Physiological, and Genetic Characterization of 2,4-D-resistant Palmer Amaranth (*Amaranthus palmeri* S. Watson) and Its Management

by

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B.Sc., Visva-Bharati, 2014
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Agronomy
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Abstract

Palmer amaranth (*Amaranthus palmeri* S. Watson) is one of the topmost troublesome, C_4 dioecious weeds in the US. Biological traits such as aggressive growth habits, prolific seed production, and the ability to withstand environmental stresses hinder control of this weed. Additionally, numerous Palmer amaranth populations across the US have been found to have evolved resistance to multiple herbicides. In 2018, a population of Palmer amaranth from a conservation tillage study from Riley County, Kansas was suspected to have evolved resistance to multiple herbicides including 2,4-dichlorophenoxyacetic acid (2,4-D) and was designated as Kansas Conservation Tillage Resistant (KCTR). 2,4-D, a synthetic auxin herbicide, is widely used for controlling broadleaf weeds in cereal crops. However, over-reliance on 2,4-D to control other herbicide-resistant weeds, along with the commercialization of 2,4-D-tolerant crop technology, has resulted in increased usage of this herbicide. The objectives of this dissertation were to 1) characterize the evolution of multiple herbicide resistance including 2,4-D in KCTR Palmer amaranth; 2) investigate the physiological mechanism of 2,4-D resistance in KCTR compared to two known susceptible Palmer amaranth populations *i.e.*, Kansas Susceptible (KSS) and Mississippi Susceptible (MSS); 3) assess the genetic basis of 2,4-D resistance in KCTR; and 4) evaluate herbicide programs that can manage glyphosate-resistant Palmer amaranth in 2,4-D-tolerant soybean. Experiments were conducted under either greenhouse or controlled growth chamber conditions. Standard herbicide dose-response, physiological, biochemical (using radiolabeled herbicides), breeding, and field experiments were designed and conducted. The results of these experiments found that KCTR Palmer amaranth had evolved resistance to six herbicide modes of action, including acetolactate synthase (ALS)-, photosystem II (PS II)-, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-, 4-hydroxyphenylpyruvate dioxygenase

(HPPD)-, protoporphyrinogen oxidase (PPO)- inhibitors, and synthetic auxins (2,4-D). Sequencing and analyses of genes coding for the herbicide targets indicated absence of all known mutations that confer resistance, except for EPSPS-inhibitor, with a massive amplification of *EPSPS* gene (up to 88 copies). Investigation of non-target site resistance mechanism(s) in KCTR confirmed the predominance of metabolic resistance to multiple herbicides mediated by either cytochrome P450 (P450) or glutathione *S*-transferase enzyme activity. Whole-plant dose-response analyses confirmed a 6- to 11- fold resistance to 2,4-D in KCTR compared to two susceptible populations (KSS or MSS). [^{14}C] 2,4-D uptake and translocation studies indicated a 10% less and 3 times slower translocation of [^{14}C] 2,4-D in KCTR compared to susceptible populations, while there was no difference in the amount of [^{14}C] 2,4-D absorbed. However, KCTR plants metabolized [^{14}C] 2,4-D much faster than the susceptible KSS and MSS, suggesting that enhanced metabolism bestows resistance to this herbicide in KCTR. Further, use of P450-inhibitor (*e.g.,* malathion) indicated that the metabolism of 2,4-D in KCTR is mediated by P450 activity. Genetic analyses of F_1 and F_2 progenies, derived from crossing between KCTR and KSS, revealed that 2,4-D resistance in KCTR Palmer amaranth is an incompletely dominant, nuclear trait. Segregation of F_2 progenies did not follow the Mendelian single gene inheritance model (3:1), suggesting the involvement of multiple genes in mediating 2,4-D resistance in KCTR. Evaluation of herbicide programs for Palmer amaranth management in the field suggested that pre-emergence herbicides with residual activity followed by post-emergence application of either 2,4-D or glufosinate or 2,4-D and glufosinate can control glyphosate-resistant Palmer amaranth in 2,4-D-tolerant soybean. Overall, the outcome of this dissertation documents the first case of a six-way resistance in a single Palmer amaranth population and also for the first time characterizes the physiological and genetic basis of 2,4-D resistance in this

weed. These findings will help in predicting and minimizing further evolution and spread of 2,4-D resistance in Palmer amaranth.

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Dedication

This dissertation is dedicated to my mother, Mrs. Kajal Shyam, my father, Mr. Madan Mohan Shyam, and my brother, Mr. Mrinal Shyam, for their immense sacrifices, hard work, and support which helped me to reach where I am today.

Chapter 1 - Literature Review

Impact of Weeds on Agricultural Production

Global crop production has always faced challenges from various biotic and abiotic stresses. Economic losses due to these stresses are significant in this era of input-intensive agricultural crop production systems (Gharde et al. 2018). Weed infestation in crop production is considered as one of the most important biotic stresses that affects crop production, by competing for resources, interfering with crop management practices, harboring pests, and reducing produce quality. Weed interference results in significant crop loss across the globe (Zimdahl 2018). Oerke (2006) reported that amongst different agricultural pests, weeds account for the highest potential for yield loss (34%), followed by animal pests (18%) and pathogens (16%). Similarly, a ten-year study conducted from 2007 to 2016 assessed that up to 47, 71, and 70% loss can occur compared to a no-weed control situation in grain sorghum (*Sorghum bicolor* L. Moench), dry bean, and sugar beet (*Beta vulgaris* var. *saccharifera*), respectively, in the US and Canada, amounting to $953, 100-622, and 22-25 million per year (Dille et al. 2020, Soltani et al. 2018a, 2018b). Such substantial economic impact makes weed management an utmost necessity. In conventional crop production systems, the most common method of weed control is use of herbicides. The first modern-day herbicide developed and used were synthetic auxins, primarily to selectively control broadleaf weeds. Since then, herbicides have become the most reliable and economical tool for controlling weeds, contributing significantly to the historical increase in crop yields over the last 75 years (Heap 2021). However, the over-reliance on herbicides as the sole method of weed control has increased selection pressure, resulting in the evolution of resistance to herbicides in several weed species.

Currently, there are 521 unique cases of herbicide resistance involving 263 weed species, distributed in 71 countries (Heap 2021). In the US, 84 weeds including monocots such as bluegrass (*Poa annua* L.), goosegrass (*Eleusine indica* L. Gaertn.), Italian ryegrass (*Lolium perenne* ssp. *multiflorum* Lam. Husnot) and dicots such as Palmer amaranth (*Amaranthus palmeri* S. Watson), common waterhemp (*Amaranthus tuberculatus* Moq. Sauer), kochia (*Bassia scoparia* L. A.J. Scott), horseweed (*Conyza canadensis* L. Cronquist) have been documented to have evolved resistance to herbicides. Within the US, specifically in the Midwestern region some of the major weeds, *e.g.*, Palmer amaranth, common waterhemp, giant ragweed, horseweed, and kochia, were found to have evolved resistance to multiple herbicides and thus driving the weed management decisions. Among these driver weeds, Palmer amaranth is found to be problematic in several cropping systems, including corn, soybean, sorghum, and cotton.

Palmer amaranth (*Amaranthus palmeri* S. Watson)

Importance

Palmer amaranth is ranked as one of the top-most troublesome weeds in the US by the Weed Science Society of America (WSSA; Van Wychen 2020). Originally considered as a native of the southwest US, this weed is now found throughout the country. Competition from Palmer amaranth can cause significant yield loss in row and vegetable crops (Table 1.1). Such yield loss is attributed to the intense competition offered by Palmer amaranth for resources (such as water, light, nutrients) and interference *via* allelopathy (Berger et al. 2015, Menges 1987).

Biological Characteristics

Palmer amaranth is a highly competitive weed owing to its aggressive growth and reproductive characteristics. Palmer amaranth is a C_4 plant (Wang et al. 1992) and can use the nitrogen and ribulose 1,5-bisphosphate carboxylase protein more efficiently than C_3 plants

(Schmitt and Edwards 1981). Such efficiency enables Palmer amaranth to have a high photosynthetic rate, especially at optimum growth conditions. With the availability of high soil moisture, the photosynthetic capacity of Palmer amaranth can be over 70 μmol CO_2 m^{-2} s^{-1} (Ehleringer 1983).

Table 1.1 Reported yield losses in row and horticultural crops due to Palmer amaranth infestation.

Crop		Infestation density (plants/ m of row)	Yield loss (%)	References
Name	Latin name			
Row crops				
Corn	*Zea mays* L.	8 plants/m	91	Massinga et al. (2001)
Sorghum	*Sorghum bicolor* L. Moench	2 plants/m	22	Unruh (2013)
Soybeans	*Glycine max* L. Merr.	8 plants/m^2	49	Basinger et al. (2019a)
		8 plants/m	79	Bensch et al. (2003)
		10 plants/m	68	Klingaman and Oliver (1994)
		0.37 plants/m^2	21	Chandi et al. (2012)
Cotton	*Gossypium hirsutum* L.	10 plants/9.1 m	54	Morgan et al. (2001)
		10 plants/6.1 m	60	MacRae et al. (2013)
Peanut	*Arachis hypogaea* L.	1 plant/m	28	Burke et al. (2007)
Horticultural crops				
Sweet potato	*Ipomoea batatas* L. Lam.	16 plants/m	79	Basinger et al. (2019b)
		6.5 plants/m	94	Meyers et al. (2010)
Watermelon	*Citrullus lanatus* Thunb.	4 plants/0.76 m	65	Bertucci et al. (2019)

Palmer amaranth plants can accumulate 32 to 83% higher dry biomass with a relative growth rate of 0.32 g g^{-1} day^{-1} compared to other *Amaranthus* species, including common waterhemp (0.31 g g^{-1} day^{-1}), redroot pigweed (*Amaranthus retroflexus* L.; 0.30 g g^{-1} day^{-1}), and tumble pigweed (*Amaranthus albus* L.; 0.26 g g^{-1} day^{-1}) (Horak and Loughin 2000).

During long-term drought conditions, despite a lower photosynthetic rate, the intercellular CO_2 concentration remains constant in Palmer amaranth indicating coordination of gas exchange parameters (Ehleringer 1983). Palmer amaranth can also maintain positive cell turgor and keep stomata open under drought conditions (Ehleringer 1985). A study conducted to evaluate the effect of water stress on Palmer amaranth seed production by Chahal et al. (2018) reported constant cumulative seed germination in female plants exposed to ≥50% soil field capacity.

A vital aspect of the reproductive biology of Palmer amaranth is its dioecious (separate male and female plants) nature, making it an obligate outcrosser. Female Palmer amaranth plants are prolific seed producers. Palmer amaranth plants, established from March through July, were reported to produce 600,000 seeds $plant^{-1}$ (Keeley et al. 1987). In another study, around 446,000 seeds were obtained from a single glyphosate-resistant (GR) Palmer amaranth plant in Georgia (Webster and Grey 2015). Outcrossing along with high fecundity ensures high genetic diversity due to sexual recombination in this weed, resulting in adaptability and weediness. Outcrossing also enables interspecific and intraspecific transfer of herbicide resistance alleles from/to Palmer amaranth. Such transfer of herbicide resistance alleles has been documented for both target-site (TSR) and non-target site (NTSR) mechanisms (explained later in section 'Evolution of Herbicide Resistance'). For instance, Oliveira et al. (2018b) observed pollen-mediated gene flow of metabolic resistance to the herbicide mesotrione, from Palmer amaranth to other susceptible populations as well as common waterhemp. In another study, acetolactate synthase (ALS)-

inhibitor resistance was transferred to spiny amaranth (*Amaranthus spinosus* L.) x Palmer amaranth and common waterhemp x Palmer amaranth hybrids (Franssen et al. 2001, Molin et al. 2021). Similarly, glyphosate resistance was transferred *via* pollen to susceptible Palmer amaranth populations and other *Amaranthus sp*. such as spiny amaranth, common waterhemp, and smooth pigweed (*Amaranthus hybridus* L.) (Gaines et al. 2012, Nandula et al. 2014, Sosnoskie et al. 2012).

Control of Palmer amaranth

Being an aggressive and highly competitive weed, the management of Palmer amaranth is crucial not only for reducing crop yield losses but also for hindering further addition of seeds to the soil seed bank. Non-chemical weed management techniques such as use of cover crops have been shown to improve Palmer amaranth control (Palhano et al. 2018). However, herbicides are the most preferred option by growers for managing this aggressive weed in crop production systems. Post-emergence (POST) herbicide options for controlling Palmer amaranth include systemic herbicides such as ALS-, photosystem II (PS II)-, 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS)-, 4-hydroxyphenyl- pyruvate dioxygenase (HPPD)-, and synthetic auxins and contact herbicides including photosystem I (PS I)- and protoporphyrinogen oxidase (PPO)-inhibitors. Pre-emergence (PRE) herbicide options include ALS-, PS II-, HPPD-, PPO-, microtubulin-, and very long-chain fatty acid (VLCFA) inhibitors. Best management practices aimed to reduce the selection pressure and evolution of herbicide resistance in troublesome weeds such as Palmer amaranth call for integrated management techniques (Aulakh et al. 2010, Norsworthy et al. 2012). Application of PREs with residual activity followed by POST herbicide applications while incorporating multiple modes of action (MOA) of herbicides have been

proven to be effective in managing herbicide-resistant Palmer amaranth populations (Chahal and Jhala 2018, Sarangi and Jhala 2019).

Evolution of Herbicide Resistance

In 1989, a Palmer amaranth population in South Carolina was first reported to have evolved resistance to the herbicide, trifluralin (a microtubule-inhibitor) (Heap 2021; Figure 1.1). Since then, Palmer amaranth populations across the US have evolved resistance to eight herbicide MOAs (Figure 1.1). These include resistance to ALS-, PS II-, EPSPS-, microtubule-, PPO-, HPPD-, VLCFA- inhibitors, and synthetic auxins (Heap 2021; Figure 1.1). Populations with resistance to more than one MOA are widespread throughout the Midwestern US, making its control more challenging. For instance, in Kansas, Palmer amaranth populations with evolved resistance to 3, 4, or 5 MOAs have been reported (Chaudhari et al. 2020, Kumar et al. 2019b,

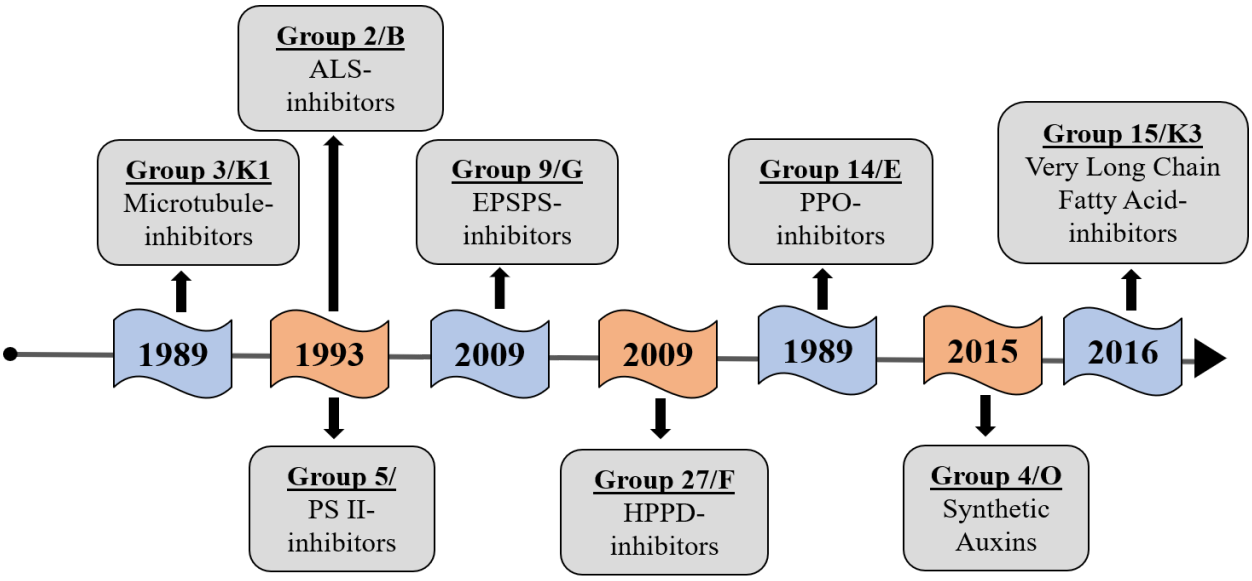

Figure 1.1 Timeline of herbicide resistance documented in Palmer amaranth; Data source: International herbicide-resistant weed database (Heap 2021).

Nakka et al. 2017a, 2017b, 2017c). Such evolution of herbicide resistance in Palmer amaranth populations occurred both *via* TSR and NTSR mechanisms.

TSR mechanisms involve alteration of the target site of herbicides, thus directly impacting the efficacy of herbicides. Known mechanisms of TSR include substitution or deletion at the target site resulting in a change in amino acids as well as an increase in copy number and/or expression of the target gene. TSR to ALS-, EPSPS-, PPO-, and HPPD-inhibitors have been reported in Palmer amaranth populations (Table 1.2). TSR mechanisms often are also known to bestow cross-resistance to different classes of herbicides within the same group of herbicides.

Table 1.2 Reported target-site resistance mechanisms in Palmer amaranth to different herbicide modes of action.

Mode of action	Target site resistance mechanism(s)	Reference
ALS-inhibitors	Substitution[a]	Chaudhari et al. (2020), Küpper et al. (2017), Nakka et al. (2017c)
EPSPS-inhibitors	Increased copy number, increased expression	Chahal et al. (2017), Chaudhari et al. (2020), Gaines et al. (2010), Koo et al. (2018)
PPO-inhibitors	Substitution, deletion[b]	Copeland et al. (2018), Salas et al. (2016), Varanasi et al. (2018b)
HPPD-inhibitors	Increased expression	Nakka et al. (2017b)

[a]Substitution refers to a mutation in the target gene caused by replacement of one or more amino acids thus significantly altering protein function.
[b]Deletion refers to a mutation in the target gene caused by deletion of one or more amino acids thus significantly altering protein function.

Unlike TSR, NTSR mechanisms do not directly affect the target site of the herbicide, rather involve altered physiological processes such as herbicide absorption, translocation, and

metabolism (Jugulam and Shyam 2019). The NTSR mechanisms such as reduced absorption and translocation, although less common, have been documented to impart glyphosate resistance in Palmer amaranth (Dominguez-Valenzuela et al. 2017). Enhanced metabolism as the primary NTSR mechanism, conferring resistance to ALS-, PS II-, PPO-, and HPPD-inhibitors has been documented in several Palmer amaranth populations (Table 1.3). NTSR mechanisms can result in cross-resistance to herbicides from the same/different MOAs (Chaudhari et al. 2020, Varanasi et al. 2019). Co-existence of TSR and NTSR has also been reported in several Palmer amaranth populations from KS (Chaudhari et al. 2020, Nakka et al. 2017a, 2017b).

Table 1.3 Reported non-target-site resistance mechanisms in Palmer amaranth to different herbicide modes of action.

