ glyphosate. All of the plants from the F3 resistant populations were resistant, proving that the resistance gene in goosegrass resides on a single locus (Chin-Hong et al. 2004).

Glyphosate resistance in horseweed (*Conyza canadensis* L.) was confirmed in 2000 in Delaware (VanGessel, 2001). Since then, resistant populations have been identified in Kentucky, Tennessee, Indiana, Maryland, Missouri, New Jersey, Ohio, Arkansas, Mississippi, North Carolina, California, Pennsylvania, and Nebraska (Heap, 2010). The total area estimated to be infested in the US exceeds 364,000 ha. Resistance to glyphosate in horseweed is significant, as it is the first weed in major agronomic crops to be selected resistant to glyphosate. Horseweed in Delaware survived 1.6 kg ai ha⁻¹ of glyphosate. Plants also survived a sequential application at the same rate. VanGessel (2001) reported that the I₅₀ of the resistant population was 8- to 13- fold greater compared to the susceptible population. Horseweed biotypes in soybean fields in Mississippi and Tennessee exhibited an 8- to 12-fold level of resistance compared to susceptible horseweed biotypes (Koger et al. 2004). Growth stage had little effect on the level of resistance. Koger and Reddy (2005) suggested the mechanism of resistance in horseweed was reduced translocation of glyphosate within the plant. Radioactive ¹⁴C-glyphosate was applied to the youngest fully expanded leaf of 23 to 29 leaf plants in populations of resistant and susceptible horseweed from Mississippi, Arkansas, Delaware, and Tennessee. Plants were harvested 48 hours after application and analyzed for the amount

of ^{14}C present in the plant. Absorption of glyphosate was similar (47 to 54%) for resistant and susceptible plants. The reduction in translocation of ^{14}C -glyphosate inside the plants ranged from 28 to 48% in resistant compared to susceptible plants. Zelaya et al. (2004) conducted inheritance studies on horseweed in 2004. Recurrent selection was used to select resistant individuals from a population, then cross selected individuals to form a new population. This technique was performed on a population of horseweed from Delaware. The population used in the studies was composed of 5% susceptible plants and 95% resistant plants. Recurrent selections were carried out by applying glyphosate at different rates to generate populations in the first and second recurrent generations. Both generations did not segregate further for resistance, suggesting that the second recurrent generation was a near homozygous population. The genetics of resistance were investigated by performing reciprocal crosses between the second recurrent generation and the original resistant and susceptible populations. The evaluations of the F1 and F2 generations suggested that glyphosate resistance was controlled by an incompletely dominant, single-locus gene. This was confirmed using backcrosses of the F1 plants to the second recurrent generations and original resistant and susceptible parents (Zelaya et al. 2004).

Weed Spread

Annual weeds are disseminated by seed, and seed spread can occur via wind, equipment, and animals. For herbicide-resistant weeds, the spread of seed plays an important role in the management of infested areas. Russian thistle (*Salsola iberica*) was first reported to be resistant to sulfonylurea herbicides in 1988. Plants can produce up to

60,000 seeds and seeds are spread by plants rolling over the ground. A study conducted in Washington revealed that the plants could move 60 to 4,000 m in 6 weeks or less, losing up to 66% of their seeds along the way (Stallings et al. 1995b).

The glyphosate-resistant weed, horseweed spreads using wind as the main vector. The seeds of horseweed are dispersed by the wind due to the structure of its seeds. A study conducted by Dauer et al. (2007) revealed that horseweed seed could be dispersed at least 500 m away from the source populations; however, 99% of the seed dispersed was found within 100 m of the source population.

Pollen

A trait such as herbicide resistance can also spread through pollen from resistant plants crossing with receptive stigmas on susceptible plants. Pollen has an integral role in all flowering plants by transferring genetic information from one plant to another. Pollen carries the male gamete from the anthers (male part) to the stigma (female part) of receiving flowers. This is most important for cross-pollinated species. Common ragweed relies on wind-dispersed pollen for successful pollination, due to the physical separation of the male and female flowers on a plant (Uva et al. 1997). In the case of herbicide-resistant weeds, pollen from resistant plants could be transmitted to susceptible plants (Stallings et al. 1995a; Sosnoskie et al. 2009). A study conducted in 1991 and 1992 on sulfonyleurea-resistant kochia (*Kochia scoparia*) revealed pollen-mediated spread of resistance was possible using rings of susceptible kochia established around a population of resistant kochia. A field in Indiana was set up with source resistant kochia.

