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Understanding the biology, inheritance and mechanism of resistance of 2,4-D resistant waterhemp (*Amaranthus tuberculatus*) - Research Proposal

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Justification and Research objectives

Waterhemp is a very well adapted summer annual weed to the U.S. Corn Belt region. Auxinic herbicides such as 2,4-D constitute effective and widely used herbicides to control waterhemp and other broadleaf species in cereal crops and turf grasses. Recently, Bernards et al. (2012) have reported a Nebraska waterhemp biotype has evolved resistant to 2,4-D. This finding represents the sixth mode-of action herbicide group to which waterhemp has evolved resistance to. Several attributes may have contributed to make waterhemp a very successful weed and prone to evolve to herbicide resistant: a high genetic variability, aggressive growth habits and high fecundity. The hypothetical scenario of a waterhemp population resistant to several herbicide mode of action constitutes a challenge on weed management, since a traditional herbicide use program could result in unmanageable situation.

Several weed species have been reported to be resistant to 2,4-D (Heap 2012), but some inconsistencies regarding the possible mechanism of resistance and their inheritance have been found among species. In the next section, several studies that have contributed to the basic understanding for auxinic herbicide resistance are presented. Overall, the complex interactions between signal receptor sites possibly involved in the mechanism of resistance- and the herbicide found in previous studies suggest the waterhemp resistance to 2,4-D has unique characteristics to be elucidated. The proposed objectives of this study are: 1) to evaluate the response of the 2,4-D resistant waterhemp population to several other herbicides mode of action,, 2) to assess the type of inheritance pattern of 2,4-D resistance in waterhemp, and 3) to determine if the mechanism of 2,4D resistance in waterhemp is due to differential absorption, translocation, or metabolism of 2,4-D.

Literature review

Herbicide resistant weed evolution

The development of herbicide-resistant weeds represents a serious worldwide threat to agricultural production. The first case of herbicide resistance was documented as early as 1957in Spreading dayflower (*Commelina diffusa* Burn.) against 2,4-D in Hawaii (Hilton, 1957). In 1963, a differential response of wild carrot (*Daucus carota* L.) to 2,4-D and other herbicides was reported in Ontario, Canada (Whitehead and Switzer 1963). The first confirmed case of herbicide resistance was reported in 1968 for common groundsel (*Senecio vulgaris* L.) against triazine herbicide in Washington (Ryan, 1970). Since then, the number of resistant weed biotypes against various herbicides has been on the rise (Figure 1).

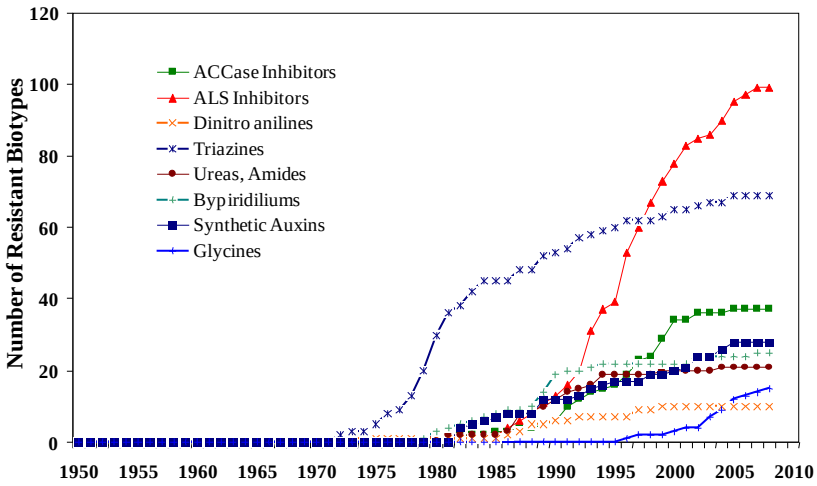

Figure 1. Number of worldwide resistant biotypes of weed species reported by International Survey of Herbicide-Resistant weeds up to 2010 (adapted from Heap 2012).

The 'International Survey of Herbicide-Resistant weeds' reports the evolution of herbicide resistant weeds in the world (Heaps 1997; 2012). When surveying began in the 1970's, weed scientists recorded only triazine-resistance weeds (simazine and atrazine herbicides principally) (Heap 1997). Between 1970 and 1977 one weed per year in average was reported to be resistant to herbicides (Heap 1997; Hatzios 2003). Since then, as herbicides with new modes of action were introduced into the market, about nine weeds per year have been reported to evolve herbicide resistant (Hatzios 2003; Heap 2012). To date, 200 species (116 dicots and 84 monocots) has been reported to be resistant to herbicides with 20 modes of action. Of the 372

herbicide resistant weed biotypes reported in the world, at least 138 biotypes have been identified in various cropping systems in the US (Heering et al. 2004; Heap 2012). In addition, the area with infected land with herbicide resistant weeds is increasing rapidly up to reach an estimated area equivalent to 570,000 fields in the current year (Heap 2012).

Some key features in Figure 1 are the starting date, the shifts in slope and the magnitude of reported herbicide resistant weeds to each group. Since the first triazine-resistant common groundsel in 1968, 69 weed species have been reported to inhibit the photosystem II represented mainly by triazine herbicide (Figure 1). It is possible that the wide use of triazine herbicides between 1978 and 1983 due to its high effectiveness has contributed to the predominance of triazine-resistant weeds (principally to atrazine and simazine) during that period. Triazine-resistant weeds were 67% of the total herbicide-resistant weeds reported until 1983 (Heap 1997). Before the middle of the 80', there was a shift in the predominance of herbicide-resistance group from triazine to ALS inhibitors. Sulfonylureas and imidazolinones, both ALS inhibitors, were introduced in 1980's and 1986's respectively (Ross and Lembi 2008). Resistance to ALS inhibitor herbicides was first reported in a prickly lecttuce (*Lactuca serriola* L.) biotype that by 1987 withstood increased doses of the sulfonylurea herbicides chlorsulfuron and metsulfuron (Mallory-Smith et al. 1990). Currently, 113 weed species have been reported resistant to ALS-inhibitors (Heap 2012). After glyphosate resistant technology was released in the market in 1996 and widely adopted in corn, soybean and cotton cropping systems, the number of glyphosate weeds has been constantly increasing until now (Heap 2012). Glyphosate resistant annual ryegrass (*Lolium multiflorum* Lam.) was suspected to be resistant in the first half of the 90's in Australia. This specie was later the first report of a glyphosate resistant weed (Pratley et al. 1996; 1999). Heap and LeBaron suggested in 2001 that more cases of glyphosate resistant weeds will be likely in the first years of the current century, but that those cases will appear less frequently than other herbicide mode of action such as ALS and ACCase-inhibitors. Actually, only 21 weed species are resistant to glyphosate being species belong to the *Conyza* sp., *Lolium* sp. and *Amaranthus* sp. genus the most frequently reported in the world (Heap 2012).