Mode of action	Non-target site resistance mechanism(s)	Reference
ALS-inhibitors	Enhanced metabolism	Nakka et al. (2017c)
PS II-inhibitors	Enhanced metabolism	Chahal et al. (2019a), Nakka et al. (2017a)
EPSPS-inhibitors	Reduced absorption and translocation	Dominguez-Valenzuela et al. (2017), Nandula et al. (2012)
PPO-inhibitors	Enhanced metabolism	Varanasi et al. (2018a)
HPPD-inhibitors	Enhanced metabolism	Küpper et al. (2018), Nakka et al. (2017b)

Synthetic Auxinic Herbicides

Importance and Classification

2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxinic herbicide (SAH), was discovered in the early 1940s, and, since then, it is being used in global agriculture for weed management (Peterson et al. 2016, Thimann and Koepfli 1935). SAHs mimic the natural plant

hormone, auxin, *i.e.*, indole-3-acetic acid (IAA) (Grossmann 2003), and are classified under group 4/O according to WSSA/Herbicide Resistance Action Committee (HRAC). Globally, SAHs are the third most used herbicides in terms of the area treated (~366 x 10^6 ha), ranking behind glyphosate (~477 x 10^6 ha), and ALS-inhibitors (~508 x 10^6 ha) (Busi et al. 2018). Amongst the SAHs, 2,4-D accounts for the highest acreage (~162 x 10^6 ha) followed by dicamba (~50 x 10^6 ha) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) (~31 x 10^6 ha) (Busi et al. 2018). Conventionally SAHs are widely used to control broadleaf weeds in cereal crops (owing to their selectivity). However, several SAHs such as quinclorac and florpyrauxifen-benzyl have activity on grasses and sedges as well (Grossmann and Kwiatkowski 1995, Koo et al. 1997, Miller and Norsworthy 2018, Teló et al. 2019).

SAHs are classified into seven sub-classes based on their distinct chemical structure, *viz*., i) phenoxy-carboxylates (*e.g.*, 2,4-D, 2,4-DB, MCPA, mecoprop-P), ii) benzoates (*e.g.,* dicamba, tricamba), iii) pyridine-carboxylates (e.g., picloram, clopyralid), iv) pyridoxyl-carboxylates (*e.g.*, fluroxypyr), v) quinolone-carboxylates (*e.g.*, quinmerac, quinclorac), vi) pyrimidine-carboxylates (*e.g.*, aminocyclopyrachlor), and vii) arylpicolinates (*e.g.*, halauxifen-methyl, florpyrauxifen-benzyl) (Grossmann 2010). Amongst these, the most recent chemistries introduced include the arylpicolinates, *i.e.*, halauxifen-methyl (2005) and florpyrauxifen-benzyl (2010) (Epp et al. 2016).

Mode of Action

The mode of action of SAHs consists of a cascade of molecular processes *i.e.,* transport, signaling, perception and physiological, and biochemical processes involving ethylene and abscisic acid (ABA) production that result in the commonly known symptoms such as leaf and stem epinasty, water stress in sensitive plants.

Auxin Transport

Directional transport is critical for maintaining the biological functions of auxins (Cho and Cho 2013). Auxin polar transport and gradient formation across tissue are catalyzed by two types of transporters functioning in opposite directions, *i.e.*, influx and efflux. Auxin influx transporters in *Arabidopsis thaliana* are attributed to the Auxin resistant 1 (AUX1)/Like AUX1 (AUX1/LAXs) family, including AUX1 and LAX1-3 transporters, which facilitate the movement of auxins within a cell (Figure 1.2). Auxin efflux transporters regulate auxin movement between cells and include transporters from ATP-binding cassette sub-family B (ABCBs) and PIN-formed (PIN) proteins (Figure 1.2) (Petrášek and Friml 2009). Some ABCB transporters such as ABCB-4 have been also reported to mediate auxin uptake (Kubeš et al. 2012). Auxin transporters act as the first target sites encountered by SAHs. Therefore, alterations in these transporters can significantly impact SAH uptake and movement, resulting in reduced sensitivity to these herbicides.

AUX1, the most widely studied influx carrier of auxins, is a member of the permease, proton co-transporter superfamily (Fischer et al. 1998). AUX1 has high selectivity for the uptake of substrates. Small molecules including natural auxin, IAA, and SAHs such as 2,4-D have a high affinity to AUX1. Hence, AUX1 (loss of function) mutant lines of *Arabidopsis* were resistant to 2,4-D (Maher and Martindale 1980, Pickett et al. 1990). In contrast, other SAHs, including dicamba, triclopyr, halauxifen-methyl, picloram, and quinclorac, have either zero or very low affinity to AUX1 (Hoyerova et al. 2018). AUX1 mutants of *Arabidopsis* lines are sensitive to dicamba (Hoyerova et al. 2018). Lack of AUX1 affinity towards other SAHs, such as dicamba, indicates that AUX1 uptake is not an absolute necessity for the functioning of SAHs (Hoyerova et al. 2018). However, information on how these SAHs are transported is elusive.

PIN proteins are a family of localized transmembrane proteins involved in auxin efflux transport (Křeček et al. 2009). 2,4-D is a substrate of PIN2 and PIN7, but not PIN1 (Yang and Murphy 2009). In contrast to AUX1/LAXs and PINs, whose role in auxin transport is dependent on an electrochemical gradient, ABCB transporters couple their transport to ATP hydrolysis and transport auxins against gradients (Geisler et al. 2017). Plant ABCB transporters also have a high degree of specificity for substrates. The *PDR9* (an ABCB transporter) gain-of-function mutant line of *Arabidopsis* was tolerant to 2,4-D, while loss-of-function mutants were hypersensitive to 2,4-D (Ito and Gray 2006). Interestingly, *PDR9* loss-of-function mutants did not impact IAA and indole-butyric acid (IBA) uptake, indicating specificity of *PDR9* on 2,4-D transport (Ito and Gray 2006). Besides these families, transporters from the major facilitator superfamily (MFS) indirectly affect polar IAA transport *via* modulation of PIN2 auxin efflux transporter abundance (Remy et al. 2013). An *Arabidopsis* line with a mutation in *PIC30*, which encodes an MFS, was found insensitive to picloram (Kathare et al. 2020).

Inhibitors of ABCB and PIN transporters are often used to mimic reduced translocation of auxins by inhibiting auxin transport. For instance, N-1-naphthylphthalamic acid, a herbicide, is a widely used inhibitor of polar transport of auxins with known activity on both ABCB and PIN transporters (Abas et al. 2021). Other commonly used PIN and ABCB inhibitors are 2,3,5-triiodobenzoic acid, verapamil, and valspodar (Johnston et al. 2020, Zhu and Geisler 2015).

Auxin Signaling and Perception

The key components of auxin signaling include, a) Skp1-cullin-F-box (SCF)-ubiquitination complex ($SCF^{TIR1/AFB}$) containing the F-box transport inhibitor response 1/Auxin signaling F-box protein (TIR1/AFB) auxin co-receptors, b) auxin/indole-3-acetic acid (AUX/IAA) transcriptional repressors, and c) auxin response factors (ARF) (Figure 1.2) (Lavy

and Estelle 2016). The E3 ubiquitin ligase $SCF^{TIR1/AFB}$ complex is essential for the polyubiquitination of TIR1/AFB-AUX/IAA complex through degradation by 26s proteasome. This $SCF^{TIR1/AFB}$ complex is composed of the scaffold protein CULLIN1 subunit, the RING-BOX protein1 (for promoting transfer of ubiquitin molecules to the substrate), *Arabidopsis* SKP1 HOMOLOG1, and an F-box protein (for providing substrate recognition) (Lavy and Estelle 2016). Auxins act as "molecular glue" and stabilize TIR1/AFB-AUX/IAA interaction by binding to a hydrophobic pocket in TIR1/AFB and provide a surface for AUX/IAA to bind (Tan et al. 2007). So far, six members of the TIR1/AFB proteins, *i.e.,* TIR1, and AFB1-5 were identified in *Arabidopsis*, which involve a high degree of functional redundancy and sequence similarity (Dharmasiri et al. 2005b). Information on specific TIR1/AFB co-receptors for different SAHs is not available yet; however, studies have pointed towards specificity. For instance, *AFB5* knockout mutants of *Arabidopsis* were resistant to picloram but sensitive to 2,4-D and IAA (Walsh et al. 2006). Similarly, selective binding of AFB5-AUX/IAA co-receptor to picloram was documented (Calderón Villalobos et al. 2012). Another co-receptor *AFB4* was also a major target of picloram (Prigge et al. 2016). Since TIR1/AFB co-receptors play a crucial role in binding with AUX/IAA repressors leading to their ubiquitination, mutations in TIR1/AFB receptors can lead to conformational changes failing herbicide binding to AUX/IAA, and ultimately resistance to SAHs. So far, no field evolved resistance to SAHs caused by mutation in TIR1/AFB co-receptors has been documented.

Aux/IAA proteins are localized in the nucleus and contain highly conserved domains encoded by auxin-responsive genes (Dharmasiri et al. 2005a). Genome-wide analysis has enabled researchers to identify Aux/IAA genes in 30 plant species (Luo et al. 2018). For example, 29 AUX/IAA genes were documented in *Arabidopsis* (Overvoorde et al. 2005).

However, many of these genes in higher plants were characterized as repeats involving tandem duplication and/or segment duplication (Singh and Jain 2015, Wu et al. 2017). ARFs are transcription factors that contain an amino-terminal DNA-binding domain (Lavy and Estelle 2016). This domain binds to auxin-responsive elements (ARE) present in the promoter of auxin-responsive genes, controlling their transcription (Ballas et al. 1993, Lavy and Estelle 2016, Teale et al. 2006). Mutations in AUX/IAA co-receptors can lead to incomplete binding of the TIR1/AFB-AUX/IAA, resulting in auxin resistance. So far, two cases of TSR have been identified leading to SAH resistance and both were identified in the AUX/IAA repressor. The first case of TSR to SAH was documented in the AUX/IAA repressor resulting in dicamba resistance in kochia (explained later) (LeClere et al. 2018). The second case was recently documented in oriental mustard (explained later) (Figueiredo et al. 2021).

At low auxin concentration (Figure 1.2), Aux/IAA transcriptional repressors interact with ARFs, resulting in suppression of their activity (Kim et al. 1997). However, at high auxin levels (Figure 1.2), auxin analogs such as 2,4-D act like "molecular glue" and help TIR1in binding to the AUX/IAA protein (Dharmasiri et al. 2005a, 2005b). This binding promotes ubiquitination of AUX/IAA proteins *via* 26s proteasome. This is followed by the activation of ARFs that involve several auxin-responsive genes such as *GH3* (Kelley and Riechers 2007).

Mechanism of Action

The mechanism of action of SAHs comprises a cascade of physiological and biochemical events that occur following SAH application. In the past two decades, transcriptomic approach has enabled researchers to study the downstream events following auxin perception. Most of these studies have focused on the involvement of *1-aminocyclopropane-1-carboxylate synthase* (*ACS*) and *9-cis-epoxycarotenoid deoxygenase* (*NCED*), which are important auxin-responsive

genes. ACS and NCED are the rate-limiting enzymes in the ethylene and ABA biosynthesis pathway, respectively (Boller et al. 1979, Qin and Zeevaart 1999). Amongst these two, historically, *ACS* has been assumed to play a key role in SAH action (Grossmann 2003). For example, in kochia, *ACS* upregulation following dicamba application has been reported (Pettinga et al. 2018). Ethylene evolution following SAH application has been extensively studied in SAH-resistant and -susceptible weeds (Hall et al. 1993, Howatt et al. 2006). Additionally, *NCED* upregulation and increased ABA accumulation following SAH application have been speculated to be triggered by *ACS* upregulation (Hansen and Grossmann 2000, Kraft et al. 2007).

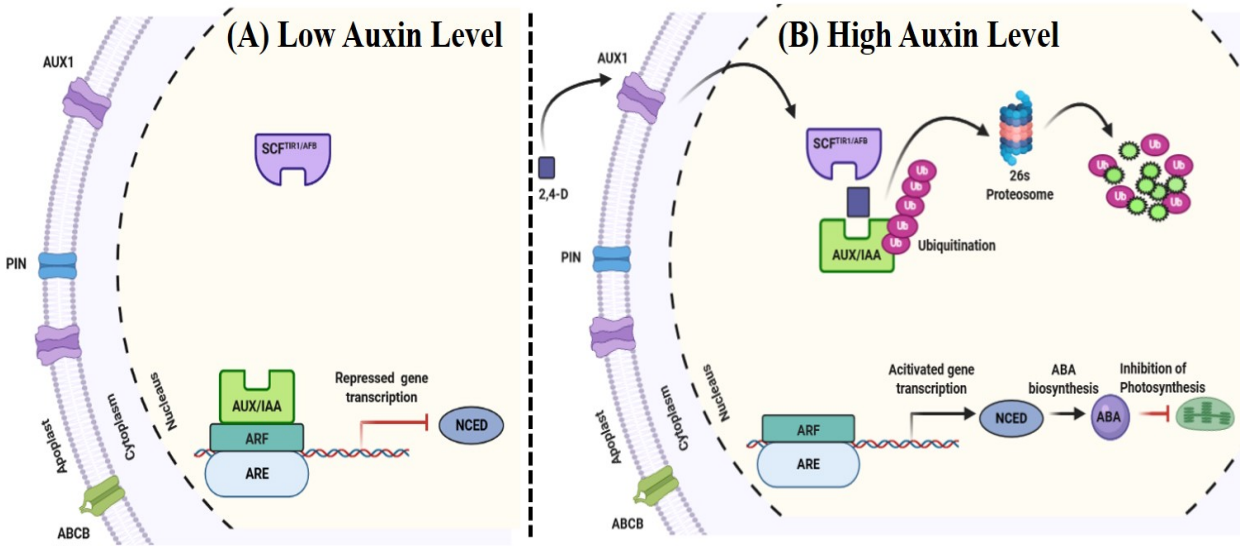

Figure 1.2 Recently proposed mode of action of synthetic auxinic herbicides (SAHs), (A) When auxin is present at low level in plants, (B) When SAHs like 2,4-D is applied and auxin level is high in plants; Adapted from: Gaines (2020); Based on findings of McCauley et al. (2020) [Created with BioRender.com].

However, lack of consensus on *ACS* upregulation following SAH application in other studies has rekindled that NCED is involved more directly in SAH action. For instance, 2,4-D application in *Arabidopsis* was documented to upregulate only *NCED* and not *ACS* (Raghavan et al. 2006).

Recently, a study was conducted in horseweed to investigate the MOA of three SAHs *i.e.,* 2,4-D, dicamba, and halauxifen-methyl (McCauley et al. 2020). Findings of the study suggest that at 6 hours after treatment (HAT), genes associated with hormonal activity, gene expression, metabolism, and drought response were upregulated, but those involved in photosynthesis, in the functioning of both PS I and PS II were downregulated. Additionally, *NCED3* (*NCED* homolog of *Arabidopsis* in horseweed) upregulation was found at 1 and 6 HAT following application of SAHs, while a slight upregulation of *ACS6* (*ACS* homolog of *Arabidopsis* in horseweed) was documented only at 6 HAT. In agreement with these findings, increased ABA accumulation was found at 6 HAT with SAH application, while increased ethylene accumulation was only shown at 24 HAT following application of 2,4-D and dicamba but not halauxifen-methyl. Levels of ABA accumulation in the plants following SAH application were also compared with plants that were kept drought-stressed. Results suggested that the level of ABA accumulation in plants treated with SAH was >3-fold than plants that were kept drought-stressed until death. Based on the findings, McCauley et al. (2020) proposed that whole-scale downregulation of photosynthetic genes (caused by both auxin and ABA) is probably the driver for the MOA of SAHs (Figure 2.2).

Basis of Selectivity

SAHs are the first selective herbicides discovered and commercialized. Most SAHs are selective against broadleaf weeds and are commonly used in cereal crops such as rice, barley, and wheat. However, over the years, SAHs with varying selectivity and chemistry have been

commercialized. The mechanism of tolerance to SAHs in monocots is attributed to i) plant anatomy, ii) detoxication pathway, and iii) induction of genes. Differential vascular bundle structures such as scattered phloem (enclosed by sclerenchyma) and lack of cambium and pericycle (auxin-sensitive) have been known to contribute to a minimal level of tolerance of grasses to SAHs (Sterling and Hall 1997). Tolerant and susceptible plants are also known to metabolize SAHs differently. Metabolism of SAHs in dicots generates amino-acid conjugates which are reversible (Sterling and Hall 1997). In contrast, tolerant monocot plants generate sugar-based conjugates that are more stable and irreversible. Moreover, SAH conjugates in dicots can retain the herbicidal activity contributing to susceptibility (Davidonis et al. 1982). Tolerant species are also known to metabolize SAHs at a faster rate than susceptible species. For instance, rapid conversion of quinmerac to inactive hydroxymethyl and dicarboxylic acid derivatives were documented in wheat (*Triticum aestivum* L.) and sugarbeet (*Beta vulgaris* L.) compared to cleavers (*Galium aparine* L.) (Grossmann and Scheltrup 1998). Additionally, the involvement of enzyme family cytochrome P450 monooxygenases (P450) in metabolizing herbicides such as 2,4-D have been documented in cereals such as wheat and corn has been studied (Frear 1995). Differential gene induction by SAHs is considered as another factor driving selectivity. For example, several *GH3* homologs were found to be strongly induced in broadleaved plants compared to weak induction in grasses following 2,4-D application (Wright et al. 1987). Similarly, assessment of *GH3* expression at the RNA and protein levels has been suggested to diagnose SAH off-target injury in broadleaves such as soybean (Kelley et al. 2006, Kelley and Riechers 2007). Rapid induction of *ACS* accompanied by higher 1-aminocyclopropane-1-carboxylate (ACC) and ABA levels in the shoot tissue was observed in

cleavers compared to oilseed rape (*Brassica napus* L.), and wheat (Grossmann and Scheltrup 1998).

Resistance to Synthetic Auxinic Herbicides

Even after >75 years of widespread usage, the number of weed species resistant to SAHs is low compared to ALS-inhibitors (166 weeds) or triazines (74 weeds), which were introduced much later (Heap 2021). Such historically low incidence of resistance to SAHs is proposed due to several factors including, i) fatality of genetic mutations in the auxin signaling pathway that affect plant ability to survive unfavorable growing conditions (Jasieniuk et al. 1995, Jasieniuk and Maxwell 1994, Mortensen et al. 2012); ii) fitness penalty associated with auxin herbicide resistance in weeds (Bourdot et al. 1996, Debreuil et al. 1996, Hall and Romano 1995, Walsh et al. 2006), iii) multiple modes of action that require multiple mutations at several loci to impart resistance (Gressel and Segel 1982), iv) relatively low-selection pressure or lower residual soil activity (Mithila et al. 2011), and v) resistance in some species being governed by recessive alleles (Sabba et al. 2003, Van Eerd et al. 2004). The first case of 2,4-D resistance was reported in 1957 in climbing dayflower (*Commelina diffusa* Burm. f.) in Hawaii and wild carrot (*Daucus carota* L.) in Canada (Heap 2021). However, in the past decade, increased reliance on SAHs for controlling ALS-and EPSPS-inhibitor resistant weeds has led to increasing selection pressure and reported cases of SAH resistant weeds. Currently, SAH resistance has been reported in 42 weeds spanning over 23 countries (Heap 2021; Figure 1.3, 1.4). Amongst these, resistance to 2,4-D has been reported in 7 weed species in the US and 25 species globally, including several economically important weeds such as common waterhemp, Palmer amaranth, wild radish, kochia, corn poppy, and common lambsquarters.

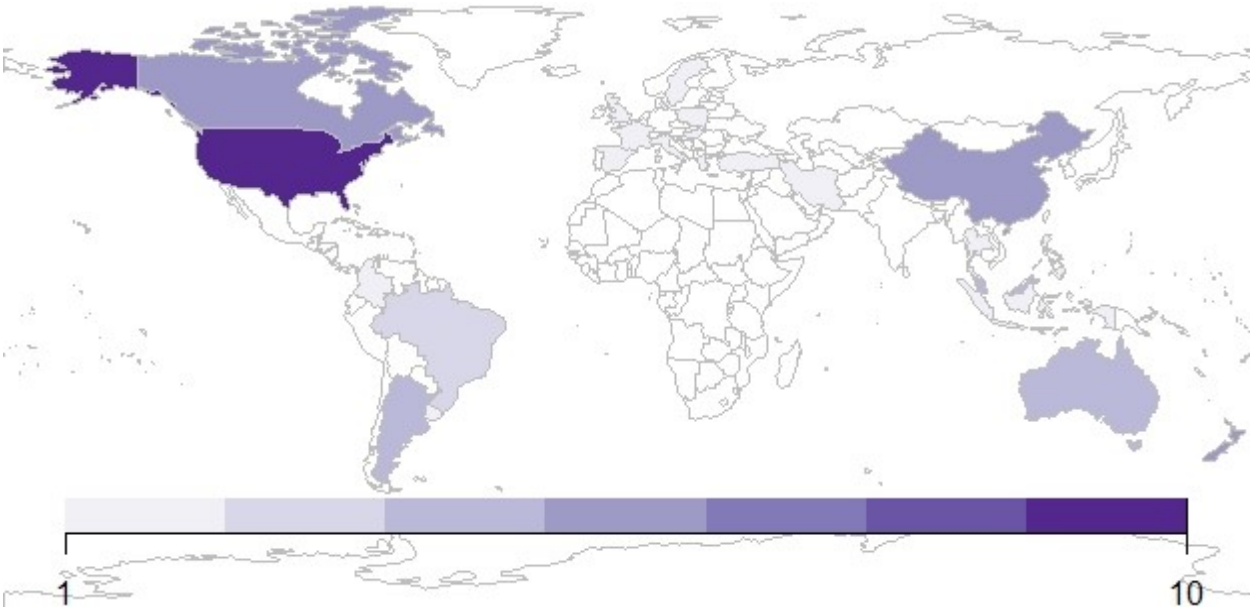

Figure 1.3 Distribution of synthetic auxinic herbicide (SAH) resistant weeds around the world; Data source: International herbicide-resistant weed database (Heap 2021).

Mechanism(s) of Synthetic Auxin Resistance in Important Weeds

Common Waterhemp (*Amaranthus tuberculatus* Moq. Sauer.)

Common waterhemp is one of the most troublesome and economically important driver weed species in the Midwestern US, where growers primarily engage in corn and soybean cultivation (Tranel 2021). The first case of 2,4-D resistance in common waterhemp was documented in Nebraska (Bernards et al. 2012, Crespo et al. 2017). Since then, evolution of 2,4-D resistance has been reported in other common waterhemp populations from Missouri (Shergill et al. 2018a, 2018b) and Illinois (Evans et al. 2019). In 2,4-D-resistant populations of Nebraska and Missouri, enhanced metabolism of 2,4-D was reported as the mechanism of resistance (Figueiredo et al. 2018, Shergill et al. 2018b). Specifically, in the Nebraska population, no differences in ^{14}C 2,4-D absorption and translocation were found between resistant and

susceptible plants, while the resistant plants metabolized 2,4-D faster than the susceptible (Figueiredo et al. 2018). Similar to the Nebraska population, 2,4-D-resistant common waterhemp from Missouri metabolized this herbicide faster than susceptible plants. Application of P450-inhibitor, malathion prior to 2,4-D treatment, significantly reduced resistance to 2,4-D in both resistant populations (Nebraska and Missouri), indicating the possible involvement of P450 enzymes in detoxifying 2,4-D in these plants. NTSR mechanisms are often affected by environmental conditions as well (Jugulam and Shyam 2019). Shyam et al. (2019) showed that 2,4-D resistant common waterhemp plants from Nebraska grown at a higher temperature (34/24 ^{o}C) metabolized 2,4-D at a faster rate compared to those grown at a lower temperature (24/14 ^{o}C). Even though 2,4-D resistance has been reported in several Midwestern populations of common waterhemp, the genes involved in conferring this resistance are still elusive. Recently, a RNA-sequencing study was performed in F_2 populations generated using 2,4-D resistant and susceptible common waterhemp plants from Nebraska and Illinois population (Giacomini et al. 2020). The results of this study indicated increased expression of the same P450 gene cluster (*CYP81A*s) in both populations. However, *in planta* role of this cluster in 2,4-D resistance has not been validated yet.

Smooth Pigweed (*Amaranthus hybridus* L.)

Smooth pigweed in Argentina was reported to have evolved resistance to 2,4-D and dicamba (Dellaferrera et al. 2018). Interestingly, the resistant plants were found to produce ~7-times higher ethylene, in response to the 2,4-D application compared to susceptible plants (Palma-Bautista et al. 2020b). Pre-treatment of resistant plants with malathion followed by 2,4-D application significantly reduced GR_{50} (amount of 2,4-D required to cause 50% reduction in growth) of resistant plants by ~50%. Both reduced translocation and enhanced metabolism of

2,4-D were attributed as the resistance mechanism in this population (Palma-Bautista et al. 2020b).

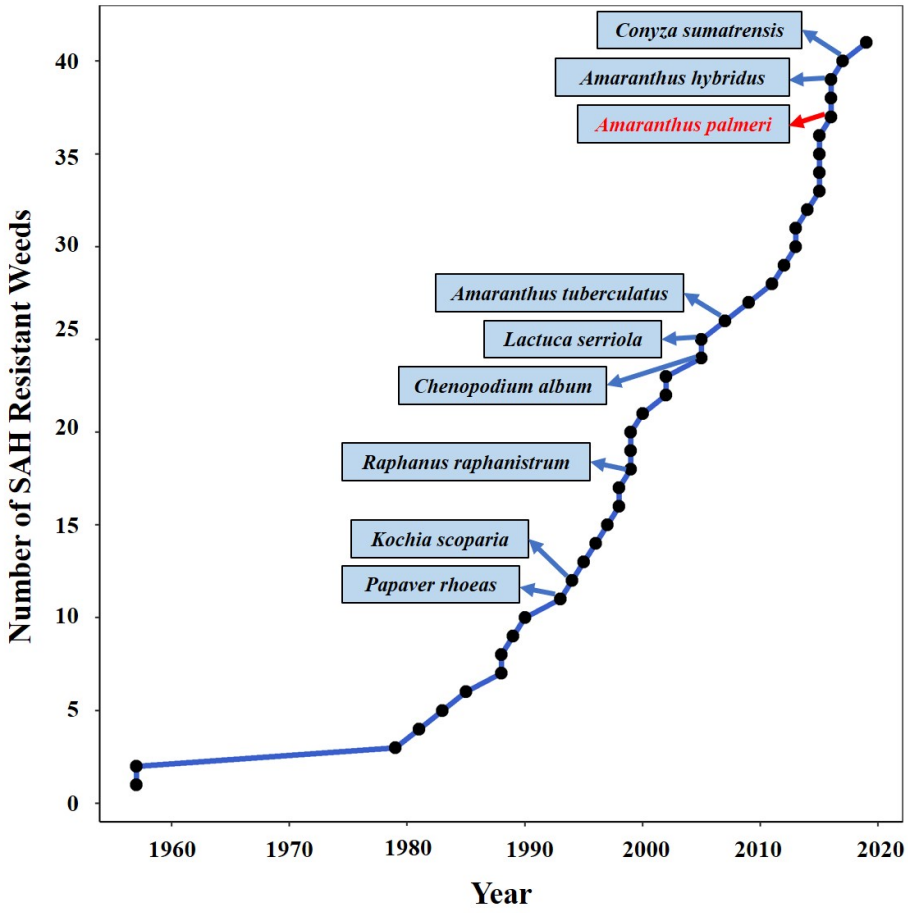

Figure 1.4 Evolution of synthetic auxinic herbicide (SAH) resistance in major weeds around the world (each dot represents a unique weed, Palmer amaranth highlighted in red); Data source: International herbicide-resistant weed database (Heap 2021).

Kochia [*Bassia scoparia* (L.) A. J. Scott]

Kochia is an invasive and troublesome summer annual weed species in the North American Great Plains (Friesen et al. 2011). To date, kochia populations across the US and Canada have been reported to have evolved resistance to SAH such as dicamba and fluroxypyr.

Dicamba-resistant populations of kochia were first reported in 1994 from Montana (Cranston et al. 2001). Since then, several populations of kochia with resistance to dicamba were documented in US Great Plains, including Kansas (Kumar et al. 2019a, Ou et al. 2021, Varanasi et al. 2015), Nebraska (Howatt et al. 2006), and Montana (Cranston et al. 2001, Goss and Dyer 2003) as well as some provinces of Canada (Beckie et al. 2019). Even though no differences were found in ^{14}C dicamba absorption and metabolism between the resistant and susceptible populations of Nebraska, the resistant population was shown to translocate significantly less dicamba. Through differential expression analysis *via* RNA-sequencing, Pettinga et al. (2018) documented induction of *ACS* and *indole-3-acetic acid amino synthetase* following dicamba application in the susceptible plants and not in the resistant. Moreover, 2-fold higher constitutive upregulation of *chalcone synthase* (*CHS*) was found in resistant plants. CHS is the rate-limiting enzyme for the flavonoid biosynthesis pathway. The authors hypothesized that increased *CHS* expression would lead to a higher synthesis of flavonols such as quertecin and kaemperfol, which will compete with auxin transport *via* ABCB-transporters. Such increased expression of *CHS* was shown to co-segregate with dicamba resistance, potentially indicating the involvement in resistance (Pettinga et al. 2018). Later, a double mutation in the AUX/IAA protein, KsIAA16, resulting in the substitution of glycine by asparagine (G73N) in dicamba-resistant kochia population from Nebraska was identified as the resistance mechanism (LeClere et al. 2018). This was the first case of TSR to SAHs ever documented. Fitness penalty was observed in resistant plants possessing the mutation (LeClere et al. 2018, Wu et al. 2021). However, it was also shown G73N variants co-evolved specific reproductive traits to compensate for the fitness penalty (Wu et al. 2021).

Contrary to the Nebraska population, no differences were found in ^{14}C dicamba absorption, translocation, and metabolism in the dicamba-resistant kochia population from Kansas (Ou 2018). Dicamba resistance in Kansas and Nebraska populations was found to evolve independently based on genetic analyses of F_2 progeny generated using dicamba-resistant and -susceptible kochia plants (Ou et al. 2021). Dicamba-resistant kochia populations from Kansas, have also been reported to be cross-resistant to fluroxypyr, another important SAH (Kumar et al. 2019a).

Oriental Mustard (*Sisymbrium orientale* L.)

Oriental mustard/Indian hedge mustard is a member of the *Brassicaceae* family found commonly distributed all over Australia. The evolution of 2,4-D resistance was reported in 2013 in an oriental mustard population from southern Australia (Preston et al. 2013). Genetic studies confirmed that a single dominant allele governs 2,4-D resistance in this population (Preston and Malone 2015). Initially reduced translocation of 2,4-D was identified as the physiological mechanism conferring resistance in this weed (Dang et al. 2018). More recently, another TSR mechanism was also identified (2nd case of TSR so far) (Figueiredo et al. 2021). Specifically, an in-frame deletion of 27-nucleotides resulting in the deletion of 9 amino acids was documented in the degron tail of AUX/IAA2 (Figueiredo et al. 2021). Such alteration resulted in reduced binding of natural auxins and SAH resulting in reduced association (Figueiredo et al. 2021). *Arabidopsis* lines transformed with this deletion were found to be resistant to both 2,4-D and dicamba (Figueiredo et al. 2021).

Corn Poppy (*Papaver rhoeas* L.)

Corn poppy is an important weed in cereal crops in southern Europe. Intensive selection pressure from 2,4-D and tribenuron-methyl has resulted in the evolution of 2,4-D and tribenuron-

methyl resistant corn poppy in Spain (Rey-Caballero et al. 2016, Torra et al. 2017). Previously, the evolution of 2,4-D resistance has also been reported in corn poppy populations from Italy, and Greece (Heap 2021, Kati et al. 2016). Reduced 2,4-D translocation was found to bestow 2,4-D resistance in the population from Spain (Rey-Caballero et al. 2016). In association with that, susceptible plants were found to produce 4-8 fold higher ethylene compared to resistant plants (Rey-Caballero et al. 2016). Later, rapid metabolism of 2,4-D in another 2,4-D and tribenuron methyl-resistant population was reported (Torra et al. 2017). More recently, the involvement of P450s in metabolizing 2,4-D in these populations was confirmed (Torra et al. 2021).

Wild Radish (*Raphanus raphanistrum* L.)

Wild radish is an important broadleaf weed in several cropping systems in southern Australia (Goggin et al. 2018). A survey conducted in 2003 indicated that ~60% of wild radish populations in western Australia were resistant to 2,4-D (Walsh et al. 2007). Such widespread occurrence of 2,4-D resistance was found to increase to 76% by 2010 owing to continued usage of 2,4-D in controlling sulfonyl-urea resistant wild radish populations (Owen et al. 2015). Goggin et al. (2016) reported that reduced translocation of 2,4-D from the treated leaf was the major mechanism of resistance in wild radish. Application of two known auxin efflux or ABCB transporters inhibitors *i.e.*, verapamil and valspodar in the 2,4-D susceptible population was found to imitate reduced translocation as seen in the resistant plants. Hence, the investigators hypothesized a possible modification in the ABCB-transporters on the plasma membrane of resistant plants inhibiting translocation of 2,4-D (Goggin et al. 2016). Following this work, whole-gene transcriptome analysis was performed using 2,4-D resistant and susceptible populations, and the role of two key genes, *i.e.*, *IAA30* and *MEKK1* in resistance to this herbicide was reported (Goggin et al. 2018). Moreover, activation of the mitogen-activated protein kinases

(MAPK) pathway was also assessed in the resistant populations (Goggin et al. 2018). The researchers found a similar level of MAPK phosphorylation in plants with reduced translocation and those with shoot-wide translocation indicating that reduced translocation plays a minor role in mediating 2,4-D resistance in wild radish (Goggin et al. 2018). Overall, they suggested that 2,4-D resistance mechanisms in different wild-radish populations across Australia might involve different alterations in the auxin signaling pathway (Goggin et al. 2018). The cost of 2,4-D resistance in the growth and reproductive traits of resistant wild radish population was also studied by growing the plants in the presence or absence of wheat (Goggin et al. 2019). Even though the resistant plants were found to produce lower biomass than susceptible plants, such a negative association was not found for seed production (Goggin et al. 2019). Recently, a study was conducted to identify key components of the plasma membrane proteome in eleven 2,4-D-resistant and two susceptible wild radish populations (Goggin et al. 2020). The study identified the association of two receptor-like kinases and an efflux transporter ABC19 with 2,4-D resistance; however, the relative contribution of these candidate genes seemed to vary in different resistant populations (Goggin et al. 2020).

Lambsquarters (*Chenopodium album* L.)

Lambsquarters/fathen is a troublesome weed in corn and brassicas in temperate countries like New Zealand (Ghanizadeh and Harrington 2017). Ghanizadeh et al. (2018) reported two populations of lambsquarters with 7-19-fold resistance to dicamba compared to susceptible populations. Interestingly, these dicamba-resistant populations were found to be cross-resistant to pyridine carboxylic acid herbicides including clopyralid and aminopyralid but not to phenoxy acetic acids, *i.e.*, 2,4-D and mecoprop-P (Ghanizadeh and Harrington 2017). Moreover, no difference in ^{14}C dicamba absorption, translocation, and metabolism was found between resistant

and susceptible populations, suggesting that a different mechanism may confer resistance in this population (Ghanizadeh et al. 2018).

Sumatran Fleabane [*Conyza sumatrensis* (Retz.) E. Walker]

Rapid necrosis from 2,4-D application is a unique symptom, recently reported in a 2,4-D resistant Sumatran fleabane population from Brazil (Queiroz et al. 2020). Light necrotic spots and wilting started to appear at 2 HAT in the resistant population, which progressed rapidly to the leaf borders, tips, and blade (Queiroz et al. 2020). These necrotic symptoms were shown in only mature leaves and meristems, while young leaves did not show these symptoms. New plant growth from auxiliary buds was observed at 21 days after treatment (DAT). Interestingly, this unique symptom was only found in response to treatment with 2,4-D but not to other SAHs such as fluroxypyr, picloram, halauxifen-methyl, and florpyrauxifen benzyl ester. 3,3′-diaminobenzidine assay indicated that H_2O_2 production in the resistant plants increased significantly within 30 minutes of 2,4-D application and remained high until 1 DAT. In contrast, H_2O_2 was maintained at a lower level in the susceptible plants up to 7 HAT, until it reached the same as in resistant plants at 1 DAT (Queiroz et al. 2020).

Ragweed Parthenium (*Parthenium hysterophorus* L.)

Parthenium is an invasive problematic weed distributed around the world. Parthenium populations from citrus orchids in the Dominican Republic have evolved resistance to SAHs such as 2,4-D, dicamba, and picloram (Mora et al. 2019, Palma-Bautista et al. 2020b, 2020a). Reduced 2,4-D translocation and enhanced metabolism were found to contribute to resistance in this population (Mora et al. 2019). Application of P450 inhibitor, malathion was found to reduce resistance to 2,4-D (Mora et al. 2019). Susceptible plants were also reported to produce a high

amount of ethylene following 2,4-D treatment compared to resistant plants (Palma-Bautista et al. 2020a, 2020b).

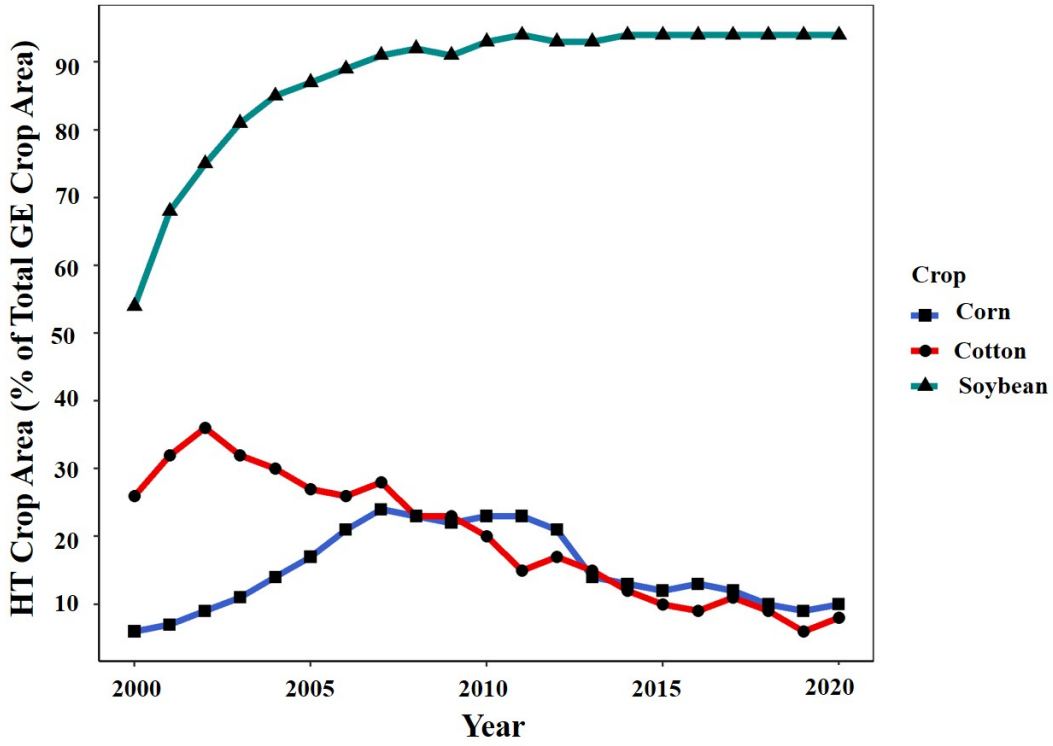

Figure 1.5 Acreage of herbicide-tolerant (HT) crops (% of total genetically engineered (GE) crop area); Data source: USDA (2021).

Synthetic Auxinic Herbicide-Tolerant Commercial Crops

Herbicide-tolerant (HT) crops have dominated the global crop production market since the introduction of GR soybeans in 1996. Nevertheless, the unprecedented adoption of this technology has not only resulted in higher crop yields by managing ALS-and PS II-inhibitor-resistant weeds but has also led to the evolution of GR weeds. According to USDA (2021), in the past two decades, the adoption of HT soybean has increased compared to corn and cotton (Figure 1.5). More than 90% acreage of genetically engineered (GE) soybean belongs to HT varieties

(USDA 2021; Figure 1.5). SAH- tolerant soybean varieties have been developed and commercialized in the US. These varieties exhibit either dicamba or 2,4-D tolerance stacked with other HT traits such as glyphosate or glufosinate resistance.