Although herbicide resistant weeds are considered an increasing problem, most of the cases of herbicide resistant weeds could be successfully managed. However, multiple herbicide resistance reports are increasing in some of the more important weed species and they can constitute a trouble in weed management (Tranel et al. 2011). Heap and LeBaron (2001) reported

that of the 152 species that have evolved resistance up to 2000, 106 species evolved to only one mode of action (MoA), 28 species to two MoA and 10 species evolved resistance to three MoAs. Moreover, two species evolve resistance to each of the four and five MoAs, three species to six MoAs and only one species *Lolium rigidum* has evolved resistance to eight MoAs (Heap and LeBaron 2001). Until middle of 2011, more weed species have evolved resistance to different herbicide MoA groups. According to Heap (2012), 35, 11, 7, 3 and 4 species have evolved resistant to two, three, four, five and six MoAs respectively. Most important grass weed species in crops such as annual ryegrass (*Lolium rigidum* L.), barnyardgrass (*Echinochloa crus-galli* L.), wild oat (*Avena fatua* L.), annual bluegrass (*Poa annua* L.), blackgrass (*Alopecurus myusoroides* L.) and goosegrass (*Eleusine indica* L.) have evolved resistant to at least five MoAs. Few dicot weed species have evolved resistant to several herbicide MoAs compared to grass species. The most important dicot species that evolved resistance to more than one MoA are common lambsquater (*Chenopodium album* L.), smooth pigweed (*Amaranthus retroflexus* L.), horseweed (*Conyza canadensis* L.), kochia (*Kochia scoparia* L.), common groundsel (*Senecio vulgaris* L.), Palmer amaranth (*Amaranthus palmeri* L.) and black nightshade (*Solanum nigrum* L.). Although Amaranthus family appears to be resistant to more than one herbicide MoA in most of the family members, waterhemp (*Amaranthus tuberculatus*) is resistant to six MoAs including ALS, triazine, PPO, ghyphosate, HPPD and synthetic auxins (Heap 2012).

Auxinic herbicides and their history about resistance in weeds

The auxinic herbicides were the first selective organic herbicides to be developed. Within this herbicide group, 2,4-D and MCPA were discovered independently by American and British scientists in the 40's. The selective control of broadleaf weeds in cereal grain crops by auxinic herbicides has made that this herbicide group one of the most widely used. Auxinic herbicides also called growth regulators mimic the action of natural plant hormones as indole-3-acetic acid (IAA) (Sterling and Hall 1997). Auxinic hormone mechanism of action was unclear during 90 years and only recent literature have clarified the way as how this hormone act in plants (Ross et al. 2002; Woodward and Bartel 2005; Vanneste and Friml 2009; Cobb and Reade 2010). The IAA and his precursor effects on plant are well known and include cell elongation, cell division, cell differentiation, root initiation, tropic response, leaf senescence, cell and organ polarity and wound responsiveness (Sterling and Hall 1997; Cobb and Reade 2010). Some aspects of the

auxinic herbicide mechanism of action has been previously reviewed by Coupland (1994), Sterling and Hall (1997) and Kelley and Riechers (2007). However, although auxinic herbicides are the oldest herbicides and have being used during more than 60 years, their mode of action is still unknown in detail.

Auxinic herbicides are in general structured similarly to IAA, but over the years, various chemical classes of auxin herbicides, with different structures, weed spectra and types of selectivity have been synthesized and commercially introduced (Grossman 2010). Some decades ago only two herbicides classes were included into the auxinic herbicides (Devine et al. (1993). Later, the auxinic herbicide classification was updated and divided in four major classes based on the position of the carboxylic acid moiety and the type of aromatic group: phenoxyalkanoic acids (e.g. 2,4-D and MCPA), benzoic acids (e.g. dicamba and cloramben), pyridine carboxylic acids (e.g. picloram, clopyralid, triclopyr and fluroxypyr) and quinolinecarboxylic acids (e.g. quinclorac and quinmerac) (Cobb and Reade 2010).

Since the first two documented 2,4-D resistant weed wild carrot (*Daucus carota* L.) (Switzer 1952) and spreading dayflower (*Commelina difussa* L.) biotypes in 1957 (Hilton, 1957), there have been a slow increase in the auxinic resistant weeds principally concentrated in the 1990's decade (Table 1) (Heap 2012). To date, 29 weed species have been reported to evolved resistance to auxinic herbicides (Table 1) (Heap 2012) after more than 60 years of use. A high selection pressure as result of repeated application of the same herbicides is the common factor that seems to be the key to the development of resistance to several herbicides classes, including auxinic herbicides (Nandula 2010). Janeiuk et al. (1996) considered the auxinic herbicides as having a lower risk of resistance development than most herbicide classes. Sterling and Hall (1997) considered that the low incidence of auxinic herbicide resistance is due to these herbicides are believed to have multiple modes and sites of action and are not persistent in the soil.

Four monocot weeds such as smooth crabgrass (*Digitaria ischaemum* Schreb.), barnyardgrass, gulf cockspur [*Echinochloa crus-pavonis* (Kunth) Schult.] and junglerice (*Echinochloa colona* L.) in the Poaceae family (also called grasses family) have evolved resistant to quinclorac which is the only herbicide belong to auxinic herbicides that is used to control some grass species (Table 1). Lack of phytotoxicity in grasses of auxinic herbicides different of quinclorac has been attributed meanly to anatomical differences in vascular structure between

monocot particularly grasses and dicot plants, and differences in ability to metabolize the herbicide (Sterling and Hall 1997; Grossman 2010). Also a grass species, *Echinochloa crus-galli* that is quinclorac resistant has also been found to be tolerant to 2,4-D in Europe (Coupland 1994; Lopez-Martinez et al. 1995). Other three monocot weed species such as spreading dayflower, globe fringerush [*Fimbristylis milicea* (L.) Vahl.] and yellow bur-haed [*Limnocharis flava* (L.) Buch.] have evolved resistance to 2,4-D.

Additionally to the herbicide resistant monocot species, twenty three dicot weeds have been reported as resistant to some of the other auxinic herbicides which are recommended to control broadleaf weeds in corn, sorghum and small grain (Table 1). Only one dicot weed species, false cleavers (*Galium spurium* L.) has been reported to be resistant to quinclorac (Table 1). Few of the weeds that have evolved resistance to auxinic herbicides have had a significant impact on the environment where they were found because of the wide array of alternative chemicals that control successfully these resistant weeds (Hatzios 2003).

Table 1. List of auxinic-resistant weeds discovered to date ordered by herbicide and reported year (adapted from Heap 2012).