Roundup Ready 2 Xtend® crops developed by Bayer Crop Science® with resistance to dicamba were deregulated by the Animal and Plant Health Inspection Service in 2015 and commercialized later in 2018 (Nandula 2019). These corn and soybean varieties possessed *dicamba monooxygenase* (*DMO*) gene from the soil bacterium *Pseudomonas maltophilia* (strain DI-6). *DMO* translates for rieske nonheme monooxygenase, an enzyme that metabolizes dicamba into 3-6-dichlorosalicylic acid (Behrens et al. 2007). Since its commercialization, growers embraced dicamba-tolerant technology, mostly because of its utility as a tool to control ALS- and EPSPS-inhibitor resistant broadleaf weeds. However, this also poses a risk of the evolution of dicamba resistance in major broadleaf weeds.

Another addition to genetically engineered SAH- tolerant crop technology is the 2,4-D-tolerant technology developed by Corteva Agrisciences® marketed as Enlist^{TM} weed management systems. These systems include the cultivation of genetically modified crops such as cotton, corn, and soybean, with the application of low volatile formulation of 2,4-D choline. 2,4-D resistance in these crops was developed through *Agrobacterium*-mediated transformation (stable insertion) of *aryloxyalkanoate dioxygenase-12* gene, which can metabolize 2,4-D, thus rendering resistance (Wright et al. 2010). Enlist corn was commercially launched in the US in 2018 and while soybean in 2019. This technology also poses the risk of the evolution of 2,4-D-resistant weeds (Egan et al. 2011).

Background of the Palmer amaranth Population used in this Research

A Palmer amaranth population termed Kansas Conservation Tillage Resistant (KCTR) was identified from a Kansas State University research field in Riley County, Kansas. This field has been part of a long-term conservation tillage study with sorghum crop grown for more than 45 years. For weed control, including Palmer amaranth, PRE herbicides that were routinely used in this field included PS II- (atrazine), VLCFA- (s-metolachlor) inhibitors, and SAH (2,4-D); whereas POST herbicides were PS II-(atrazine) inhibitor and SAH (2,4-D). Recent lack of control with 2,4-D and some other herbicides has indicated that this population might have evolved resistance to multiple herbicides. In summer 2018, ten plants which survived the application of 2,4-D at the field recommended dose (560 g ae ha^{-1}) were uprooted from the field and brought to the greenhouse. These plants were transplanted in big pots and kept in isolation in the greenhouse to set seeds. Simultaneously, vegetative clones were generated from each of these plants by nodal cuttings with axillary buds from the matured plants followed by treating them with rooting hormone (IBA) and transplanting them in small pots. Once these clones established and grown to 10-12 cm tall, they were treated with 2,4-D at 1120 g ae ha^{-1} to get a preliminary assessment of resistance to 2,4-D in KCTR plants. Seeds from the female plants that survived 2,4-D application were grown in isolation to generate more homogeneous seed for using in various experiments conducted in this research.

Objectives of Research

Even though 2,4-D resistance has been reported previously in another Palmer amaranth population from Kansas (Kumar et al. 2019b), information on the mechanism of 2,4-D resistance in Palmer amaranth is not available. Knowledge of different aspects of 2,4-D resistance in Palmer amaranth will help understand how 2,4-D resistance will spread and can aid in

formulating management decisions. Therefore, the overall goal of this dissertation was to characterize and investigate physiological, and genetic aspects of 2,4-D resistance in Palmer amaranth. Additionally, herbicide programs to manage GR Palmer amaranth in 2,4-D-tolerant soybean to safeguard the longevity of 2,4-D tolerance trait were also evaluated. To achieve the overall goal, the dissertation was divided into four chapters:

Chapter 2: Characterization of multiple herbicide-resistance including 2,4-D in Palmer amaranth from Riley County, Kansas

Chapter 3: Physiological basis of 2,4-D resistance in Palmer amaranth

Chapter 4: Genetic basis of 2,4-D resistance in Palmer amaranth

Chapter 5: Management of glyphosate-resistant Palmer amaranth in 2,4-D tolerant soybean

Note: The chapters were written in the format of manuscripts for publication in peer-reviewed journals. Therefore, there is some redundancy in the background information in several chapters.

Chapter 2 - Characterization of multiple herbicide-resistance including 2,4-D in Palmer amaranth from Riley County, Kansas

Manuscript Information

Manuscript Title: Predominance of Metabolic Resistance in a Six-Way-Resistant Palmer Amaranth (*Amaranthus palmeri*) Population

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Abstract

Evolution of multiple herbicide resistance in Palmer amaranth across the US is a serious challenge for its management. Recently, a Palmer amaranth population (KCTR; Kansas Conservation Tillage Resistant) from a long-term conservation tillage research project in Kansas, US, was found uncontrolled by several commonly used herbicides. Importantly, this field did not have a history of repeated use of some of the herbicides for which the KCTR Palmer amaranth population showed a lack of control. The objectives of this study included confirming the evolution of multiple resistances, determine the possible mechanism(s) of resistance, and characterize 2,4-D resistance in KCTR Palmer amaranth plants. KCTR plants were treated with field recommended dose of common post-emergence herbicides and percent survival was assessed. Plants surviving ALS- and PS II-inhibitors were tested for the presence of previously reported mutations in the *ALS* and *psbA* genes. Similarly, glyphosate survivors were tested for *EPSPS* amplification. KCTR plants were also pre-treated with P450-inhibitor (malathion) and GST-inhibitor (NBD-Cl) to investigate the presence of metabolic resistance to imazethapyr, atrazine, 2,4-D, mesotrione, and lactofen. Dose-response assays were conducted with varying doses of 2,4-D to evaluate the degree of resistance compared to susceptible populations. In response to post-emergence application, 28-100% of KCTR Palmer amaranth survived field recommended doses of 2,4-D, ALS-, PS II-, EPSPS-, PPO-, HPPD-inhibitor herbicides, or tank- or pre-mixture of PS II- and HPPD-inhibitor herbicides, confirming evolution of six-way resistance in this Palmer amaranth population. However, this population was found susceptible to the PS I- and glutamine synthetase inhibitor herbicides. Chlorsulfuron-, imazethapyr-, and atrazine-resistant plants did not show any previously reported mutation in *ALS* and *psbA* genes, the target sites of these herbicides, respectively. However, the survivors of glyphosate treatment

showed amplification of *EPSPS* gene (up to 88 copies). The KCTR plants pretreated with cytochrome P450 or GST inhibitors along with atrazine, 2,4-D, lactofen, or mesotrione had significantly less dry weight accumulation than those treated with herbicides alone. Plants treated with P450 inhibitor followed by imazethapyr showed moderate reduction of dry weight in KCTR which was statistically similar to a susceptible Palmer amaranth population treated with imazethapyr. These results suggest a predominance of metabolic resistance possibly mediated by cytochrome P450 and GST enzyme activity that may have predisposed the KCTR Palmer amaranth population to evolve resistance to multiple herbicides. Dose-response analysis further confirmed that KCTR population is 6 to 11 fold resistant to 2,4-D compared with susceptible populations. This is the first report of the evolution of six-way resistance in a single Palmer amaranth population. Appropriate management strategies, including integration of cultural, and mechanical, and herbicide mixtures, are warranted to control such Palmer amaranth populations.

Introduction

Palmer amaranth (*Amaranthus palmeri* S. Watson) is a top-ranked troublesome weed in the US (Van Wychen 2020). It is a summer annual C_4 eudicot species, with a fast growth rate and ability to accumulate biomass (Horak and Loughin 2000, Sellers et al. 2003). These biological attributes make Palmer amaranth a highly competitive species and if uncontrolled can contribute to drastic yield losses of up to 91% in corn, 79% in soybean, 59% in cotton, and 50% in sorghum (Bensch et al. 2003, Massinga et al. 2001, Moore et al. 2004, Morgan et al. 2001). Palmer amaranth has currently evolved resistance to eight herbicide modes of action (MOAs) in the US, including acetolactate synthase- (ALS-), photosystem II- (PS II-), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS-), 4-hydroxyphenylpyruvate dioxygenase- (HPPD-), protoporphyrinogen oxidase- (PPO-), very long-chain fatty acid- (VLCFA-), microtubule-inhibitor herbicides, and synthetic auxins (Heap 2021). Previously, resistance to up to five MOAs, *i.e.*, ALS-, EPSPS-, PS II-, HPPD-inhibitor herbicides, and synthetic auxins, was reported in different populations of Palmer amaranth from Kansas (Chaudhari et al. 2020, Kumar et al. 2019b, Nakka et al. 2017a, 2017b, 2017c).

Weed resistance to herbicides, especially multiple-herbicide resistance, poses a serious threat to global food production. Both target-site (TSR) and non-target-site (NTSR) resistance mechanisms have been found to confer resistance to herbicides in Palmer amaranth. TSR mechanisms involving alterations in the target site of the herbicide such as amino acid substitutions or deletions and increased copy number and/or expression of the target gene have been reported in this species (Gaines et al. 2010, Nakka et al. 2017b, 2017c). Mutations in the gene encoding herbicide-targeted enzymes can reduce herbicide-binding activity leading to resistance. Specifically, in Palmer amaranth single amino acid substitutions, *i.e.*, A122S or

A122T, P197S or P197A, T574L, and S653A were reported to confer resistance to ALS-inhibitors in Palmer amaranth (Küpper et al. 2017, Nakka et al. 2017c, Singh et al. 2019). Palmer amaranth populations resistant to PPO-inhibitor herbicides were found to have the amino acid substitutions R128M/G (also referred to as R98), and G399A, as well as a codon (glycine) deletion at the position 210 (Δ210) in *PPX2* gene coding for the target enzyme of PPO-inhibitor herbicides (Giacomini et al. 2017, Rangani et al. 2019, Salas et al. 2016, Salas-Perez et al. 2017, Varanasi et al. 2018b). Another commonly identified TSR mechanism in Palmer amaranth is the amplification of the *EPSPS* gene, the molecular target of glyphosate (Chahal et al. 2017, Gaines et al. 2010, Molin et al. 2018, Singh et al. 2018). Furthermore, the amplified *EPSPS* gene copies are present in the form of extrachromosomal circular DNA, with an autonomous replication site, and are randomly inherited during cell division (Koo et al. 2018, Molin et al. 2020). After the first case of glyphosate-resistant Palmer amaranth from Georgia in 2005 (Culpepper et al. 2006), it has rapidly spread throughout the US becoming a serious challenge for agriculture. Apart from *EPSPS* amplification, a mutation in the *EPSPS* gene leading to P102S substitution has also been reported in Palmer amaranth (Kaundun et al. 2019).

In contrast to TSR, NTSR mechanisms do not directly alter the target site but reduce the amount of active herbicide reaching the target site due to either reduced absorption, translocation, or increased metabolism of the herbicide. Specifically, in metabolic resistance, the active herbicide is broken down into non-toxic forms before it reaches the target site, thus reducing its efficacy. Reduced absorption and translocation imparting glyphosate resistance in a Palmer amaranth population from Argentina have been reported (Palma-Bautista et al. 2019). However, enhanced herbicide detoxification *via* cytochrome P450 monooxygenase (P450s) and glutathione S-transferase (GSTs) activity is the most common NTSR mechanism reported in

ALS-, PS II-, HPPD-, and PPO-inhibitor resistant Palmer amaranth (Nakka et al. 2017a, 2017b, 2017c, Varanasi et al. 2018b). Additionally, GSTs were found to be involved in s-metolachlor resistance in this species (Brabham et al. 2019).

P450s and GSTs are groups of enzymes important to the catalysis of several xenobiotic compounds in living organisms, including herbicides in many crops and weeds (Dixon et al. 2010, Pandian et al. 2020). Importantly, metabolic resistance can confer cross- or multiple-resistance (Jugulam and Shyam 2019). The P450s from *CYP81A* subfamily have been shown to impart cross-resistance to several herbicide classes including ALS-, ACCase-, PS II-, phytoene desaturase-, PPO-, HPPD-, and 1-deoxy-D-xylulose 5-phosphate synthase- inhibitor herbicides in late watergrass (*Echinochloa phyllopogon* Stapf. Koso-Pol) (Dimaano et al. 2020). Likewise, a phi class *GST*, *AmGSTF1*, was shown to detoxify multiple herbicides in blackgrass (*Alopecurus myosuroides* Huds.) (Cummins et al. 2013).

2,4-D resistance in Palmer amaranth was first reported globally from Kansas in 2015 (Heap 2021, Kumar et al. 2019b). Dose-response analysis has confirmed that the 2,4-D resistant Palmer amaranth population from Kansas is 3-4 fold resistant compared to susceptible population. Prior to Palmer amaranth, 2,4-D resistance has been reported in other *Amaranthus sp.* such as common waterhemp (*Amaranthus tuberculatus* Moq. Sauer.) populations from Missouri (Shergill et al. 2018a, 2018b), Illinois (Evans et al. 2019), Nebraska (Figueiredo et al. 2018, Shyam et al. 2019) and smooth pigweed (*Amaranthus hybridus* L.) population from Argentina (Palma-Bautista et al. 2020b). In the common waterhemp populations from Nebraska and Missouri, enhanced metabolism is documented as the resistance mechanism (Figueiredo et al. 2018, Shergill et al. 2018b). Application of P450-inhibitor malathion reversed such metabolic resistance in these resistant populations, implying P450 based metabolism (Figueiredo et al.

2018, Shergill et al. 2018b). In 2018 and 2019, crops with genetically engineered 2,4-D tolerance were commercialized. Widespread adoption of this technology poses the risk of increased selection for 2,4-D resistance (Egan et al. 2011). Therefore, characterization of 2,4-D resistance in other weed species or populations is critical to formulating management decisions.

In 2018, a Palmer amaranth population (KCTR: Kansas Conservation Tillage Resistant) from a long-term conservation tillage experimental field (Department of Agronomy, Kansas State University) grown with continuous sorghum for over 45 years was found to survive post-emergence (POST) application of several commonly used herbicides, including 2,4-D and atrazine. These herbicides have been routinely used in this field to control broadleaf weeds. Since Palmer amaranth has evolved resistance to eight MOAs (Heap 2021) and there was a predominance of metabolic resistance in other Palmer amaranth populations in Kansas, the KCTR Palmer amaranth was used in this research to confirm and characterize resistance. The objectives of this research were to i) confirm evolution of resistance in KCTR to several post-emergence (POST) herbicides, ii) determine if TSR or NTSR mechanisms confer resistance to multiple herbicides, and iii) characterize 2,4-D resistance in KCTR through dose-response assay.

Materials and Methods

Plant Material and Growing Conditions

Ten KCTR plants that survived 2,4-D treatment (560 g ae ha^{-1}) and showed active growth after herbicide application were collected (summer 2018) and brought to the weed science greenhouse at Kansas State University. These plants were transplanted into pots (15 × 10 × 15 cm) for seed production. Seeds produced from several female plants were harvested, cleaned, and pooled to evaluate their response to multiple herbicides. Susceptible populations including one from Kansas (KSS: Kansas Susceptible) and one from Mississippi (MSS: Mississippi

Susceptible) were used for comparisons. All experiments were conducted in the above greenhouse maintained at 30/23°C ± 2°C (d/n temperatures) with 60% ± 10% relative humidity, and 14/10 h d/n photoperiod supplemented with 250 μmol m^{-2} s^{-1} illumination provided by sodium vapor lamps.

Screening with Post-emergence (POST) Herbicides

Seeds of KCTR, KSS, and MSS populations were germinated in plastic trays (21 × 6 × 4 cm) filled with a commercial potting mixture (Pro-Mix® premium potting mix, Premier Tech Home and Garden Inc., Ontario, Canada). After emergence, seedlings were individually transplanted into pots (6 × 6 × 6.5 cm) and grown under greenhouse conditions as previously described. This experiment was performed in a completely randomized design with 18 treatments including field recommended doses of 17 POST herbicides (Table 2.1) and non-treated control. Twenty-five replicates were maintained for each treatment, and the experiment was repeated. In total, 50 plants (from two runs) from each of the KCTR and KSS or MSS populations were treated with these POST herbicides (Table 2.1). Adjuvants were included following manufacturer instructions (Table 2.1). Herbicides were applied using a bench-track sprayer (Generation III, DeVries Manufacturing, RR1 Box 184, Hollandale, Minnesota, US) equipped with a flat-fan nozzle tip (8002 Teejet, Spraying Systems Co., Wheaton, Illinois, US) calibrated to deliver a spray volume proportional to 187 L ha^{-1} at 4.77 km h^{-1}. Plant survival (alive or dead) was assessed at 2 weeks after treatment (WAT) with PPO-inhibitor herbicides; 3 WAT for glyphosate, ALS-, HPPD-, and PS II-inhibitor herbicides; and 4 WAT for 2,4-D. The percent survival (Table 2.2) was calculated by dividing the number of plants that survived herbicide by total number of plants treated, considering both experimental runs.

Table 2.1 Post-emergence herbicide treatments used for screening KSS (susceptible), MSS (susceptible), and KCTR (resistant) Palmer amaranth populations.

WSSA group	MOA	Herbicides	Dose[a] (g ai ha^{-1} or g ae ha^{-1})	Product	Manufacturer
2	ALS-inhibitors	chlorsulfuron	18	Glean®[b]	Corteva Agriscience, Willington, DE
		thifensulfuron	36	Harmony SG [b]	Corteva Agriscience
		imazamox	35	Beyond[b]	BASF Corp., Research Triangle Park, NC
		imazethapyr	36	Pursuit[b]	BASF Corp.
4	Synthetic auxins	2,4-D	560	2,4-D 4L Amine	Winfield Solutions, LLC, St. Paul, MN
5	PS II-inhibitors	atrazine	2240	Aatrex 4L[c]	Syngenta Crop Protection, LLC., Greensboro, NC
		metribuzin	140	Sencor 75[c]	Bayer Crop Science, Centreway Green Way, NC
9	EPSPS-inhibitors	glyphosate	840	Roundup WeatherMAX[d]	Bayer Crop Science
10	Glutamine synthetase inhibitors	glufosinate	655	Liberty 280 SL[d]	BASF Corporation
14	PPO-inhibitors	lactofen	175	Cobra[c]	Valent U.S.A. Corp., Walnut Creek, CA
		fomesafen	264	Flexstar[b]	Syngenta Crop Protection
22	PS I-inhibitors	paraquat	560	Gramaxone SL 2.0 [c]	Syngenta Crop Protection
27	HPPD-inhibitors	mesotrione	105	Callisto[c]	Syngenta Crop Protection
		tembotrione	92	Laudis[de]	Bayer Crop Science
5 + 27	PS II- + HPPD-inhibitors	atrazine + mesotrione	1120 + 105	Aatrex 4L + Calisto[c]	Syngenta Crop Protection
		bromoxynil + pyrasulfotole	288	Huskie[bd]	Bayer Crop Science

[a]Field recommended dose to control Palmer amaranth.
[b] non-ionic surfactant at 0.25% v/v; [c] crop oil concentrate at 1% v/v; [d] ammonium sulfate (34%) at 2% v/v; methylated seed oil at 1% v/v.