	Triclopyr	Clopyralid	Picloran	2,4-D	MCPA	Dicamba	Fluroxypyr	Mecoprop	Quinclorac
Commelina diffusa Spreading dayflower				USA 1957					
Daucus carota Wild carrot				Canada 1957					
Convolvulus arvensis Field bindweed				USA 1964					
Matricaria perforate Scentless chamomile				France 1975					
Cirsium arvense Canada thistle					Sweden 1979				
Carduus nutans Musk thistle				New Zealand 1981					
Sphenoclea zeylanica Gooseweed				Philippines 1983					
Stellaria media Common chickweed								UK 1985	
Centaurea solstitialis Yellow starthistle			USA 1988						
Ranunculus acris Tall buttercup					New Zealand 1988				
Fimbristylis miliacea Globe fringerush				Malaysia 1989					
Sinapis arvensis Wild mustard			----------Canada----------					Canada 1990	
					1990				
Kochia scoparia Kochia						----------USA----------			
						1995			
Papaver rhoeas Corn poppy				Spain 1993					
Limnocharis flava Yellow bur-haed				Indonesia 1995					
Galium spurium False Cleavers									Canada 1996

Table 1. Continued

	Triclopyr	Clopyralid	Picloran	2,4-D	MCPA	Dicamba	Fluroxypyr	Mecoprop	Quinclorac-
Carduus pycnocephalus Italian Thistle				New Zealand 1997					
Echinochloa crus-galli Barnyardgrass									USA 1998
Galeopsis tetrahit Common Hempnettle					----------Canada----------	1998			
Echinochloa crus-pavonis Gulf cockspur									Brazil 1999
Soliva sessilis Carpet Burweed	----------New Zealand---------- 1999								
Echinochloa colona Junglerice									Colombia 2000
Digitaria ischaemum Smooth crabgrass									USA 2002
Limnophila erecta Marshweed				Malaysia 2002					
Chenopodium album Lambsquarters						New Zealand 2005			
Sisymbrium orientale Indian Hedge Mustard				----------Australia---------- 2005					
Raphanus raphanistrum Wild radish				Australia 2006					
Lactuca serriola Prickly Lettuce				----------USA---------- 2007					
Amaranthus rudis Common waterhemp				USA 2009					

Waterhemp Biology and Ecology

The genus *Amaranthus* is integrated by approximately 75 species which are part of Amaranthaceae family including waterhemp. Most of these *Amaranthus* species are distributed worldwide and 40 of them are considered native from North America (Pratt and Clark 2001). The *Amaranthus* species have had nomenclatural and taxonomic divergences since the first *Amaranthus* was named in the first half of the 1800's (Riddell 1835). In the U.S., Sauer (1955) identified two waterhemp species, *A. tuberculatus* in Indiana and Ohio, and *A. rudis* in the states between Nebraska and Texas. Both species were overlapped in other states as Missouri, Illinois and Iowa. Recently, Pratt and Clark (2001) included to *A. rudis* as a synonymous of *A. tuberculatus*, and proposed that only a single, highly variable species of waterhemp should be recognized and named as *Amaranthus tuberculatus* (Moq.) Sauer. Later, Costea and Tardif (2003) and Costea et al. (2005) agreed with identifying a single species as Pratt and Clark (2001) proposed, but suggested to differentiate both at the varietal level. Some differences in the seedlings between both *A. rudis* and *A. tuberculatus* are significant. However, where *A. rudis* and *A. tuberculatus* occupy the common area, hybridization is possible and therefore diagnostic traits may be unique and morphological, biological and genetically inseparable (Costea et al. 2005). Based in the above discussion, in the present proposal I will refer to waterhemp as *Amaranthus tuberculatus* without distinction between varietal levels.

According to Hager et al. (2002a), in the Great Plains region of the U.S., approximately 10 *Amaranthus* species are troublesome weeds in crop systems. *Amaranthus* family include some species that are monoecious (bisexual, with male and female in separate flowers) such as redroot pigweed (*A. retroflexus* L.), smooth pigweed (*A. hybridus* L.), Powell amaranth (*A. powellii* S. Wats.), tumble pigweed (*A. albus* L.), prostrate pigweed (*A. blitoides* S. Wats.), and spiny amaranth (*A. spinosus* L.), and other one that are dioecious (unisexual, with separate male and female plants) such as common waterhemp (*A. rudis* Sauer), tall waterhemp (*A. tuberculatus* (Moq.) J.D. Sauer), Palmer amaranth (*A. palmeri* S. Wats.), and sandhills waterhemp (*A. arenicola* I.M. Johnst.) (Gleason and Cronquist 1991; Horak et al. 1994). Both varieties of *A. tuberculatus* have become a major troublesome in corn (*Zea mays* L.) and soybean (*Glycine max* L. Merr.) over the last three decades in the Midwest (Hager and Sprague 2002; Webster 2005; Steckel et al. 2007). Besides being found in croplands, *Amaranthus* species can be found in pastures and rangelands, fence-rows, river and pond margins and waste areas (Sauer 1957).

Anatomic differences between *Amaranthus* species are divided in two categories: vegetative and floral/seedhead characters (Pratt et al. 1999). Although vegetative characters are less reliable than floral and seedhead characters, they are important in identifying immature plants and some general trends can be noted. Within vegetative characters are included seedling shapes, hair and leaf shapes. Waterhemp plants are erect herbs that are typically, though not always, hairless. The plants range between 5 cm and 2 m in height, and the stems vary from green to red to a striate of the two colors. The leaves of waterhemp are long and narrow and the petioles are in general shorter than the leaves (Pratt et al. 1999; Costea et al. 2005).

At the same way that others dioecious *Amaranthus* species, waterhemp has pentamerous staminate flowers together with complex terminal inflorescences, often called spikes. Flowering structures are narrow, branched and range between 3 and 35 cm long. *Amaranthus* species identification is based meanly on flower characters. Because *Amaranthus* flowers are very small (1-4 millimeters), the identification process requires magnification to see clearly this structure. Male flowers have five petals, are very similar across all *Amaranthus* species, and only vary in size. Female flowers have one or none petal and are much more different between species of this family. Both male and female flowers are greenish and are found on long, slender seedheads. In addition, both male and female flowers have short bracts (modified leaves), the size and shape of which can alter the appearance of the terminal inflorescences (Sauer 1955; Gleason and Cronquist 1991; Horak et al. 1994; Pratt et al. 1999).

Amaranthus seed including waterhemp are small and easily transported by wind, water, birds and human activities. Waterhemp seeds are small, round, black and shiny and can persist in the soil and remain viable for four or more years (Buhler and Hartzler 2001; Sellers et al. 2003; Nordby et al. 2007; Steckel et al. 2007). According to Tranel (2011) the adoption of no-tillage and reduced-tillage cropping systems also has favored to waterhemp given than his small seeds can germinate most effectively when they are at or near the soil surface.

As a dioecious species, waterhemp is an obligate outcrosser and has the capacity to cross with other *Amaranthus* species such as smooth pigweed (*Amaranthus hybridus* L.) and Palmer amaranth (*Amaranthus palmeri* S. Wats) (Fransses et al. 2001; Costea et al. 2005; Trucco et al. 2006). It confers an increased genetic and phenotypic diversity of the species and effective move genes within and among populations (Costea et al. 2005). Other notable biological features of waterhemp that have contributed to the unequal capability to be successful under variable

environmental conditions, including: rapid growth rate (in part due to its use of the C_4 photosynthetic pathway), short life cycle, prolific seed production (> 1 million seed per female plant) and extended emergence window along with the growing season (Costea et al. 2005).

Waterhemp is summer annual that emerges throughout the growing season (Nordby et al. 2007). Higher percentage of plants emerges later in the season than most other summer annual weeds (Hartzler et al. 1999). The irregular emergence patterns of waterhemp can be difficult to control waterhemp in crop. In Iowa, Hartzler et al. (1999) determined that waterhemp emerged between 5 and 25 days later than other summer annual weeds such as giant foxtail (*Setaria faberi* Herrm.), woolly cupgrass (*Erichloa villosa* Thunb. Villosa) and velvetleaf (*Abutilon theophrasti* Medicus). However, waterhemp had a longer emergence period than the other three species (Hartzler et al. 1999). In Illinois, Hager et al (2002a) observed the same emergence pattern of waterhemp in corn (*Zea mays* L.) and soybean production fields. When waterhemp plants emerge late in the season, usually it does not affect crop yields. However, seed that can not be controlled late in the season will contribute with a significant amount of seed to the soil seedbank (Cordes et al. 2004; Hartzler et al. 2004; Nordby et al. 2007).

Herbicide resistance in waterhemp

One of the most recently paper review about herbicide resistant in waterhemp is from beginning of 2011 (Tranel et al. 2011). This paper summarize about the waterhemp response to five herbicide mode of action groups to which waterhemp has had the ability to rapidly evolve resistant: PSII inhibitors, ALS inhibitors, PPO inhibitors, glyphosate and HPPD inhibitors (Heap 2012). One months after this review was published a very recent report showed the waterhemp resistant evolution to the 2,4-D in Nebraska (Bernards et al. 2012). It represents the sixth site-of action group to which waterhemp has evolved resistance. With this just two literature reports it is possible to shows the dynamic situation in which waterhemp is. In part, as it was mentioned above session, this capacity of herbicide resistance evolution is given because waterhemp is a dioecious species (Costea et al. 2005). Thus, outcrossing is assured and gene flow among and within populations occurs readily (Trucco et al. 2006). This attribute, together with a high seed production, provide large genetic variability and enough genetic material to the selection pressure take place. The potential for long-distance dispersal of resistance via wind-borne pollen is another important biological characteristic of waterhemp that helps to herbicide resistance

easily spread and stack with other herbicide resistant traits. This situation has lead populations with multiple herbicide resistance that limits chemical options for managing waterhemp (Tranel et al. 2011). In addition, where herbicide use is the primary weed control method, stacking results in additional selection pressure for the few herbicides that are still effective (Tranel et al. 2011).

The first herbicide resistant waterhemp biotype was reported to be resistant to PSII-inhibiting herbicides such as atrazine in Nebraska in 1990 (Anderson et al 1996). Subsequently triazine-resistant waterhemp biotypes were reported in several other midwestern states and also Canada. One year later, in 1991 waterhemp biotypes were sampled and reported to be resistant to ALS-inhibiting herbicides (Horak and Peterson 1995). A few years later, in 1996, multiple resistance to both triazines and ALS-inhibitor herbicides was identified in Illinois by Foes et al. (1998). Two decades after the first ALS-inhibiting resistant waterhemp was reported, Tranel et al. (2011) stated that the resistance to this herbicide mode of action in waterhemp is more common that finding susceptible waterhemp populations.

At the end of the century 20th, PPO-inhibiting herbicides started to be extensively used as an alternative way to control ALS-inhibiting resistant waterhemp, and the first biotype of PPO-inhibiting resistant waterhemp was documented in waterhemp populations collected in 2000 in Kansas (Shoup et al. 2003). In addition, this population showed to have multiple resistances to both PPO- and ALS-inhibitors. A few years after this first report, several other scientists reported to have populations of waterhemp with variable levels of resistance to PPO-inhibitor in Illinois, Missouri and Iowa (Hager et al. 2002b; Heap 2012). In 2005 Patzoldt et al. (2005) reported the first three-way resistant waterhemp population from Illinois which was resistant to ALS-, PPO-inhibitors and triazines.

In 2004 a waterhemp sample from a field where glyphosate-resistant soybean grew at least for six years in Missouri was screened as being suspected resistant to glyphosate. A few years after Legleiter and Bradley (2008) reported the first waterhemp glyphosate-resistant biotype. In addition, the same waterhemp population was resistant to ALS- and PPO-inhibiting herbicides (Legleiter and Bradley 2008).

In 2009, a farmer from Nebraska contacted to scientists from University of Nebraska-Lincoln and reported that waterhemp plants survived and recovered after being treated with a recommended rate of 2,4-D. The grower also reported that suspected 2,4-D resistant population

was in a field were warm season grass was growing since 1996 with annual applications of 2,4-D, atrazine and metolachlor to control grasses and broadleaf weeds (Bernards et al. 2011; 2012). After greenhouse and field experiments, Bernards et al. (2012) confirmed that the waterhemp population was resistant to 2,4-D. It is the sixth herbicide mechanism-of-action group reported to which waterhemp has evolved resistance.

2,4-D background and mode of action

Research about 2,4-D started during the World War II, and first 2,4-D was synthesized in 1941 by Pokorny (1941). Two research groups from British and the U.S. were simultaneity working on the 2,4-D development. Both research groups worked under wartime regulations and secrecy. Although the scientists aimed to increase crop yields for a nation at war, both research groups had the developing of potent chemical warfare agents as a primary goal. Fortunately, 2,4-D was not available as a weapon in this context and the agriculture potential was soon released. The original patent was not issued until 1945 to Dr. Franklin Jones, a plant physiologist, in the U.S. It is considered the beginning of the modern herbicide technology (Troyer 2001; Senseman 2007; Cobb and Reade 2010).

2,4-D (2,4-dichlorophenoxy acetic acid) is an active ingredient belong to phenoxyalkanoic acids (also called phenoxy acids) in the synthetic auxins. 2,4-D is a selective herbicide to control many annual and perennial broadleaf weeds and is meanly used in postemergence in grass crops such as corn, grain sorghum and small grain. This herbicide also has some specialized uses such as preharvest in small grain, preemergence soil application for corn between corn planting and emergence and early preplant before planting soybean. In addition, 2,4-D can be used in turf grasses, pastures, rangeland, rice, sugarcane and forest management (Senseman 2007; Ross and Lembi 2008).

The Herbicide Handbook lists 28 different 2,4-D forms which are grouped as acids, amine salts (i.e. dimethylamine salt) and esters such as butoxyethyl and isooctyl esters (Senseman 2007). The ester and amine salt forms are the more currently used (Wilson et al. 1997). All these forms vary in solubility and volatility. In general, 2,4-D is highly soluble in water. The salt and acid forms have the highest solubility in water (796 g L^{-1} and 900 mg L^{-1}, respectibely), while butoxyethyl and isooctyl ester forms have the lowest solubility in water (100 and 0.0324 mg L^{-1}, respectively) (Senseman 2007).

One of the most important processes which can lead to injury of non-target plants is the volatility. 2,4-D is considered highly volatile, but this is variable with the chemical form (Grover et al. 1972; Senseman 2007). In general, most of the forms in the butoxyethyl ester group have high volatility, while the forms within the isooctly ester group have low volatility. The salt forms are considered non-volatile. Vapor drift of high volatile 2,4-D esters increase when environmental temperatures are high and relative humidity is low. Volatile products can "evaporate" from soil or plant surfaces, up to 48 hours after application (Grover et al. 1972; Que Hee and Sutherland 1974; Wilson et al. 1997; Campbell 1997; Liu et al. 2011).