Assessment for Prevalent TSR Mechanisms

DNA Isolation and Sequence Comparisons of *ALS* and *psbA* Genes in KCTR, KSS, and MSS Palmer Amaranth

Approximately 100 mg of young leaf tissue was collected from the survivors of chlorsulfuron (n = 3; n: number of plants) and imazethapyr (n = 16) and atrazine (n = 22) treated KCTR, and non-treated KSS (n = 1) and MSS (n = 5) plants for DNA isolation. After collection, leaf tissue was homogenized using a prechilled mortar and pestle with liquid nitrogen. Total genomic DNA was extracted using a genomic DNA extraction kit (Thermo Fisher Scientific, Waltham, Massachusetts, US). DNA was quantified using Nanodrop 1000 (Thermo Fisher Scientific), and quality was verified using 0.8% agarose gel electrophoresis prior to further steps. Polymerase chain reactions (PCR) were performed using T100TM Thermal Cycler (Bio-Rad Inc., Hercules, California, US) to amplify the *ALS* and *psbA* genes, the target site of these herbicides. Individual reactions included 80 ng of DNA, 2 µL of each forward and reverse primers (5 µM), 10 µL of PCR master mix (GoTaq Green Master Mix, 2×, Promega PCR Master Mix, Fisher Scientific Company, Ontario, Canada), and molecular-grade water totalizing 25 µL per reaction. Primer sets used to amplify the *ALS* and *psbA* genes were designed by Mengistu et al. (2005) and Whaley et al. (2007), respectively, and have previously been used in our lab (Nakka et al. 2017a, 2017b). For *ALS* gene amplification, the following PCR conditions were maintained: 95°C for 5 min and 35 × 95°C for 1 min, 57°C for 30 s, 2 min at 72°C, and 10 min at 72°C. PCR conditions consisted of 95°C for 5 min for initial denaturation and 35 cycles of 95°C for 30 s for denaturation, 55°C for 30 s for annealing, 72°C for 45 s for extension, and 10 min at 72°C for final extension for *psbA*. PCR products were purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific) following the manufacturer instructions and sent for Sanger

sequencing at the Genewiz facilities (Genewiz Inc., South Plainfield, New Jersey, US). For *ALS* sequencing, along with the forward and reverse primers used for PCR, an internal primer (ALS_F2-5′-AACAGCCCATTAAATTGGGTG-3′) was used. The *psbA* gene was sequenced with the same forward primer used for PCR. Multiple alignments of *ALS* and *psbA* gene sequences of KCTR, KSS, and MSS sequences were performed using Geneious Prime® software (Biomatters Inc., Newark, New Jersey, US).

Relative *EPSPS* Genomic Copy Number Estimation

Leaf tissue of KCTR plants (n = 13) that survived glyphosate treatment was collected to estimate the *EPSPS* copy number relative to *β-Tubulin* using a real-time quantitative PCR (qPCR). DNA extraction was performed as described above, and qPCR was performed using a StepOnePlus™ real-time detection system (Applied Biosystems, Waltham, Massachusetts, US). Each qPCR reaction consisted of 8 µL of PowerUp™ SYBR™ Green master mix (Applied Biosystems), 2 µL each of forward and reverse primers (5 µM) (Gaines et al. 2010), and 2 µL of gDNA (20 ng μL^{-1}) with 14 µL total. *β-Tubulin* was used as an endogenous control as described by Godar et al. (2015). Individual reactions were performed with DNA collected from thirteen different KCTR plants that survived glyphosate application as biological replicates, with three technical replicates per DNA sample for both target and endogenous control genes. This experiment was repeated, and data was combined. To determine the specificity of the qPCR products, a melt curve profile was included following the thermal cycling. The *EPSPS* copy number in KCTR was estimated using the formula for fold induction ($2^{-\Delta\Delta Ct}$) (Pfaffl 2001) relative to the reference sample, *i.e.*, glyphosate-susceptible KSS or MSS plants, in each run. The mean *EPSPS* copy number of KCTR plants along with susceptible KSS and MSS were plotted along with standard error of mean calculated from two experimental runs.

Assessment for Metabolic NTSR Mechanism in KCTR Palmer amaranth

Whole-plant bioassays were conducted to investigate the presence of P450- and GST-mediated metabolic resistance to the herbicides (imazethapyr, atrazine, 2,4-D, mesotrione, and lactofen) for which Palmer amaranth and common waterhemp has been reported to have evolved such resistance. This experiment was performed under a completely randomized design with a factorial arrangement and was repeated. Treatments included a combination of Palmer amaranth populations (resistant and susceptible) and chemical treatments (described below), with at least 8 replicates. Resistant (KCTR) and susceptible (KSS or MSS; based on the availability of seeds) Palmer amaranth populations were compared. Chemical treatments included a) herbicide only, b) enzymatic inhibitor only (either P450 and/or GST inhibitor, depending on the herbicide), c) combination of enzymatic inhibitor with a herbicide, and d) a non-treated control. Herbicide doses included application of field recommended doses of imazethapyr, mesotrione, atrazine, lactofen, and 2,4-D (Table 2.1). Based on published literature, both P450 and GST inhibitors were included to evaluate the metabolic resistance to lactofen for a total of six treatments, *i.e.*, (a) lactofen only, (b) malathion only, (c) NBD-Cl only, (d) combination of malathion with lactofen, (e) combination of NBD-Cl with lactofen, and (f) non-treated control. The treatments were applied using a bench-track sprayer with appropriate adjuvants as described before. Malathion (Spectracide®, Spectrum Group, St. Louis, Missouri, US), a P450 inhibitor, was applied at 2,000 g ai ha^{-1} at least 30 min prior to herbicide application followed by soil application (5 mM, 50 mL solution pot^{-1}) at 48 h after herbicide application as described by Ma et al. (2013) in combinations with or without imazethapyr, lactofen, and mesotrione. Our preliminary study (Shyam et al. unpublished) to test the effect of malathion at 1,500 and 2,000 g ai ha^{-1} on 2,4-D efficacy in 2,4-D-resistant common waterhemp from Nebraska indicated that malathion at 1,500

g ai ha^{-1} was sufficient to increase susceptibility of common waterhemp to 2,4-D. Palmer amaranth is a close relative of common waterhemp; therefore, for assessing 2,4-D metabolism in KCTR, malathion was used at 1,500 g ai ha^{-1}, followed by soil application as described above. The GST inhibitor, NBD-Cl (Sigma Aldrich, St. Louis, Missouri, US), was applied at 270 g ai ha^{-1}, 48 h before atrazine or lactofen applications (Ma et al. 2013). Experiments were performed twice. Aboveground biomass was harvested at 2 WAT for lactofen; 3 WAT for glyphosate, imazethapyr, mesotrione, and atrazine; and 4 WAT for 2,4-D, oven-dried at 65°C for 72 h, and quantified. Dry weight data were converted to relative dry weight relative (% of non-treated control) using this formula (Equation 2.1):

$$RDW\ (\%) = [(DW \times 100)/ADW]$$

In equation 2.1, RDW is relative dry weight (% of non-treated control), DW is dry weight of the sample and ADW is the average dry weight of non-treated control.

Dose-response with 2,4-D

Whole-plant dose-response with 2,4-D was conducted to characterize the level of 2,4-D resistance in KCTR compared to susceptible KSS, and MSS populations. This experiment was performed under a completely randomized design in growth chambers and was repeated. Growth chambers were maintained at 32.5/22.5 ºC (d/n) temperature with a photoperiod of 15/9 h (d/n) and 60±10% relative humidity. Lighting inside the growth chambers was provided by fluorescent and incandescent bulbs (750 µmol m^{-2} s^{-1}). For the experiment, KCTR, KSS, and MSS seedlings were germinated in greenhouse and transplanted into small pots as described previously. After transplanting, the seedlings were transferred to growth chambers. Ten-12 cm seedlings were treated with 2,4-D (2,4-D 4L Amine, Winfield Solutions, LLC, St. Paul, Minnesota, US) at the following doses *i.e.*, 0, 140, 280, 560 (field recommended dose, Table 2.1), 1120, 2240, 4480,

8690 g ae ha^{-1}. Seedlings were returned to growth chambers within 30 min of treatment. At 4 WAT, above-ground biomass was harvested from these seedlings, oven-dried, weighed, and converted into relative dry weight (% of non-treated) as described previously (Equation 2.1).

Data Analysis

Levene's test ($\alpha = 0.05$) was conducted to compare runs of whole-plant inhibitor bioassay experiment, and if no significance was identified, relative dry weight data were combined. Normality of residuals and homoscedasticity of variances were verified prior to ANOVA, and relative dry weight data was square root-transformed. Data were subsequently fitted to a linear mixed effect model using the '*nlme*' package and the function '*lme*' available in R (version 4.0.3, R Core Team, 2020) with the R-Studio 9.4 interface (R Studio, PBC, Boston, Massachusetts, US) considering Palmer amaranth populations and chemical treatments as a fixed effect and experimental runs as a random effect (Pinheiro et al. 2021). If interaction across populations and treatments was significant, the means were separated using Tukey's test using the '*multcompView*' and '*lsmeans*' packages at $\alpha = 0.05$ (Graves et al. 2019, Length 2016). Data for adjusted means were back-transformed to calculate percent reduction in dry weight with application of inhibitors in comparison to herbicide alone. These results were plotted using '*ggplot2*' package for graphical visualizations (Wickham 2016).

Levene's test ($\alpha = 0.05$) was also conducted to compare two runs of 2,4-D dose-response analysis. Pooled relative dry weight and visual injury data from the 2,4-D dose-response assays were analyzed using log-logistic regression in R using package '*drc*' (Knezevic et al. 2007, Ritz et al. 2015). The three-parameter regression model had the following formula (Equation 2.2):

$$Y = d + \exp [b (\log x - \log e)]$$

where Y is the response variable (relative dry weight), x is the applied herbicide dose, d is the upper limit, b is the relative slope around e and e is GR_{50} (amount of 2,4-D required to reduce shoot dry weight by 50%). Using the function '*compParm*' in '*drc*' package, estimated GR_{50} of different Palmer amaranth populations were further tested for difference *via* t-test (Shyam et al. 2019). In-built '*plot*' function in the '*drc*' package was used to obtain the dose-response curves. Resistance index (RI) was computed as a ratio of GR_{50} of KCTR with GR_{50} of KSS or MSS populations.

Results

Percent Survival of KCTR and KSS or MSS in Response to POST Herbicide Treatment

The percent survival of KCTR plants to different herbicides was highly variable indicating the considerable genetic variability KCTR Palmer amaranth population. Overall, >28% of KCTR plants survived field-recommended doses of all herbicides tested, except paraquat and glufosinate, for which this population was found to be susceptible (Table 2.2). Following 2,4-D treatment, KSS plants (~10%) were recorded with green tissue, weak twisted stem, and dried meristem (Figure 2.1). However, since no growth, as well as presence of dried meristem, was observed following 2,4-D treatment, these plants were considered as dead. Overall, > 95% control of either KSS or MSS plants was recorded with all herbicide treatments (Table 2.2). The lowest percent survival of KCTR plants was found for glyphosate (28%). In response to several ALS-inhibitor herbicides, the KCTR plants showed variation in % survival as follows: 34% for chlorsulfuron, 60% for thifensulfuron and imazethapyr, and 70% for imazamox suggesting that KCTR has evolved resistance to both sulfonylureas and imidazolines.

Figure 2.1 The response of susceptible (MSS or KSS) and resistant (KCTR) Palmer amaranth to (A) chlorsulfuron, (B) 2,4-D, (C) atrazine, (D) glyphosate, (E) glufosinate, (F) paraquat, (G) lactofen, and (H) mesotrione application 3 to 4 weeks after treatment.

In response to PS II-inhibitor (*e.g.*, atrazine and metribuzin) application, all KCTR plants survived atrazine but only 36% survived treatment with metribuzin, confirming the evolution of resistance to PS II-inhibitor herbicides (Table 2.2). Eighty-four and 90% of KCTR plants survived mesotrione and tembotrione treatments, respectively, suggesting a prevalence of resistance to HPPD-inhibitor herbicides (Table 2.2). Nonetheless, 42% of KCTR plants survived the tank mixture of atrazine and mesotrione (Table 2.2). Additionally, 98% of KCTR

Table 2.2 Percent survival of KCTR (Kansas Conservation Tillage Resistant) Palmer amaranth population to different post-emergence herbicides.

WSSA group	MOA	Herbicides[a]	Survival[b] (%)
2	ALS-inhibitors	Chlorsulfuron	34
		Thifensulfuron	60
		Imazamox	70
		Imazethapyr	60
4	Synthetic auxins	2,4-D	84
5	PS II-inhibitors	Atrazine	100
		Metribuzin	36
9	EPSPS-inhibitors	Glyphosate	28
10	Glutamine synthetase inhibitors	Glufosinate	0
14	PPO-inhibitors	Lactofen	84
		Fomesafen	29
22	PS I-inhibitors	Paraquat	0
27	HPPD-inhibitors	Mesotrione	90
		Tembotrione	84
5 + 27	PS II-inhibitors + HPPD-inhibitors	Atrazine + Mesotrione	42
		Bromoxynil + Pyrasulfotole	98

[a] A total of 50 plants from each population (KCTR and KSS or MSS) were sprayed with each herbicide.

[b] KSS and MSS were controlled (≥95%) with all the herbicides.

plants also survived the commercial premix of bromoxynil (PS II-inhibitor) + pyrasulfotole (HPPD-inhibitor), one of the widely used POST herbicides for Palmer amaranth control in grain sorghum production. In response to PPO-inhibitor applications, 29 and 84% of KCTR plants

survived treatment with fomesafen and lactofen, respectively, confirming evolved resistance to PPO-inhibitor herbicides (Table 2.2). Also, 84% of KCTR plants survived 2,4-D treatment at the field recommended dose.

Assessment of TSR Mechanisms in KCTR Palmer Amaranth

Nucleotide sequence alignment of the *ALS* gene of the KCTR, KSS, and MSS plants showed a lack of the four previously reported mutations (Chaudhari et al. 2020, Küpper et al. 2017, Nakka et al. 2017c, Singh et al. 2019) known to confer resistance ALS-inhibitor herbicides in Palmer amaranth (Figure S2.1). Even though some nucleotide polymorphisms were detected, none of them were consistent among the resistant plants or resulted in amino acid substitution (Figure S2.1). No nucleotide polymorphisms were detected in the *psbA* sequence of KCTR plants (Figure S2.2). Our qPCR results indicated that glyphosate-resistant KCTR plants had an increased number of *EPSPS* copies, ranging from 20 to 88, compared to KSS or MSS (Figure 2.2).

Assessment of NTSR Mechanisms in KCTR Palmer Amaranth

Malathion treatment alone did not significantly impact dry weight accumulation in either KCTR, KSS, or MSS plants (Figures 2.3A, C–E). Contrary to that, imazethapyr treatment resulted in significantly ($p < 0.0001$) lower dry weight accumulation in MSS plants (9%) compared to KCTR plants (23%) (Figure 2.3A). Treatment of malathion with imazethapyr did not reduce the relative dry weight accumulation of KCTR plants compared to KCTR plants treated with imazethapyr alone (Figure 2.3A). Interestingly, there was no significant difference between KCTR plants treated with malathion with imazethapyr (15%) in comparison to MSS plants treated with either imazethapyr alone (9%) or malathion with imazethapyr (8%; Figure 2.3A).

Similar to malathion, NBD-Cl treatment alone did not significantly affect dry weight accumulation in either KCTR, KSS, or MSS plants (Figures 2.3B, E). Atrazine treatment at the

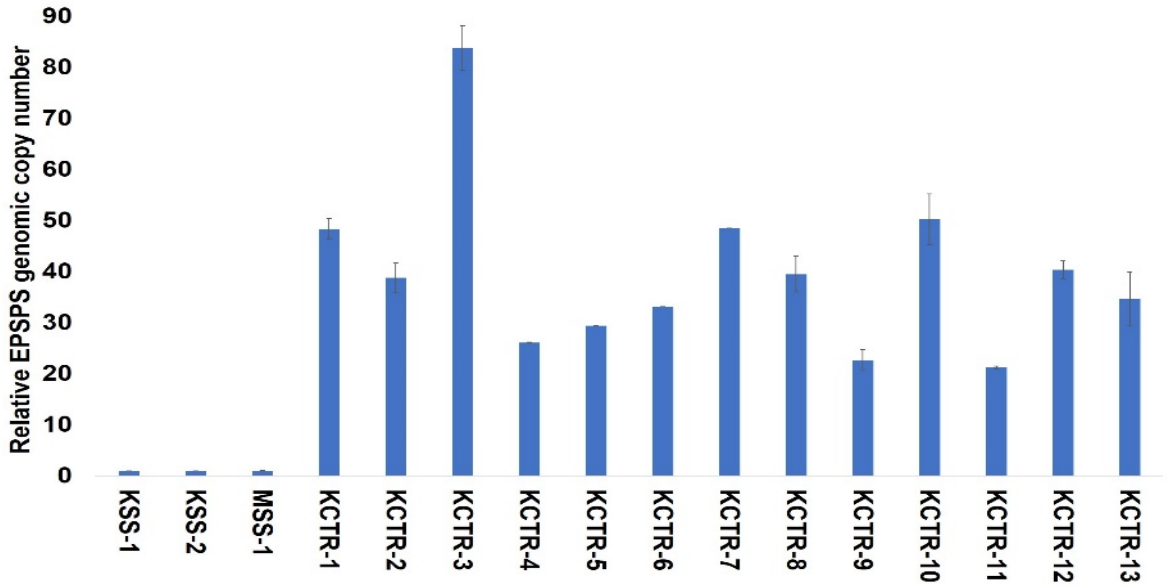

Figure 2.2 *EPSPS* genomic copy number in glyphosate-susceptible (MSS and KSS) and glyphosate-resistant (KCTR) Palmer amaranth plants relative to the susceptible plants. KCTR plants were treated with field recommended dose of glyphosate. Error bars represent the standard error from the mean (2 runs and in each run 3 technical replicates). The qPCR data was normalized using *β-tubulin* as the reference gene.

field-recommended dose resulted in 58% relative dry weight accumulation in KCTR, whereas MSS plants had 3% dry weight accumulation (Figure 2.3B). This is similar to the results of the herbicide screening experiment (Table 2.2). Treatment of NBD-Cl 48 h prior to atrazine resulted in only 33% relative dry weight accumulation in KCTR plants which was significantly lower

than atrazine only treatment (58%; $p < 0.0001$). Such effect of malathion was not observed with pre-treatment of NBD-Cl in MSS plants (Figure 2.3B).

Highly variable dry weight accumulation was observed in KCTR plants treated with 2,4-D; however, relative dry weight accumulation in 2,4-D-treated KCTR plants (81%) was higher than 2,4-D-treated KSS plants (21%) (Figure 2.3C). Treatment of malathion with 2,4-D resulted in statistically lower dry weight accumulation (45%) than only 2,4-D-treated KCTR plants ($p = 0.0005$). However, such impact of malathion was not observed in KSS plants (Figure 2.3C).

Mesotrione treatment resulted in significantly lower dry weight accumulation in KCTR plants (65%) compared to KSS plants (8%) (Figure 2.3D). Nonetheless, treatment of malathion with mesotrione resulted in lower dry weight accumulation in KCTR plants (31%) than mesotrione treatment alone ($p < 0.0001$) (Figure 2.3D). Contrary to that, malathion- and mesotrione-treated KSS plants (8%) showed no difference in dry weight accumulation compared to mesotrione-only treatment (Figure 2.3D).

Lactofen-only treatment resulted in significantly lower ($p = 0.0418$) relative dry weight accumulation in KCTR plants (26%) compared to KSS plants (11%). Even with significant dry weight reduction in the resistant population, several plants survived this herbicide application (Table 2.2 and Figure 2.3E). The KSS Palmer amaranth was susceptible to all treatments containing lactofen (Table 2 and Figure 2.3E). Treatment of NBD-Cl fb lactofen did not result in increased sensitivity of KCTR (Figure 2.3E). However, combination of malathion with lactofen significantly reduced dry weight accumulation in KCTR plants (8%), compared to the lactofen-only treatment. Such impact of malathion was not observed in KSS plants (Figure 2.3E).

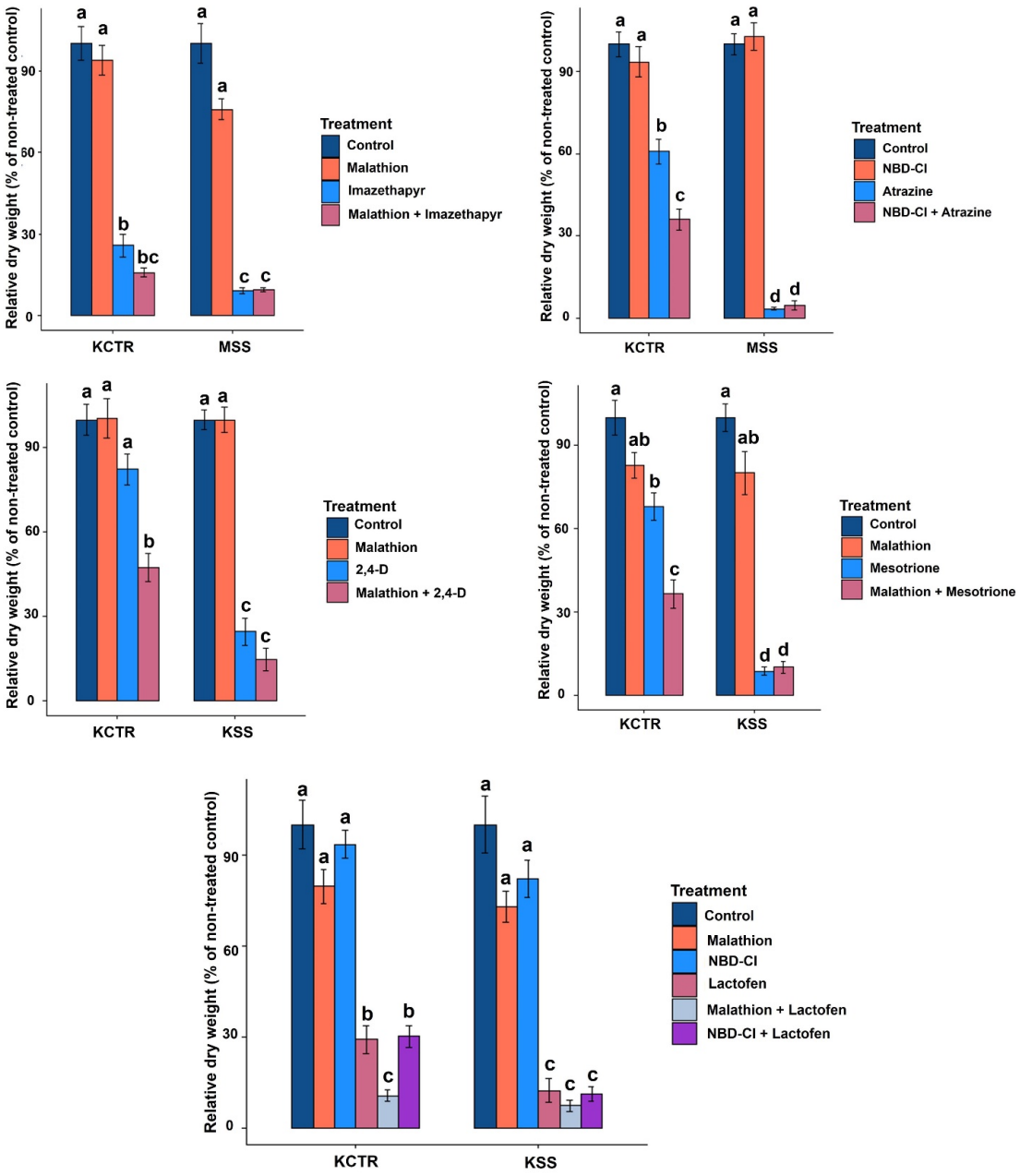

Figure 2.3 Effect of P450- (malathion) and GST-(NBD-Cl) inhibitors on efficacy of (A) imazethapyr, (B) atrazine, (C) 2,4-D, (D) mesotrione, and (E) lactofen in susceptible (KSS or MSS) and resistant (KCTR) Palmer amaranth populations. Error bars represent standard error of mean. Letters represent significant differences identified by separation of means using Tukey's test (5%).

2, 4-D Dose-Response Assay

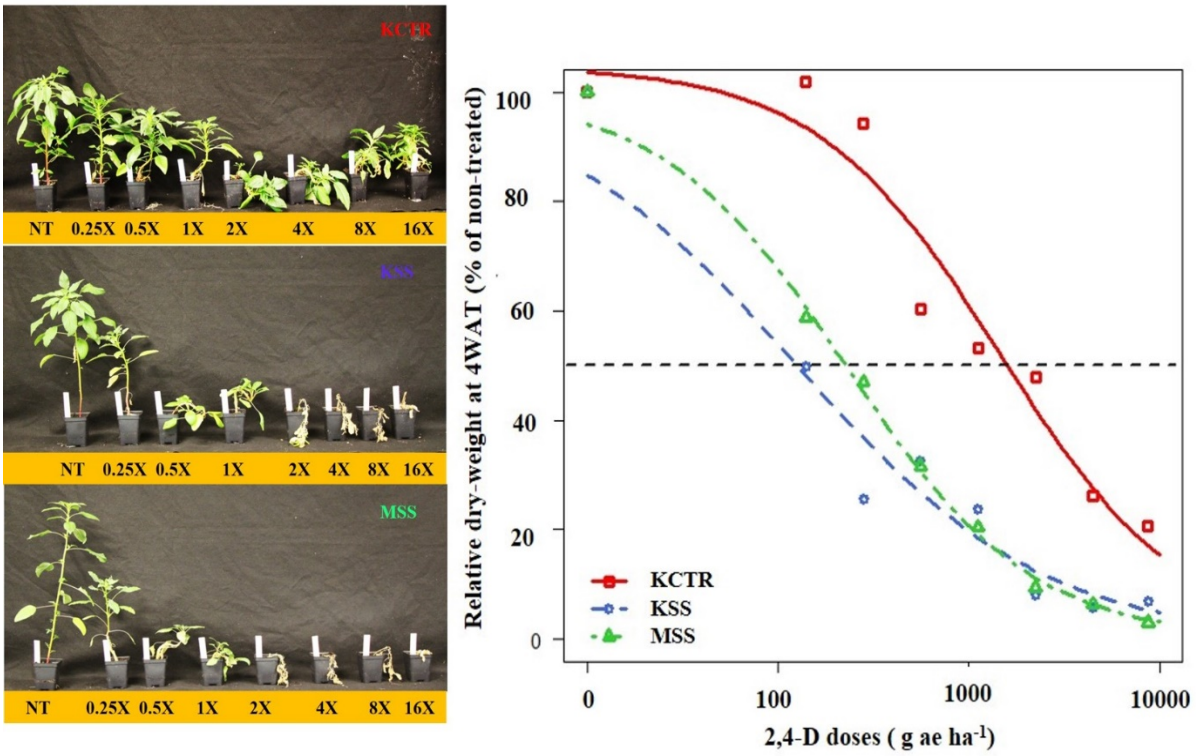

Figure 2.4 (A) Response of KSS (susceptible), MSS (susceptible), and KCTR (resistant) to varying doses of 2,4-D ranging from non-treated (NT) to 16X, where 1X is the field recommended dose of 560 g ae ha^{-1} at 4 weeks after treatment (WAT). (B) Dose-response curves representing relative dry weight (% of non-treated) of KSS, MSS, and KCTR Palmer amaranth using the three-parameter log-logistic regression model (Equation 2.2) at 4 WAT. Dotted arrow at the center of the plot represents the 50% of relative dry weight.

Dose-response analysis revealed that the amount of 2,4-D required to reduce above-ground dry weight by 50% (GR_{50}) at 4WAT was ~1441g ae ha^{-1} for KCTR compared to 127 and 227 g ae ha^{-1} for MSS and KSS, respectively (Figure 2.4, Table 2.3). Even though at controlled environment growth chamber conditions susceptible plants survived the field recommended ate of 2,4-D (560 g ae ha^{-1}) but the plants showed a high level of epinasty and stunting up to 4 WAT. Absolute discrimination in terms of alive and dead plants between KCTR and susceptible populations was observed at 1120 g ae ha^{-1}. Based on GR_{50} values, KCTR was ~11 and ~6 times more resistant compared to KSS and MSS, respectively (Fig. 2.4, Table 2.3). T-test analysis using the '*comParm*' function in '*drc*' package in R showed that there is a significant difference in the estimated GR_{50} of KCTR compared to KSS and MSS.

Table 2.3 Regression parameters (Equation 2.2) describing the response of KSS (susceptible), MSS (susceptible) and KCTR (resistant) Palmer amaranth to 2, 4-D under growth chamber conditions (b: relative slope, d: upper limit; e or GR_{50}: dose required for 50% dry weight reduction; SE: standard error; RI: resistance index).

Population	b (SE)	d (SE)	e (SE)	RI
KSS	0.67 (0.18)	99.94 (7.41)	126.71 (62.43)	11.37
MSS	0.90 (0.20)	99.82 (7.41)	227.49 (72.80)	6.33
KCTR	0.91 (0.15)	104.75 (6.47)	1441.08 (342.53)	-

Discussion

Palmer amaranth is a dioecious species with prolific seed production that has already evolved resistance to eight herbicide MOAs (Heap 2021). In this research, we report for the first time the evolution of six-way resistance in a single Palmer amaranth population, *i.e.*, KCTR with

a predominance of metabolic resistance mechanisms. Previously Palmer amaranth populations with resistance to three and five MOAs have been reported (Chaudhari et al. 2020, Kumar et al. 2019b, Nakka et al. 2017a, 2017b, 2017c). Similar to our findings, the six-way resistant common waterhemp population was also documented in Missouri (Shergill et al. 2018a). Our results confirm the evolution of resistance to ALS-, PS II-, HPPD-, PPO-, EPSPS-inhibitor herbicides, and synthetic auxins in the KCTR population (Table 2.2). KCTR plants survived (28–100%) application of these herbicides, while satisfactory control (0% survival) was achieved only with PS I- (*e.g.*, paraquat) and glutamine synthetase inhibitor herbicides (*e.g.*, glufosinate) (Table 2.2). Such a wide range of survival for different MOAs indicates that KCTR is likely to be not genetically homogeneous, and possibly there is ongoing segregation for resistance to these MOAs, especially ALS- and EPSPS-inhibitor herbicides (Table 2.2). This is not unusual considering the amount of genetic variability offered by an outcrossing weed species like Palmer amaranth. Improved control of KCTR plants (58%) was observed with a tank mixture of atrazine (half of the recommended dose: 1,120 g ai ha^{-1}) and mesotrione (recommended dose: 105 g ai ha^{-1}) (Table 2.2) compared to atrazine alone (0%) or mesotrione alone (16%). However, some plants were not effectively controlled by this tank mixture. Such improvement in control can be attributed to the synergism often observed with tank mixing PS II- and HPPD-inhibitor herbicides (Abendroth et al. 2006, Chahal et al. 2019a). Additionally, the commercial pre-mixture of bromoxynil (PS II-inhibitor) and pyrasulfotole (HPPD-inhibitor) performed poorly in comparison to a tank mix of atrazine and mesotrione.

Although mutations in herbicide target genes conferring resistance are rare evolutionary events, mutations in the *ALS* gene at eight positions (four positions in Palmer amaranth) were found to confer resistance to several classes of ALS inhibitors in weeds (Heap 2021, Tranel and

Wright 2002, Yu and Powles 2014). Several amino acid substitutions at the *ALS* gene have been documented in Palmer amaranth populations from Kansas (Chaudhari et al. 2020, Nakka et al. 2017c). Interestingly, upon sequencing the whole *ALS* gene, the KCTR plants showed no presence of any of these substitutions (Figure 2.2). Malathion has been shown to increase the metabolic half-life of herbicides by inhibiting P450-dependent metabolism (Kreuz and Fonné-Pfister 1992). Therefore, malathion treatment was done prior to imazethapyr to test the involvement of P450-based detoxification mechanisms in KCTR. Even though imazethapyr treatment was not significantly different from the combination of malathion with imazethapyr in reducing KCTR dry weight, malathion with imazethapyr-treated KCTR plants produced the same level of dry weight as imazethapyr-treated MSS plants (Figure 2.3A). This indicated a moderate reduction of dry weight in KCTR with malathion treatment. Moreover, since only 60% of KCTR plants were found to be resistant to imazethapyr (Table 2.2), sensitive plants in KCTR may have contributed to the lack of differences between imazethapyr only and combination of malathion with imazethapyr treatment. Different P450 inhibitors have been observed to have varying levels of synergistic effect in resistant weeds depending on the herbicide as well as P450 isozyme. For instance, Preston et al. (1996) observed piperonyl butoxide (PBO; another P450 inhibitor) inhibiting chlorotoluron and simazine resistance in rigid ryegrass (*Lolium rigidum* Gaudin), but malathion failed to do so. Similarly, Oliveira et al. (2018b) observed that malathion improved the efficacy of tembotrione but not of mesotrione in HPPD-inhibitor-resistant common waterhemp from Nebraska. Therefore, it is possible that different P450s, which are not completely inhibited by malathion, are involved in imparting resistance to imazethapyr in KCTR.

All KCTR plants survived atrazine application (Table 2.2), and the lack of any known mutations in the *psbA* suggests an NTSR mechanism to atrazine as reported in other Palmer

amaranth populations (Chaudhari et al. 2020, Nakka et al. 2017a). A V219L mutation in the *psbA* gene was found in atrazine-resistant Powell amaranth (*Amaranthus powellii* S. Watson) (Dumont et al. 2016) but not in common waterhemp or other Amaranthus species closely related to Palmer amaranth (Chahal et al. 2019b, Ma et al. 2013, Shergill et al. 2018b). NBD-Cl derivatives have been shown to give strong GST inhibition in human tumor cells and are termed as suicide inhibitors of GSTs (Ricci et al. 2005). Therefore, NBD-Cl treatment prior to atrazine application was given to KCTR plants to determine the involvement of GSTs in imparting resistance. This treatment significantly reduced dry weight accumulation in KCTR plants indicating the involvement of GST enzymes in metabolizing atrazine. Previously, pre-treatment with NBD-Cl has reversed atrazine resistance in common waterhemp (Ma et al. 2013, Shergill et al. 2018b). Metabolic resistance to atrazine *via* glutathione conjugation mediated by GST activity has been reported in several Palmer amaranth and common waterhemp populations in the US Midwest (Chahal et al. 2019b, Ma et al. 2013, Shergill et al. 2018b). KCTR plants are also resistant to metribuzin, another PS II inhibitor. Metribuzin resistance mediated by enhanced metabolism was reported in wild radish (*Raphanus raphanistrum* L.) (Lu et al. 2019) and rigid ryegrass (Ma et al. 2020).

Treatment of malathion with application of mesotrione, or lactofen significantly reduced dry weight accumulation in KCTR plants (Figures 2.3E), suggesting P450-mediated detoxification of these herbicides in KCTR plants. Previously, malathion-induced reversal of weed resistance to carfentrazone, and fomesafen (Obenland et al. 2019, Varanasi et al. 2018a), and mesotrione (Ma et al. 2013) has been reported. However, NBD-Cl failed to impact dry weight accumulation in KCTR when applied prior to lactofen treatment. This indicates that certain P450 enzymes and not GSTs, or potentially specific GSTs not inhibited by NBD-Cl, may

be involved in imparting resistance to lactofen in KCTR plants. In contrast to our findings, in the PPO-inhibitor-resistant Palmer amaranth population from Arkansas, the use of NBD-Cl caused the reversal of resistance to fomesafen (Varanasi et al. 2018a).

EPSPS amplification (up to 88 copies) was found to contribute to glyphosate resistance in KCTR plants. Gaines et al. (2011) have shown that > 30 *EPSPS* copies are needed to withstand the field recommended dose of glyphosate application (840 g ai ha^{-1}) in Palmer amaranth. Despite a low percentage (28%; Table 2.2) of glyphosate survivors in this population, lack of fitness penalty associated with this resistance mechanism and the obligate outcrossing nature of Palmer amaranth can lead to the rapid spread and transfer to other susceptible populations *via* pollen (Giacomini et al. 2014, Jhala et al. 2021, Sosnoskie et al. 2012).

KCTR Palmer amaranth is 6-11 fold resistant to 2,4-D compared to KSS and MSS. So far, the evolution of 2,4-D resistance has been reported from three *Amaranthus sp. i.e.,* Palmer amaranth, common waterhemp, and smooth pigweed. Mechanism of 2,4-D resistance in the common waterhemp populations of Nebraska and Missouri has been confirmed to be enhanced metabolism possibly mediated by P450s (Figueiredo et al. 2018, Shergill et al. 2018b). In smooth pigweed, both reduced translocation and enhanced metabolism have been reported as the 2,4-D resistance mechanism (Palma-Bautista et al. 2020b). Treatment of malathion prior to 2,4-D, increased susceptibility in KCTR implying involvement of P450s. This indicates that 2,4-D resistance in KCTR is metabolic and mediated by P450s. However, presence of other NTSR mechanisms such as reduced absorption and translocation and presence of TSR also needs to be investigated on KCTR, to rule out or confirm their presence.

Based on previous research in our laboratory, the coexistence of both TSR and NTSR for the same herbicide can occur in a single population or individual plant of Palmer amaranth

(Chaudhari et al. 2020, Nakka et al. 2017b, 2017c). Research is in progress to determine if such a scenario is present in KCTR as well. Future investigations focused on identifying specific P450s and GSTs involved in herbicide detoxification in this population will also be investigated. It is important to understand what predisposes a population to develop metabolic resistance to several MOAs, including those with no history of use and absence of selection pressure.

Because pre-emergence (PRE) herbicide treatments are regarded as one of the best strategies to manage herbicide resistance in weeds (Norsworthy et al. 2012), information on response of KCTR to PRE herbicides can help in formulating viable options to manage this population. Therefore, experiments are in progress to investigate the response of KCTR to several soil-applied residual PRE herbicides (*e.g.*, atrazine, mesotrione, flumioxazin, and s-metolachlor) commonly used to control germinating and emerging seedlings of Palmer amaranth. Previously, there has been a fitness penalty associated with multiple-herbicide resistance in weed species such as rigid ryegrass (Vila-Aiub et al. 2005). Studies will also be conducted to assess whether any fitness penalty is present in this population as a result of evolution of six-way herbicide resistance, which can help in understanding the spread of resistance traits to other populations.

Conclusion

Evolution of resistance to six herbicide MOAs in the KCTR population leaves very few POST herbicide options for its control. Moreover, such accumulation of resistance traits in a single Palmer amaranth population poses serious questions on the effectiveness of stacked resistance traits in crops, such as 2,4-D + glyphosate + glufosinate or dicamba + glyphosate resistance in corn and beans. Considering the lack of introduction of new MOAs, it is crucial to conserve currently available MOAs to effectively manage weeds. Growers should be encouraged

to adopt integrated weed management techniques to reduce selection pressure by herbicides and discourage the further selection of the evolution of multiple resistance.