In general, the auxinic herbicides such as 2,4-D are foliar applied. However, 2,4-D salt has soil activity and can be absorbed by roots more than ester forms which are more rapidly absorbed in foliar application (Senseman 2007). Basically, 2,4-D moves primarily via the symplastic pathway when it is foliar applied. This movement includes the plant phloem in both acropetally (up) and basipetally (down), and concentrates in growing points of shoots and roots (Sterling and Hall 1997). Sterling and Hall (1997) suggested that some auxinic herbicides such as 2,4-D are ambimobile which means that this herbicide can move from the phloem to the xylem and vice versa. When the 2,4-D is absorbed by roots, it is partially translocated in the transpiration stream by the apoplastic pathway. After that, 2,4-D move acropetally into the xylem and redistribute to the phloem when it reach the top of the plant as consequence of the transpiration gradient. Once in the phloem, 2,4-D moves throughout the plant in any direction, but basically to the growing points (Sterling and Hall 1997; Senseman 2007).

Although 2,4-D's mode of action is not completely understood, it is believed that acts in a similar way that endogenous auxin hormones in plants. At the same way that in the natural hormone indole-3acetic acid (AAI), the specific molecular binding site and auxin receptor/s of 2,4-D has not been identified. According to Grossmann (2010), the principal candidates as auxin receptors are the auxin-binding protein 1 (ABP1) and the transport inhibitor response 1 (TIR1) protein. The binding of 2,4-D to some of this two proteins (i.e. ABP1 and/or TIR1) leads to the succeeding series of biochemical and physiological events associated with herbicide action. It has been found that these events affect the cell wall plasticity and nucleic acid metabolism (Senseman 2007). After the binding occurs, it is followed by a cell wall acidification by stimulating the activity of a membrane-bound ATPase proton pump. Then, the acidification of

cell wall induces cell elongation because it is thought that increase the activity of enzymes responsible for cell wall loosing (Senseman 2007).

In addition, as a consequence of treating plants with auxinic herbicides such as 2,4-D, the biosynthesis of ethylene and abscisic acid (ABA) hormones are stimulated (Sterling and Hall 1997). Ethylene overproduction causes swelling of stems and roots and leaf abscission and epinasty as the AAI effects. Moreover, the overproduction of ethylene inhibits its own transport in the plant and consequently, contributes to growth abnormalities and senescence (Grossmann 2001). Together with the ethylene effects, ABA functions as a hormonal second messenger in the mode of action of auxinic herbicides. The increased ABA accumulates in the shoot tissue, translocates within the plants and induces stomatal closure. Consequently, ABA also promotes leaf senescence and control of the transpiration and carbon assimilation by photosynthesis. In this context, the latest effect is the accumulation of hydrogen peroxide (H_2O_2) resulting in oxidative damage by phytotoxicity (Grossmann et al. 2001; 2009; Cobb and Reade 2010).

2,4-D resistance in crops

One of the first and more adopted herbicide-resistant crops was those resistant to glyphosate. Glyphosate-resistant soybean was the first crop that started to grown commercially in 1996, followed by cotton in 1997, corn in 1998 and canola in 1999. All of them were rapidly adopted by farmers basically because the weed control program with glyphosate was effective, easy-to-use, economical, safe, and novel (Green and Owen 2010). Unfortunately, this approach with only one herbicide used up to 3-4 times per crop season every year has contributed to the increasing in the number of glyphosate-resistant weeds. Actually, farmers need to use different herbicide mode of actions to avoid or decrease the evolution of glyphosate resistant weeds (Nandula 2010).

Since middle 90's several nontransgenic and transgenic herbicide-resistant crops have been available for farmers. However, no all of them had the same grade of adoption; even some of these herbicide-resistant crops are not longer in the market. Although some nontransgenic herbicide-resistant crops such as imidazolinone or sulfunylurea resistant canola, sunflower and wheat were partially adopted by farmers, the new generation of transgenic crops released after 1996 were rapid and massively adopted by farmers (Green and Castle 2010). Along with glyphosate-resistant crops, glufosinate-resistant canola, corn and cotton have been commercially

available in the market. However, these crops have not had too much success particularly because glufosinate is more expensive than glyphosate and also has some restrictions in the application timing (Green and Owen 2010).

New transgenic technologies conferring resistance to dicamba, 2,4-D and ALS-inhibitor herbicides are being developed to replace or complement the glyphosate-resistance traits. Two genes from the aad family that code for the enzyme complex of aryloxyalkanoate dioxygenase and provide resistance to some auxin herbicides were aisolated (Müller et al. 2006; Schleinitz et al. 2004). Called DHT (for Dow AgroSciences Herbicide Tolerance) is a trait being developed by Dow AgroSciences that confers resistance to some auxinic herbicides and in addition to a AACase- inhibitor herbicide as aryloxyfenoxyproprionic acids (herbicides ending in "fop"), such as quizalofop. DHT is divided in two traits: DHT 1 and DHT 2. The DHT 1 trait is being developed in corn. The aad-1 gene was isolated from *Sphingobium herbicidovorans* and inactivates auxins such as 2,4-D and ACCase-inhibiting herbicides in only the fop's class. In the DHT 2 is being developed in soybean and cotton. The aad-12 gene was isolated from the bacterium *Delftia acidovorans* and codes for a 2-ketoglutarate dependent dioxygenase that inactivates phenoxyacetate auxins (e.g., 2,4-D) and pyridinyloxyacetate auxins (e.g., triclopyr and fluoroxypyr) (Wright et al. 2010).

2,4-D resistance in weeds

Only 29 weed biotypes have been reported resistant to auxinic herbicides in the world. Of those, 16 biotypes are resistant to 2,4-D (Table 1) (Heap 2012). Since the first two documented 2,4-D resistant weed wild carrot (*Daucus carota* L.) (Switzer 1952) and spreading dayflower biotypes in 1957 (Hilton, 1957), there have been a slow increase in the auxinic resistant weeds principally concentrated in the 1990's decade (Table 1) (Heap 2012). Resistance to auxinic herbicides has been considered low risk occurrence compared to the resistance to other groups such as ALS-inhibitors or triazinas. Gressel and Segel (1982) considered that this low risk is due to auxinic herbicides have multiples possible sites of action. Resistance to auxinic herbicides in particular to 2,4-D has been minimally studied and few reports described inheritance and/or mechanism of resistance. Up to date, it has been found inconsistencies in identifying a inheritance pattern and mechanism of resistance between species that are resistant auxinic herbicides (Kohler et al. 2004; Walsh et al. 2004; Jugulam et al. 2005; Riar et al. 2011).