Chapter 3 - Physiological Mechanism of 2,4-D Resistance in Palmer amaranth

Abstract

A Palmer amaranth population (KCTR: Kansas Conservation Tillage Resistant) collected from a conservation tillage field was confirmed to have evolved resistance to at least six herbicide modes of action including 2,4-D, a widely used synthetic auxinic herbicide (Chapter 2). Results of dose-response experiments indicated a 6-to 11-fold resistance to 2,4-D in KCTR compared to two susceptible Palmer amaranth populations (KSS: Kansas Susceptible and MSS: Mississippi Susceptible; Chapter 2). This chapter was focused on the investigation of the physiological basis of 2,4-D resistance in KCTR Palmer amaranth based on the hypothesis that non-target site resistance mechanisms such as differential absorption, translocation, or metabolism of 2,4-D may impart resistance. The objectives of this research were, using KCTR, KSS, and MSS Palmer amaranth and wheat (naturally tolerant to 2,4-D) i) investigate the 2,4-D absorption, and translocation profiles and ii) evaluate the rate of 2,4-D metabolism, and iii) determine the role of cytochrome P450 (P450)- inhibitors [malathion and piperonyl butoxide (PBO)] in conferring metabolic resistance to 2,4-D. Experiments were carried out by treating plants grown under controlled environment growth chamber conditions with [^{14}C] 2,4-D to determine differential absorption, translocation, and metabolism in KSS, MSS, and KCTR. P450s are known to metabolize pesticides including herbicides in plants. Therefore, whole-plant dose-response assays were conducted by treating KCTR and KSS plants with P450 inhibitors (malathion or PBO) prior to the application of 2,4-D. Results of these experiments showed no difference in percent of [^{14}C] 2,4-D absorption among the populations. However, approximately 10% less and 3 times slower

[^{14}C] 2,4-D translocation was found in KCTR compared to susceptible populations (KSS and MSS). Importantly, [^{14}C] 2,4-D was metabolized faster in KCTR than KSS and MSS Palmer amaranth. Specifically, at 24, 48, and 72 h after treatment (HAT), KCTR metabolized ~ 20-30% more [^{14}C] 2,4-D than KSS and MSS. Wheat is naturally tolerant to 2,4-D due to the rapid metabolism of this herbicide by P450 enzyme activity. Therefore, the retention time of polar metabolites of [^{14}C] 2,4-D and quantity of parent [^{14}C] 2,4-D were compared between wheat and KCTR Palmer amaranth. Even though KCTR and wheat generated the same types of polar metabolites of [^{14}C] 2,4-D, nonetheless, at 24 HAT, ~70% of [^{14}C] 2,4-D was metabolized in wheat, compared to only 30% in KCTR Palmer amaranth. Application of malathion prior to 2,4-D treatment was found to decrease dry weight accumulation in KCTR suggesting involvement of P450s in mediating 2,4-D metabolism. However, no such impact with the use of PBO was found. Overall, the findings of this chapter confirm that enhanced metabolism, mediated by P450 enzyme activity, is the primary physiological mechanism imparting 2,4-D resistance in KCTR Palmer amaranth.

Introduction

2,4-dichlorophenoxy acetic acid (2,4-D) a synthetic auxinic herbicide (SAH) has been in use to control broadleaf weeds since 1945 (Peterson et al. 2016). Based on mode of action (MOA), SAHs are classified as group O (Herbicide Resistance Action Committee: HRAC) or group 4 (Weed Science Society of America: WSSA) herbicides. When used at ultra-low doses, the SAHs imitate the physiological and biochemical responses as that of naturally occurring phytohormone auxins, such as indole acetic acid (IAA), and indole butyric acid (IBA). However, at high doses they are herbicidal. SAHs are the most used herbicide group globally in terms of acreage, and amongst SAHs, 2,4-D usage is the highest (Busi et al. 2018).

Conventionally, SAHs such as 2,4-D are widely used to control broadleaf weeds in cereals crops such as sorghum, wheat, and corn; however, over the years, SAHs, *viz*., quinclorac and halauxifen-methyl, have been found to have the ability to control certain grass weeds as well. Despite extensive use, the evolution of resistance to SAH is relatively low compared to other herbicide groups such as triazines or acetolactate synthase (ALS)-inhibitors. More importantly, the evolution of 2,4-D resistance in weeds has also been low. However, in the past two decades, increased reliability and usage of 2,4-D for controlling ALS- and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor-resistant weeds has led to a rapid increase in resistance to 2,4-D in weeds. Currently, 2,4-D resistance has been documented in 42 weeds globally (Heap 2021). In the US alone, the number of 2,4-D resistant weeds stands at 7, including important broadleaf weeds like smooth pigweed (*Amaranthus hybridus* L.), common waterhemp (*Amaranthus tuberculatus* Moq. Sauer), kochia (*Kochia scoparia* L. Schrad.), and Palmer amaranth (*Amaranthus palmeri* S. Watson), and grasses such as barnyard grass (*Echinochloa crus-galli* var. *crus-galli*) (Heap 2021). Moreover, 2,4-D tolerant crops, *i.e.*,

soybean, corn, and cotton, were recently commercialized, which can aid in controlling troublesome weeds such as glyphosate-resistant Palmer amaranth (Shyam et al. 2020). Intensified adoption of this technology is also expected to cause a surge in 2,4-D use, resulting in increased selection pressure, and potentially leading to 2,4-D resistance evolution (Egan et al. 2011). This scenario calls for a thorough investigation of prevalent mechanisms bestowing 2,4-D resistance in weeds, which in turn can help formulate sustainable weed management programs and increasing the longevity of 2,4-D-tolerant crop technology.

Resistance to 2,4-D in weeds has been reported to be conferred either by target-site (TSR) or non-target site (NTSR) -based mechanisms. TSR to herbicides involves alterations of the herbicide target impacting the binding of the herbicide and reducing its efficacy. Potential target sites for 2,4-D in weeds are auxin receptor and transporter proteins (Todd et al. 2020). However, not many instances of TSR to SAHs have been found, owing to the complexity of the mechanism of action of these herbicides. In 2018, the first confirmed case of TSR was documented in a kochia population from Nebraska where a mutation in the auxin receptor protein KS18A resulted in dicamba resistance (LeClere et al. 2018). More recently, a case of TSR mechanism with a 20 amino acid deletion in the auxin co-receptor *IAA2* was reported to cause 2,4-D resistance in an Indian hedge mustard population (*Sisymbrium orientale* L.) from Australia (Figueiredo et al. 2021). In contrast, a multitude of weeds has been documented with NTSR involving either reduced absorption (Kohler et al. 2004), translocation (Dang et al. 2018, Kohler et al. 2004, Riar et al. 2011), and/or enhanced metabolism (Figueiredo et al. 2018, Torra et al. 2017) of SAHs. For instance, reduced 2,4-D translocation was reported in Indian hedge mustard (Dang et al. 2018), ground ivy (*Glechoma hederacea* L.) (Kohler et al. 2004), prickly lettuce (*Lactuca serriola* L.) (Riar et al. 2011), wild radish (*Raphanus raphanistrum* L.) (Goggin

et al. 2016), and corn poppy (*Papaver rhoeas* L.) (Rey-Caballero et al. 2016). Enhanced metabolism of 2,4-D was also documented in common waterhemp populations across US Midwest (Figueiredo et al. 2018, Shergill et al. 2018b, Shyam et al. 2019) and corn poppy populations in France (Torra et al. 2017). Likewise, natural tolerance to 2,4-D in grasses is often attributed to the rapid metabolism of this herbicide compared to susceptible broadleaved weeds. Metabolism of 2,4-D is well characterized in naturally tolerant cereal crops such as corn (*Zea mays* L.) (Fang and Butts 1954), wheat (*Triticum aestivum* L.) (Bristol et al. 1977, Fang and Butts 1954, Hamburg et al. 2001, Scheel and Sandermann 1981), and sorghum (Morgan and Hall 1963). Cytochrome P450 monooxygenases (P450s) have been known to metabolize herbicides including SAHs imparting selectivity in tolerant crops (Frear 1995, McFadden et al. 1989, Xiang et al. 2006). P450s are one of the major families of enzymes involved in phase I herbicide metabolism, thus offering herbicide selectivity to some plants (Pandian et al. 2020). Application of P450-inhibitors such as malathion, piperonyl butoxide (PBO), tetracylis, *etc*. is widely practiced in the field of weed science as a preliminary assessment of P450-based metabolic resistance to herbicides.

Palmer amaranth, one of the top-ranked troublesome weeds in the US (Van Wychen 2020), is a C_4 plant that can accumulate biomass rapidly and has a high growth rate compared to other *Amaranthus sp*. (Horak and Loughin 2000, Wang et al. 1992). Being a dioecious plant, Palmer amaranth is an obligate out crosser and possesses high genetic diversity. This allows this weed to adapt quickly to challenging abiotic and biotic stress conditions and the ability to evolve resistance to herbicides. So far, Palmer amaranth has evolved resistance to eight herbicide MOAs involving acetolactate-synthase (ALS)-, photosystem II (PS II)-, 5-enolpyruvylshikimate-3-

phosphate synthase (EPSPS)-inhibitor, protoporphyrinogen oxidase (PPO)-inhibitor, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor, and synthetic auxins (Heap 2021).

KCTR (Kansas Conservation Tillage Resistant) Palmer amaranth population was found to be resistant to ALS- (chlorsulfuron, and imazethapyr), PS II- (atrazine, and metribuzin), EPSPS- (glyphosate), PPO- (lactofen, and fomesafen), HPPD-inhibitors (mesotrione, and tembotrione), and SAH (2,4-D) (Chapter 2). Eighty-four percent of KCTR plants (collected from the field which survived 2,4-D treatment at 560 g ae ha^{-1}) survived 2,4-D treatment at the field recommended dose of 560 g ae ha^{-1}, confirming the evolution of resistance (Chapter 2). In addition to the KCTR population, evolution of 2,4-D resistance has been reported in another Palmer amaranth population in Kansas (Kumar et al. 2019b); nonetheless, information on the physiological mechanism of 2,4-D resistance in Palmer amaranth is lacking. Therefore, the objectives of this chapter were using KCTR and known susceptible Palmer amaranth to investigate i) 2,4-D absorption, and translocation profiles and ii) evaluate the rate of 2,4-D metabolism, and iii) determine the role of cytochrome P450 (P450)-inhibitors [malathion and piperonyl butoxide (PBO)] in metabolizing 2,4-D.

Materials and Methods

Plant Material and Growing Conditions

2,4-D-resistant KCTR and susceptible *i.e.,* KSS (Kansas Susceptible), and MSS (Mississippi Susceptible) Palmer amaranth populations were used in this research. KCTR population was collected from a conservation tillage field and has been characterized previously (Chapter 2). KSS population was collected from a nearby field in Ashland Bottoms, Kansas, and is susceptible to all commonly used herbicides used to control Palmer amaranth (Chapter 2;

Nakka et al. 2017a, 2017b, 2017c). Seeds of the MSS population were purchased from a company (Azlin Seed Services, Leland, Mississippi, US) and is susceptible to common herbicides used to control Palmer amaranth (Chapter 2; Nakka et al. 2017a, 2017b, 2017c). Experiments were conducted in either greenhouse or controlled environment growth chamber conditions. The greenhouse was maintained at 30/23 ºC d/n temperature with 16/8 h photoperiod (d/n) and 60 ± 10% relative humidity. Natural light in the greenhouse was supplemented with 250 µmol m^{-2} s^{-1} illumination provided by sodium vapor lamps. Growth chambers were maintained at 32.5/22.5 ºC (d/n) temperature with a photoperiod of 15/9 h (d/n) and relative humidity of 60 ± 10%. Lighting intensity of 750 µmol m^{-2} s^{-1} was provided inside the growth chambers through fluorescent and incandescent bulbs. KCTR, KSS, and MSS seedlings were germinated in trays (21 × 6 × 4 cm) filled with commercial pre-mix (Pro-Mix® premium potting mix, Premier Tech Home and Garden Inc., Ontario, Canada). Three-four leaf seedlings were transplanted to individual pots (6 × 6 × 6.5 cm) and allowed to grow in either greenhouse or growth chambers.

Absorption and Translocation of [^{14}C] 2,4-D in KCTR, KSS, and MSS Palmer amaranth

KCTR, KSS, and MSS seedlings were grown (as described above), transplanted in the greenhouse, and then moved to growth chambers (conditions mentioned in the 'Plant Materials and Growth Conditions' section). Ten-12 cm seedlings of the three populations were treated with a stock solution containing a total of 3.3 kBq of [^{14}C] radio-labeled 2,4-D (American Radio Chemicals, Inc., St. Louis, Missouri, US) with a specific activity of 50 mci $mmol^{-1}$. For preparing the stock solution, [^{14}C] radio-labeled 2,4-D was mixed with commercial 2,4-D amine (2,4-D Amine 4L, Winfield United Solutions, Arden Hills, Minnesota, US) to obtain a dose

equivalent to the field recommended dose (560 g ae ha^{-1}) in a carrier volume of 187 L or 20 gallons per acre (GPA). Each plant was treated with 10 µL (20,000 dpm μL^{-1}) of the stock solution on the upper surface of the 3rd or 4th fully opened young leaf. The treated plants were returned to their respective growth chambers after 30 minutes (min) and plant parts were harvested at four time-points: 6, 24, 48, and 72 HAT (h after treatment). At each harvesting time point, the treated plants were trisected into three parts, namely, TL (treated leaf), ATL (area above-treated leaf), and BTL (area below-treated leaf), to investigate the amount of 2,4-D absorbed and translocated within the plant. The treated leaf of each sample was washed with 5 ml wash solution (10% ethanol and 0.05% Tween-20) twice for 1 minute, to remove excess unabsorbed herbicide. Scintillation cocktail (Ecolite (+)™ (MP Biomedicals, LLC, Solon, Ohio, US) was added to this leaf-rinsate for measuring the radioactivity in a liquid scintillation counter (LSC: Beckman Coulter, LS 6500 Liquid Scintillation Counter, Beckman Coulter, Brea, California, US). Once washed, the trisected plant parts were individually wrapped in wipes (Kim wipes) and packed in brown paper envelopes for drying in an oven at 65 ^{O}C for 72 h. These dried plant parts were combusted using a biological oxidizer (OX-501, RJ Harvey Instrument, Tappan, New York, US), and [^{14}C] CO_2 generated from the combustion was trapped in a scintillation cocktail (RJ Harvey Instrument). Radioactivity of this scintillation cocktail was measured using LSC. Three replications were maintained for each treatment and the experiment was repeated. The following equations were used to convert LSC data into percent [^{14}C] 2,4-D absorbed and translocated (Equation 3.1, 3.2, 3.3, 3.4, 3.5, and 3.6):

$$\text{Abs. (\%)} = [(R_{Applied} - R_{Rinsate}) / R_{Applied}] \times 100$$

$$\text{Trans. (\%)} = 100 - [R_{TL}/(R_{Applied} - R_{Rinsate})\text{x } 100]$$

$$\text{TL (\%)} = R_{TL}/(R_{Applied} - R_{Rinsate})\text{x } 100$$

$$ATL\ (\%) = R_{ATL}/(R_{Applied} - R_{Rinsate})x\ 100$$

$$BTL\ (\%) = R_{BTL}/(R_{Applied} - R_{Rinsate})x\ 100$$

$$Recovery\ (\%) = [(R_{Rinsate} + R_{TL} + R_{ATL} + R_{BTL} / R_{Applied}) \times 100]$$

In the above equations (3.1 through 3.6), $R_{Applied}$ is the total amount of radioactivity in disintegration per minute (dpm) applied in each plant, Abs. (%) is the percent absorption, $R_{Rinsate}$ is the amount of radioactivity recovered (dpm) in the treated leaf wash rinsate, Trans (%) is the percent translocation, R_{TL} (%) is the percent radioactivity recovered in the treated leaf, R_{ATL} (%) is the percent radioactivity recovered in the plant tissue above treated-leaf, R_{BTL} (%) is the percent radioactivity recovered in the plant tissue above, below treated-leaf, and recovery (%) is the total amount of radioactivity in the experiment.

Metabolism of [^{14}C] 2,4-D in KCTR, KSS, MSS Palmer amaranth, and wheat

KCTR, KSS, and MSS seedlings were grown as described earlier for absorption and translocation experiments in growth chambers under the same conditions as described above. Additionally, wheat seedlings (winter wheat variety KS Western Star) were grown as a positive control because of their natural ability to metabolize 2,4-D.

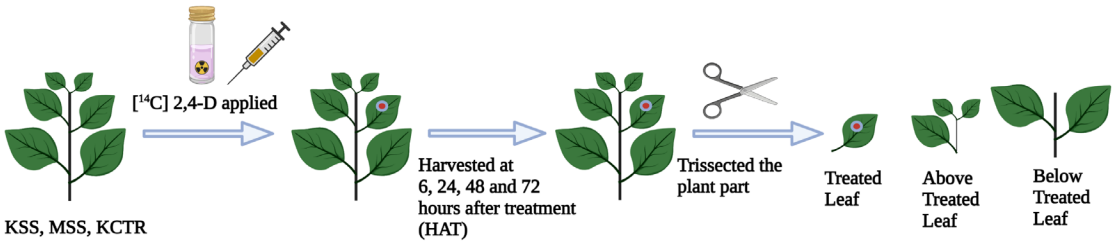

Figure 3.1 Illustration of application of [^{14}C] 2,4-D for investigating absorption, translocation, and metabolism of 2,4-D in KSS, MSS, and KCTR seedlings [Created with BioRender.com].

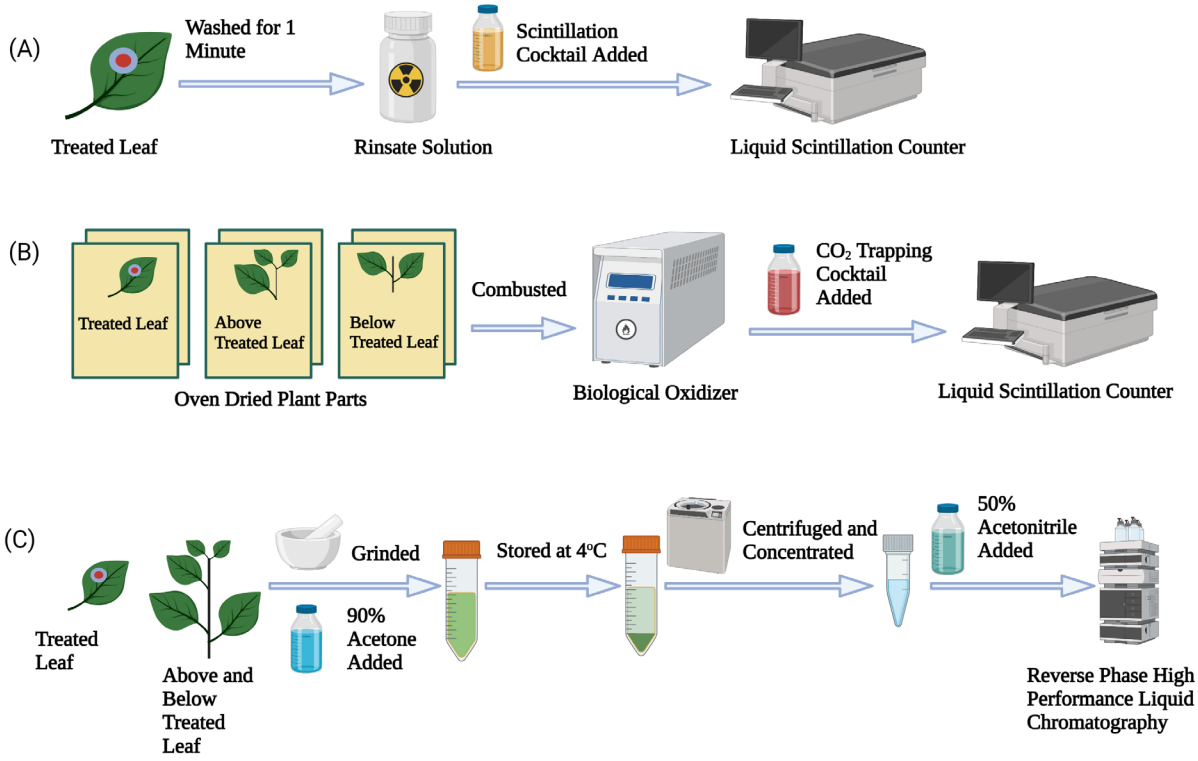

Figure 3.2 Steps involved in estimation of [^{14}C] 2,4-D (A) absorption, (B) translocation, and (C) metabolism of 2,4-D in KSS, MSS and KCTR seedlings [Created with BioRender.com].