Although it was not reported as a resistant biotype, Bell et al. (1972) reported kochia biotypes from four U.S. states which showed differential susceptibility to 2,4-D after repeated use of this herbicide in wheat. The long term use of the same single herbicide or herbicides belonging to the same group of mode of action has been the most common key factor to speeding the evolution of herbicide resistance as the case of 2,4-D resistant weed spreading dayflower biotypes in sugar cane fields in Hawaii (Hilton, 1957) Peniuk et al. (1993) stated that the resistance to dicamba, 2,4-D and MCPA in wild mustard was not due to altered uptake, translocation or metabolism. The same study and other later ones showed that auxinic herbicide susceptible plants treated with some auxinic herbicides such as picloram increased the ethylene production compared to the resistant plants. In this context, ethylene could be linked to the abnormal plants response (i.e. epinasty and senescence) after being treated with some auxinic herbicide (Zheng et al. 2001; Grossman 2010). In a recent study, Jugulam et al. (2005) showed that dicamba, 2,4-D and picloram resistance in wild mustard is conferred by a single and dominant gene. In addition, their study suggested that the genetic loci conferring resistance to these three herbicides which belong to different auxinic families are closely linked and the alteration of a single locus is enough to cause the cross resistance to several auxinic herbicides in wild mustard (Jugulam et al. 2005).

In the last de decade, also wild radish was reported resistant to 2,4-D amine in a lupin (*Lupinus angustufolius* L.) and wheat (*Triticum aestivum* L.) crop rotation in Australia which was treated 2,4-D in wheat during more than 17 years (Walsh et al. 2004). These authors are cautioned because the 2,4-D resistant level in wild radish is relatively low (around 2.5-fold). However, because this wild radish biotype has been reported to be resistant to other herbicide mode of action groups, Walsh et al. (2004) brought out that under this scenario the continuous use 2,4-D could increase the frequency of 2,4-D resistant wild radish in the population and turn unmanageable with a traditional herbicide use program (Walsh et al. 2004).

Kohler et al. (2004) suggested that the difference in foliar uptake to 2,4-D resistant and susceptible ground ivy (*Glechoma hederacea* L.) populations may partially contribute to differences in response to 2,4-D between these two populations. A 2,4-D susceptible population of ground ivy in lawn turf from Ohio absorbed 37% more 2,4-D than tolerant populations from Nebraska. However, both Ohio and Nebraska populations translocated a similar amount of 2,4-D (5%). Lastly, Kohler et al. (2004) reported that the Ohio population translocated 42% more toward the apical meristem of the primary stolon than the Nebraska population. Riar et al. (2011) showed the inheritance and resistant mode of

action in 2,4-D resistant prickly lettuce which constitutes one of the most recent studies. Their results suggest that 2,4-D resistance trait in prickly lettuce is single and dominant gene and nuclear-encoded in a F1 population. In addition, it was showed that resistant and susceptible biotypes metabolized the 2,4-D in a similar rate (Riar et al. 2011). Finally, this study showed that susceptible prickly lettuce biotype appeared to have absorbed and translocated more 2,4-D that the resistant biotype (Riar et al. 2011). As a conclusion, this researchers suggested that lower absorption and translocation should be expected in resistant biotypes because these individuals would have an altered signal receptor site which does not allow to receive the 2,4-D signal by overdose.

In the summer of 2009 a grower contacted to experts from the University of Nebraska-Lincoln and reported an inability to control waterhemp using 2,4-D. At this time, waterhemp population was growing in a warm season grass-production field established in 1996. Annual applications of 2,4-D were made to control annual grasses and broadleaf weeds since the grass crop was planted. Subsequently, dose-response experiments in greenhouse and in the field showed that the putative 2,4-D resistant waterhemp population from southeast of Nebraska was 10-fold more tolerant to 2,4-D than a susceptible population based in injury and dry weight (Bernards et al. 2012). The 2,4-D dose required to reach 50% of dry weight reduction was 995 and 109 g ha^{-1} in the resistant and susceptible populations respectively. To reach 90% of visual injury was needed 19 times more 2,4-D in the resistant population than the susceptible one. In addition, the 2,4-D resistant waterhemp population showed an low level of resistance to dicamba in order to 3-fold based in visual injury (Bernards et al. 2012).

Waterhemp has evolved resistance to PSII-inhibitors, ALS-inhibitors, PPO-inhibitors, glyphosate and HPPD-inhibitors (Hausman et al. 2011; McMullen and Green 2011; Tranel et al. 2011). Tranell et al. (2011) identified individual waterhemp plants that were resistant to four different mechanism-of-action groups. The accumulation of multiple herbicide resistant traits in waterhemp limits chemical options for managing waterhemp. In addition, if a waterhemp population with multiple resistance is identified, the few herbicide options that could applied would result in additional selection pressure for the evolution of resistance to these few herbicides.

Materials and Methods

The main objective of this proposal is to contribute to the understanding of waterhemp biology, inheritance and mechanism/s of resistance to 2,4-D. To accomplish this objective, the activities will be divided accordingly into three parts each having its own hypotheses and objectives.

Cross and multiple herbicide resistance in 2,4-D resistant waterhemp population

Objectives:

1) Evaluate the reported 2,4-D resistant waterhemp population (collected in 2010 - FS) response to several herbicide modes of action important in corn and soybean production systems compared to the Auburn (SE) and Clay Center (SCAL) populations.

2) Evaluate the reported 2,4-D resistant waterhemp population (FS) response to herbicide belonging to different families of the auxinic herbicide modes of action group compared to the Auburn (SE) and Clay Center (SCAL) populations.

Hypotheses:

1) More than 50% of the individuals of the 2,4-D resistant population will survive recommended field rate applications of atrazine and imazethapyr.
2) Less than 5% of individuals in the 2,4-D resistant population will survive recommended field rate applications of lactofen, mesotrione, glufosinate, and glyphosate.
3) Less than 5% of individuals in the 2,4-D resistant population will survive applications of dicamba, picloram, aminopyralid, clopyralid and aminocyclopyrachlor.

Procedures:

Plants from two waterhemp biotypes identified by Bernards et al. (2012) to be resistant and susceptible to 2,4-D will be used in this study. Plants of the 2,4-D resistant waterhemp population were collected from a native-grass (little bluestem [*Schizachyrium scoparium* (Michx.) Nash 'Camper']) field in southeast Nebraska in Fall 2010. Plants from a susceptible waterhemp population were collected from a soybean field near Auburn, NE in Fall 2010. In addition, a second 2,4-D susceptible waterhemp population was sampled in a field from the

South Central Agricultural Laboratory of University of Nebraska-Lincoln in Clay Center, NE in Fall 2010. Each sample was a composite of 40 or more plants. Waterhemp seed was cleaned and then stored at 4 C.

The experiments to determine cross and multiple resistance in the 2,4-D resistant waterhemp population will be carry out in greenhouse facilities located on East Campus of University of Nebraska-Lincoln in Lincoln, NE. Greenhouse area is equipped with sodium halide lamps that provide supplemental lighting to ensure a 15-hr photoperiod. Daytime temperatures are set at 24 ± 2 C, and nighttime temperatures are set at 19 ± 3 C.

Seed from each of the three waterhemp populations will be placed in moist paper in petri dishes into a dark oven at 35 C during 24 - 72 h to break the dormancy and get uniform germination. Two to three small seedlings will be selected and transplanted into 0.9 L black plastic pots filled with potting mix (BM1® Growing Mix, Berger Peat Moss LTD, Saint-Modeste, Quebec, C-anada). After transplanting, seedlings will be covered with transparent plastic film in groups of 50-60 pots during 5 to 10 days after transplanting to assure the appropriate moist and temperature conditions. After removing the plastic film, the plants will be watered as needed. Waterhemp plants will be thinned at 15 - 21 days after transplanting and only one plant will be left per pot. At thinning time, plants will be fertilized every 10 days with 50 ml/pot solution of 24-8-16 fertilizer. Waterhemp plants will be treated with herbicide when reach 8 - 12 cm in height and have 5 - 8 fully expanded leaves.

Screening for multiple resistance

Experiments will be divided in two parts. In the first part only one dose of the eight herbicides listed in Table 2 will be applied to 50 pots of each waterhemp population (FS, SE, and SCAL) in separated experiments. This first experiment will permit to evaluate if the populations are resistant to any of the recommended field rate of each herbicide. Ten pots of each population per herbicide treatment will be maintained as an untreated control. Visual injury and plant survival at 7, 14, 21, and 28 DAT will be recorded based on the expected symptoms for each herbicide mode of action compared to the untreated control plants on a scale of 0 (no injury) to 100 (dead plants). For any herbicide mode of action that the 2,4-D resistant waterhemp population shows more that 50% survival, the FS population will be suspected resistant and that herbicide mode of action will then be selected for the next step of the experiment.

Dose-response studies

In the second part of the experiment, the selected herbicides will be evaluated in a dose response experiment for the three waterhemp populations. Visual injury estimates will be recorded at 7, 14, 21, and 28 DAT. At the end of the experiment waterhemp plants will be harvested and dried until constant weight in a forced air dryer at 65 C. Dose response experiments will be arranged in a randomized complete block design with five repetitions and each experiment will be replicated in time (2 runs total). At least one plant per block will be harvested prior to the application of herbicide treatments in each experiment to have an average dry weight before treatment. Treatments will be prepared in distilled water and applied in a single-tip chamber sprayer (DeVries Manufacturing Corp, Hollandale, MN 56045) using an 8001E nozzle (Spraying Systems Co., Wheaton, IL 60187) calibrated to deliver 190 L ha^{-1} carrier volume at a pressure of 207 kPa.

Table 2. Details of the herbicide treatments for evaluating cross herbicide resistance (first part of the greenhouse experiments).

Herbicide	Brand	Rate (g ha^{-1})	Adjuvant
Atrazine	Aatrex Nine-O®	1.1 lb Aatrex/A	1 qt COC/A
Imazethapyr	Pursuit®	4 fl oz /A	1.25% (v/v) COC 12 lb AMS/100 gal
Lactofen	Cobra®	8 fl oz /A	1% (v/v) COC 2 lb AMS/A
Glyphosate	Roundup PowerMAX®	22 fl oz ae /A	17 lb AMS/100 gal
Mesotrione	Callisto®	3 fl oz /A +	1% (v/v) COC 8.5 lb AMS/100 gal
Glufosinate	Ignite®	22 fl oz /A	17 lb AMS/100 gal

Statistical analysis

Visual injury estimate, dry weight and survival data will be analyzed using a nonlinear regression model in statistical software. Dose-response models will be constructed using a four-parameter log-logistic equation (Equation 1).

$$y = c + (d - c / 1 + \exp (b (\log x - \log e))) \quad [1]$$

In this four-parameter log-logistic model, y is the response based in visual injury estimate, dry weight or plant survival, c is the lower limit, d is the upper limit, x is the herbicide dose, e is the herbicide dose giving a 50% response (growth reduction, GR_{50}, injury estimation, I_{50} or survival, S_{50}) between the upper and lower limit, and it also represents the inflection point, and b is the slope of the regression curve at the inflection point. The herbicide dose required to achieve 50, 80 and 90% visual injury, reduction in dry weight or plant survival will calculated for all the herbicides and populations using the log-logistic models fitted to the data. The R:S ratios will be calculated by dividing the GR_{50}, I_{50}, or S_{50} of the resistant population by the same values of the susceptible population.

2,4-D resistance inheritance

Objectives:

1) Determine the inheritance of 2,4-D resistance in waterhemp.

Hypotheses:

1) Inheritance of 2,4-D resistance follows Mendel Inheritance.
2) Inheritance of 2,4-D resistance will be inherited both maternally and through pollen.
3) Waterhemp 2,4-D resistance is conferred by a single and dominant gene.

Procedures:

Obtaining the F1 lines

Plants from two waterhemp biotype identified by Bernards et al. (2012) to be resistant (R-) and susceptible (S-) to 2,4-D will be planted and growth in separated bays in the greenhouse and under the same condition and procedure decrypted before. When the plants from the R-population reach 8 - 12 cm in height and 5 - 8 fully expanded leaves, they will be treated with 280 g ha^{-1} of 2,4-D. Plants that survived at 14 days after treatment will be scored by visual injury and categorized as resistant or partially resistant according to the epinastic effects, regrowth and how healthy the plant looks like. Male and female plants in both R (resistant only)- and S-populations will be identified when the flowers are visible. Identified plants will be placed in a cage and crossed in all combinations for a total of 100 crosses. Caging techniques will consist in a PVC tube prism shape frame (70cm x 70cm x 150cm – W x L x H) covered with a pollination

mesh bag which must have pores small enough to prevent passage of insects or pollen, but still allow for sunlight to pass. Crosses using R-plants as females and males, and S-plants as females and males will be made of each pairing of R- / S- plants using just one male per cage. Before placing the female plants into the tent, each of them will be checked and all flowers/inflorescence will be removed by hand. In the same way, the flower head of the male plants will be removed in all male before placing them into the tent to stimulate the growing of branches and pollen production. Male plants inside the cages will be shaken every day over the female plants to ensure the maximum pollen transfer. Seed produced (F1) from each individual plant inside the cage will be collected, identified and kept separated from the others plants in the cage, dried to room temperature during at least 3 weeks and stored at 4 C until next step of the study.

F1 inheritance

To determine the mode of inheritance and level of dominance of resistance to 2,4-D, a total of 12 F1 individual crosses (F1 lines) will be selected and evaluated. F1 lines selection is as follow: three R-males crossed with two S-female per each R-male will be selected, and three S-males crossed with two R-females per S-male will be selected. In all cases, the R-plants will be those categorized as having high level of resistance. Ninety pots will planted with seed from each selected F1 line, and 90 pots will be planted with seed from each R- and S- parental populations will be planted. Plants will be planted and grown following the procedures explained in the cross resistance experiments. In this first step F1 lines will be evaluated to see the importance of male and female parent in herbicide resistance. Ten plants for each F1 crossing and S-population will be treated with one of the following 2,4-D doses: 0, 70, 140, 280, 560, 1120, 2240 and 4480 g ae ha^{-1}. In addition, 10 plants of the R-population will be treated with one of the following 2,4-D doses: 0, 140, 280, 560, 1120, 2240, 4480 and 8960 g ae ha^{-1}. Plant survival and visual injury estimation will be recorded at 7, 14, 21 and 28 DAT. At 28 DAT dry weight of the plants will be determined. According to the visual injury estimates at 28 DAT, plants will be classified as resistant (Visual injury $\leq$ 35%), intermediate (Visual injury between 35 and 75%) or susceptible (Visual injury $\geq$ 75%). In addition, the ratio between resistant, intermediate and susceptible (R:I:S) plants will be calculated.

Obtaining F2 lines and backcrosses

For a complete understanding of inheritance and dominance of the resistance, F2 and backcrossed lines will be created. From the previous experiment the F1 lines that show the relative more uniform response to 2,4-D dose will be selected. Seed from the selected F1 lines will be planted and grow under the same condition detailed previously. When the plants reach 8 - 12 cm in height and 5 - 8 fully expanded leaves, they will be treated with the maximum equivalent 2,4-D dose where differences between R- and S-population could be observed in the previous experiment. All treated F1 plants that survived to the 2,4-D application and are in healthy condition (≤ 50% of visual injury estimate) at 28 DAT will be selected for crossings. Crosses will consist in all the options between selected F1 and parental S-population as male, and selected F1 and parental S-population as females (i.e. selected F1 as male x selected F1, parental S-population, and parental R F1-population as females in a cage; parental S-population as male x selected F1 and x parental S-population as females in other cage). THIS SECOND CROSSING WILL NOT BE NEEDED IF I DETERMINE FROM ANALYSIS OF F1s THAT THE TRAIT IS NUCLEAR INHERITED. The crossing that includes the selected F1 population as male and female will create the F2 line. On the other hand, the crossing that includes both the selected F1 population and the parental S-population as male and female will create the backcrossed progeny in either direction. When the male plants do not produce more pollen and/or mature seed is observed in female plants, seed from these female plants will be harvested and stored as previously described.

F2 and backcrosses inheritance

At the same way that F1 line, seed from F2 lines, backcrossed lines and parental R- and S-populations will be planted and grown 100 pots per line/population in greenhouse under the same conditions as previously described experiments. All 100 plants for each line/population will be treated with 1120 g ae ha^{-1} of 2,4-D. Plant survival and visual injury estimation will be recorded at 7, 14, 21 and 28 DAT. According to the visual injury estimates at 28 DAT, plants will be classified as resistant (Visual injury ≤ 35%), intermediate (Visual injury between 35 and 75%) or susceptible (Visual injury ≥ 75%). In addition, the segregation ratios between R:I:S plants will be calculated.

Analysis

Results from F1, F2 and backcrossed lines will be tested using chi-square (χ^2) test to evaluate the observed segregation ratios versus the hypothesized segregation rations that 2,4-D resistant is conferred by a single dominant or partially dominant gene. In these cases the expected segregation ratios will be 3:1 (R:S) or 1:2:1 (R:I:S) for F2 lines, and 1:1 (R:S) or 0:1:1 (R:I:S) for backcrossed lines.

2,4-D resistance mechanism of action

Objectives:

1) Develop a uniform R-population to ensure the maximum homozygous plants possible.
2) Determine if differential absorption, translocation and/or metabolism are possible mechanisms of resistance to 2,4-D of the R-population compared to a 2,4-D susceptible waterhemp population.

Hypotheses:

1) The 2,4-D resistance of waterhemp is due to at least one of the three following possible mechanism: reduced absorption, reduced translocation, or herbicide metabolism.

Procedure:

Obtaining the purified lines

Plants from two waterhemp biotype identified by Bernards et al. (2012) and used in inheritance study will be planted and grown in separated greenhouse bays under the same conditions and procedures described for previous experiments. To determine the mechanism of resistance, F1 resistant lines originated from the same three R-males selected in inheritance study will be selected, but in this case these three R-males will be crossed with two R-females (that show a high level of resistance in the screening) per R-male. A total of six F1 resistant lines will be created.

Sixty pots will be planted with seed from each selected F1 resistant line, and additionally 60 pots of the each R- and S- parental populations will be planted. Plants will be planted and grown in the same condition as previous experiments in order to get uniform (i.e homozygous) populations after to be treated with the following 2,4-D doses: 0, 2240, 4480 and 8960 g ae ha^{-1}.

Between 10 and 12 plants will be assigned to each dose. Plant survival and visual injury estimate will be recorded at 7, 14, 21 and 28 days after treatment. The F1 resistant line that shows the most uniform resistance response will be selected. From this selected line, five treated males and ten treated females that survived the higher dose will be placed in a cage to cross between themselves. Plants will be irrigated and fertilized as needed to insure abundant seed production. When male's plants do not produce more pollen and/or when female plants start to senesce, the plants will be harvested and the seed will be individually collected for each female plant to be used in all the studies of resistance mechanisms.

Absorption and translocation experiment

This experiment will be carried out using the purified R- and S-populations. Non-formulated 2,4-D with ^{14}C labeled benzene ring and non-formulated 2,4-D amine will be used (obtained from Dow AgroSciences). Seed from R- and S- purified populations will be planted and grown under similar conditions than previous experiments.

When plants reach between 10 and 13 cm in height and 7 and 10 leaf stage, a portion in the center of the adaxial side of the second fully expanded leave will be covered with a plastic tag to intercept the herbicide spray. The plant will then be treated with 560 g ae ha^{-1} of nonlabeled 2,4-D. After the no labeled herbicide on the plant dries out, the plastic tag will be removed an labeled 2,4-D will be applied in the center of the previously covered portion of the leaf. Plants will be harvested at several intervals (hours after treatment) and dissected into five parts: marked portion treated with labeled 2,4-D, the remaining portion –no labeled – of the leaf including the petiole, the portion of the plant above the treated leaf, the portion of the plant below the treated leaf, and the plant root.

The portion of the leaf treated with labeled 2,4-D will be rinsed with a methanol:water solution to remove unabsorbed 2,4-D. The rinsed solution will be collected for determination of the nonabsorbed labeled herbice by liquid scintillation spectrometry. The labeled herbicide absorbed by the different plant parts will be analyzed by liquid scintillation spectrometry after oxidation of the sample.

Because regrowth is expected to be observed in the R-biotype, a second part of the absorption and translocation experiment will include studying of adsorption and translocation of labeled 2,4-D at 21 days after treatment. The materials and methods will be the same that the

used in the short time 2,4-D adsorption and translocation study, however the plants will be harvested at longer intervals (days after treatment). Plant harvest, dissection, and conditioning of sample will be similar to the short term study. In addition, survival, visual injury estimation and whole plant dry weight will be recorded at 21 days after treatment.

Metabolism experiment

All procedures for growing, treating, and harvesting plants will be similar to those of the absorption and translocation experiment. An extra harvest interval (168 hours after treatment) will be included. The difference is that the treated leaf will not be dissected into two parts, but the entire labeled leaf will be processed for analysis. After harvest and dissection all plant parts will be wrapped in aluminum foil and stored in −20 °C until extraction. The extracts will be analyzed by thin-layer chromatography (TLC) to separate labeled 2,4-D from labeled metabolites using a radiochromatogram scanner.

Statistical analysis

Absorption and translocation over time will be analyzed using non-linear regression to assess any differences between R- and S- purified populations. For the metabolism experiment the different metabolites amounts over time will be analyzed using non-linear regression.

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