Ten-12 cm tall Palmer amaranth plants along with wheat plants at 3-4 leaf stage were treated with [^{14}C] 2,4-D stock solution. The stock solution contained 7.2 kBq [^{14}C] 2,4-D with a concentration of 30,000 dpm μL^{-1}. For treatment, 10 µL of this [^{14}C] 2,4-D stock solution was applied on the adaxial surface of the 3rdor 4th youngest leaf of Palmer amaranth seedlings and the second fully opened leaf of wheat plants. Palmer amaranth plants were harvested at 4 time-points *i.e.*, 6, 24, 48, and 72 HAT; while wheat seedlings were harvested only at 24 HAT (because wheat is known to rapidly metabolize 2,4-D soon after application; (deBoer et al. 2006, 2011, Tanetani et al. 2013). At each harvest time, treated leaves were washed as described previously to remove unabsorbed

herbicide. After washing, the whole seedlings were clipped individually above-ground and then flash-frozen in liquid nitrogen along with the washed treated leaf. The frozen seedlings were homogenized using mortar and pestle, and [^{14}C] 2,4-D and its metabolites were extracted using 15 ml of 90% acetone at 4 ºC for 16 h. This was followed by centrifugation at 5,000 X g for 10 min and concentrating the supernatant at 45 ºC for 2 h with a rotary evaporator (Centrivap, Labconco Corporation, Kansas City, Missouri, US) to obtain a final volume of around 600-1000 µL. The supernatant was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 10,000 X g for 10 min at room temperature to precipitate the waste and supernatant was collected. After centrifuging, the radioactivity of each sample was measured using LSC by adding 2 µL of extract (supernatant) in 15 ml scintillation fluid (Ecolite$^{(+)TM}$, MP Biomedicals). The data were used to normalize the sample to a standard concentration of 3000 dpm per 50 µL by adding 50% acetonitrile (HPLC-grade, Fischer-Scientific). About 500 µL of normalized extract from each sample was run through reverse-phase high-performance liquid chromatography (HPLC) (1260 Infinity II LC System, Agilent, Santa Clara, California, US) to quantify the parental compound and metabolites of 2,4-D.

Effect of P450 inhibitors in metabolizing 2,4-D in KCTR and KSS Palmer amaranth

To confirm the involvement of P450s in mediating the metabolism of 2,4-D in KCTR, dose-response experiments were conducted in a greenhouse (conditions mentioned in 'Plant and Growing conditions') with KCTR and KSS seedlings using P450-inhibitors. These experiments were conducted separately with two P450 inhibitors, *i.e.*, malathion and PBO. For these experiments with each inhibitor, treatments were arranged in a factorial structure that included three factors, *i.e.*, Palmer amaranth populations (2 levels: KCTR, KSS), inhibitor doses (2 levels: non-treated, treated), and 2,4-D doses (5 levels: non-treated, 140, 280, 560, 1120, 2240 g ae ha^{-}

[1]). Malathion (Spectracide®, Malathion Concentrate, St. Louis, Mo), a commonly used P450 inhibitor was applied 30 minutes prior to 2,4-D treatment at 1500 g ai ha^{-1} and was reapplied as a soil drench (50 mM) at 48 HAT as described by Ma et al. (2013). Similar to malathion, PBO was applied 30 mins prior to 2,4-D treatment at 2100 g ai ha^{-1} as described in previous studies (Shergill et al. 2018b, Zhao et al. 2019). All inhibitor and 2,4-D applications were done using a bench-track sprayer (Generation III, DeVries Manufacturing, RR1 Box 184, Hollandale, MN) that is equipped with a flat-fan nozzle tip (8002 Teejet, Spraying Systems Co., Wheaton, IL). The sprayer was calibrated to deliver a spray volume proportional to 187 L ha^{-1} or 20 GPA at a speed of 4.77 km h^{-1}. Eight replications were maintained for each treatment and the experiment was repeated. Plants were harvested at 4 weeks after treatment (WAT) by clipping the above-ground part, followed by oven-drying at 65 ºC for 72 h and measuring dry weight. For data analysis, the dry weight data were converted to relative dry weight (% of non-treated control) using the following formula (Equation 3.7):

$$RDW = [(DW \times 100)/ ADW]$$

In equation 3.7, RDW is the relative dry weight (% of non-treated control), DW is the dry weight of the sample and ADW is the average dry weight of non-treated control.

Data Analysis

Percent [^{14}C] 2,4-D absorbed and translocated in the treated plants was determined and the data were fitted with asymptotic regression, a rectangular hyperbola (RHB), and linear model as suggested by (Kniss et al. 2011). The analysis was done using '*drc*' and '*qPCR*' packages in R (version 4.0.3, R Core Team, 2020) with the R-Studio 9.4 interface (R Studio, PBC, Boston, MA) (Ritz et al. 2015, Spiess 2018). Following the model fitting of the data, the bias-corrected

Akaike information criteria (AICc) of each model were compared, and based on the lowest AICc value, the RHB model was selected for analyzing both the absorption and translocation data. The following RHB models were fitted (Kniss et al. 2011, Equations 3.8 and 3.9):

$$\text{Abs.} = (A_{max} \times t) / [(10/90) \times A_{90} + t]$$

$$\text{Trans.} = (T_{max} \times t) / [(10/90) \times T_{90} + t]$$

In equations 3.8 and 3.9 Abs. is percent herbicide absorbed, expressed as percentage herbicide applied to the plant (Equation 3.1), and Trans. is the percent herbicide translocated expressed in terms of percentage herbicide absorbed in the plant (Equation 3.2). A_{max} and T_{max} are the maximum herbicide absorption and translocation, respectively, in time t, whereas A_{90} and T_{90} are the time required for 90% of the absorption and translocation to occur. Percent [^{14}C] 2,4-D translocated in TL, ATL, and BTL (Equation 3.5, 3.6, 3.7) were plotted as line graphs using '*ggplot2*' (Wickham 2016).

In metabolism experiments, HPLC chromatographs showing separated parent [^{14}C] 2,4-D and polar metabolites of the different Palmer amaranth populations and wheat at different time points were used for visual assessment of 2,4-D metabolism. Percent parent [^{14}C] 2,4-D present in each sample was determined based on the area of the chromatographs. Levene's test was conducted using '*Car'* package in R-studio to compare the two runs of metabolism and since it was non-significant, data were pooled (Fox and Weisberg 2019). Percent parent [^{14}C] 2,4-D data was analyzed using two-way ANOVA in R with population (3 levels: KSS, MSS, and KCTR) and time-points (4 levels: 6, 24, 48, and 72 HAT) being the two factors. Since the interaction between the two factors was significant, the data was plotted based on each time-point for better visualization.

Relative dry weight data from two runs of the dose-response experiments were compared *via* Levene's test ($\alpha = 0.05$). Since the test was non-significant, the data were pooled. Pooled data was analyzed using three-parameter log-logistic regression in R utilizing package '*drc*' (Knezevic et al. 2007, Ritz et al. 2015). The following three-para meter regression model (Equation 3.10) was fitted:

$$Y = d + \exp [b (\log x - \log e)]$$

In equation 3.7, Y is the response variable *i.e.*, relative dry weight, x is the applied 2,4-D dose, d is the upper limit, b is the relative slope around e and e is GR_{50} *i.e.*, amount of 2,4-D required to reduce above-ground dry weight by 50%. Using the function '*compParm*' in '*drc*' package, estimated GR_{50} of Palmer amaranth populations were further tested for difference *via* t-test (Shyam et al. 2019). The in-built '*plot*' function in the '*drc*' package was used to obtain the dose-response curves.

Results

Absorption and translocation of [^{14}C] 2,4-D in KCTR, KSS, and MSS Palmer amaranth

A rectangular hyperbola was utilized to analyze percent [^{14}C] 2,4-D absorption and translocation at 6, 24, 48, and 72 HAT in the three Palmer amaranth populations, i.e., KCTR, KSS, and MSS. Overall, in all the populations, a maximum of ≥89% [^{14}C] 2,4-D was found to be absorbed (A_{max}; Figure 3.3, Table 3.1). There was no statistical difference in the model that estimated A_{max} (maximum percent absorption) of KCTR, KSS, and MSS (Table 3.1, 3.3). The time required to achieve A_{max} *i.e.*, A_{90} in the three populations varied from 5-9 h (Figure 3.3, Table 3.1). Even though MSS took a longer time to achieve maximum absorption of [^{14}C] 2,4-D

compared to KCTR and KSS, the t-test analysis showed no statistical difference in estimated A_{90} of KCTR, KSS, and MSS (Table 3.3).

Table 3.1 Parameters describing percent absorption of [^{14}C] 2,4-D in KSS, MSS, and KCTR Palmer amaranth plants calculated (Equation 3.8). A_{max} refers to the maximum limit of [^{14}C] 2,4-D absorption over time t in each population and A_{90} refers to the amount of time required for that maximum absorption to occur. Data combined over two runs. SE is the model estimated standard of error.

Population	A_{max} (SE)	A_{90} (SE)
KSS	90.41 (1.81)	5.21 (2.34)
MSS	89.27 (1.84)	8.78 (2.69)
KCTR	88.77 (1.79)	5.70 (2.42)

Analysis of percent translocation showed that KSS, MSS, and KCTR translocated a maximum (T_{max}) of 93.9, 94.2, and 84.6% [^{14}C] 2,4-D, respectively (Table 3.2). Hence, there was ~10% higher translocation of [^{14}C] 2,4-D in KSS and MSS compared to KCTR which was statistically significant (Table 3.2, 3.3). Overall, >84% of absorbed [^{14}C] 2,4-D was translocated in all three populations (Figure 3.3, Table 3.2). Time required to translocate the maximum amount of [^{14}C] 2,4-D (T_{90}) in KCTR was much higher (~89 h) than KSS and MSS (~30 h), indicating slower translocation of 2,4-D compared to susceptible plants (Figure 3.3, Table 3.2). The difference in T_{90} between KCTR and KSS was significant at α=0.1, while KCTR and MSS was significant at α=0.05 level (Table 3.3). For all populations, a higher quantity of [^{14}C] 2,4-D was translocated towards tissue below the treated leaf compared to tissue above treated leaf (Figure S 3.1). No other population-specific pattern was observed in [^{14}C] 2,4-D movement in tissue above or below treated leaf (Figure S 3.1).

Table 3.2 Parameters describing percent translocation of [^{14}C] 2,4-D in KSS (susceptible), MSS (susceptible), and KCTR (resistant) Palmer amaranth populations calculated (Equation 3.9). T_{max} refers to the maximum limit of [^{14}C] 2,4-D translocation over time t in each population and T_{90} refers to the amount of time required for that maximum translocation to occur. Data combined over two runs. SE is the model estimated standard of error.

Population	T_{max} (SE)	T_{90} (SE)
KSS	93.91 (4.67)	31.87 (11.12)
MSS	94.21 (4.18)	30.11 (8.67)
KCTR	84.63 (6.39)	89.17 (26.44)

Table 3.3 Comparison of parameters estimating [^{14}C] 2,4-D (a) absorption and (b) translocation in KSS (susceptible), MSS (susceptible) and KCTR (resistant) Palmer amaranth plants (using equation 3.8, 3.9) when grown under growth chamber conditions. T-test was performed to compare parameters of each population. Data combined over two runs. *P-value <0.1, **P-value <0.05, ***P-value <0.01, ^{NS}p-value= non-significant indicates the level of significance of difference in means).

Parameters	Population comparisons (p-value)		
	KSS vs. MSS	KCTR vs. MSS	KSS vs. KCTR
A_{max}	0.6596^{NS}	0.8479^{NS}	0.5526^{NS}
A_{90}	0.3188^{NS}	0.3968^{NS}	0.8835^{NS}
T_{max}	0.3049^{NS}	0.2153^{NS}	0.2465^{NS}
T_{90}	0.9013^{NS}	0.0389**	0.0514*

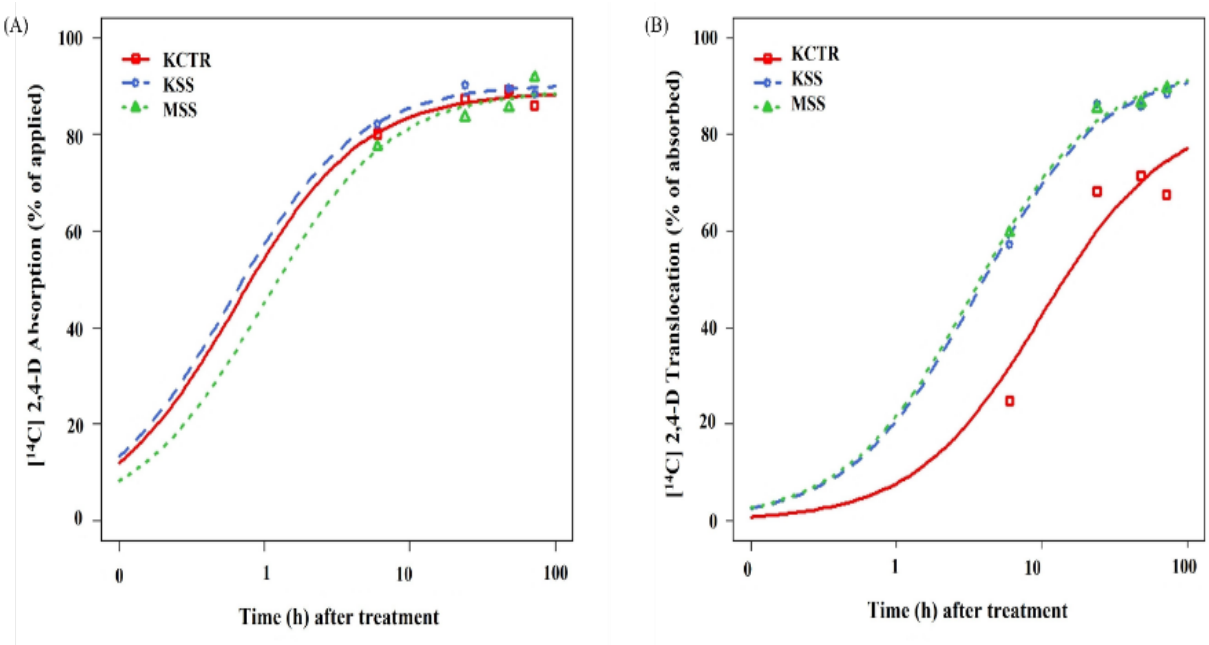

Figure 3.3 [^{14}C] 2,4-D (A) absorption and (B) translocation in KSS (susceptible), MSS (susceptible) and KCTR (resistant) plants grown under growth chamber conditions as determined using equation 3.4.

Metabolism of [^{14}C] 2,4-D in KCTR, KSS, MSS Palmer amaranth, and wheat

To investigate if enhanced metabolism of 2,4-D confers resistance in KCTR, the rate of [^{14}C] 2,4-D metabolism over time was assessed in KSS, MSS, and KCTR seedlings. The extracts of [^{14}C] 2,4-D treated KSS, MSS, and KCTR seedlings were run through reverse-phase HPLC to resolve parent [^{14}C] 2,4-D and its polar metabolites. Wheat seedlings were used as a positive control because of their natural ability to metabolize 2,4-D. In the chromatographs, a single peak with a retention time (RT) of the parent [^{14}C] 2,4-D was found at ~10.5 min. Apart from parent [^{14}C] 2,4-D, three major polar metabolites were identified from these samples *i.e.*, metabolite 1 (M1, RT= ~5.5 min), metabolite 2 (M2, RT= ~6.3 min), and metabolite 3 (M3, RT= ~7.9 min) respectively (Figure 3.5). As expected, wheat seedlings rapidly metabolized 2,4-D with ~70% of

[^{14}C] 2,4-D degraded by 24 HAT (Figure 3.5D). Based on RT, chromatographs of wheat samples at 24 HAT showed the presence of the same polar [^{14}C] 2,4-D metabolites as seen in KCTR (Figure 3.4). There was no difference in the amount of [^{14}C] 2,4-D metabolized in KSS or MSS, and KCTR at 6 HAT (Figure 3.6A). However, at 24, 48, and 72 HAT there was ~20-30% significantly higher parent [^{14}C] 2,4-D in KSS and MSS compared to KCTR (Figure 3.6B, C, D).

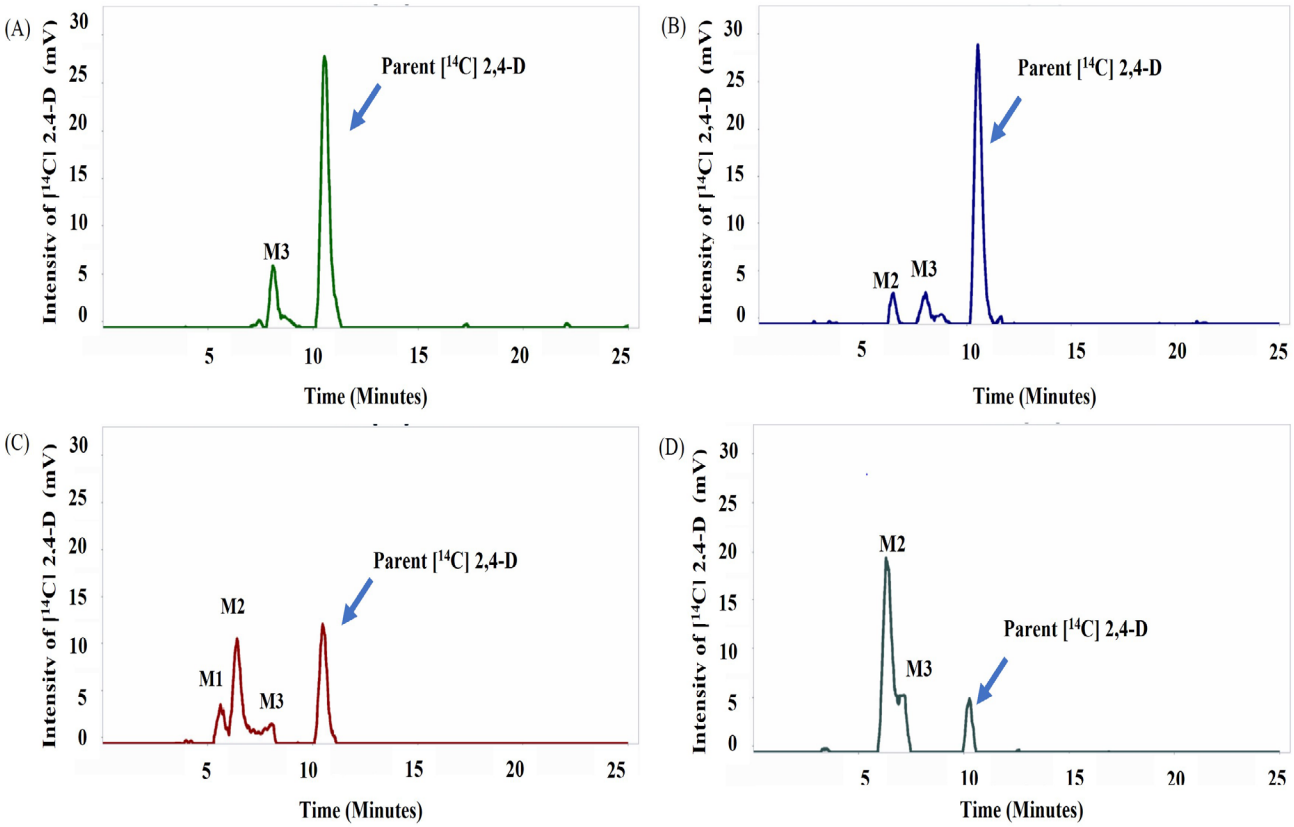

Figure 3.4 HPLC chromatographs depicting resolved [^{14}C] 2,4-D parent compound and its metabolites at 24 h after treatment (HAT) in (A) KSS (susceptible), (B) MSS (susceptible), (C) KCTR (resistant), and (D) wheat (naturally tolerant) seedlings, grown under growth chamber conditions.

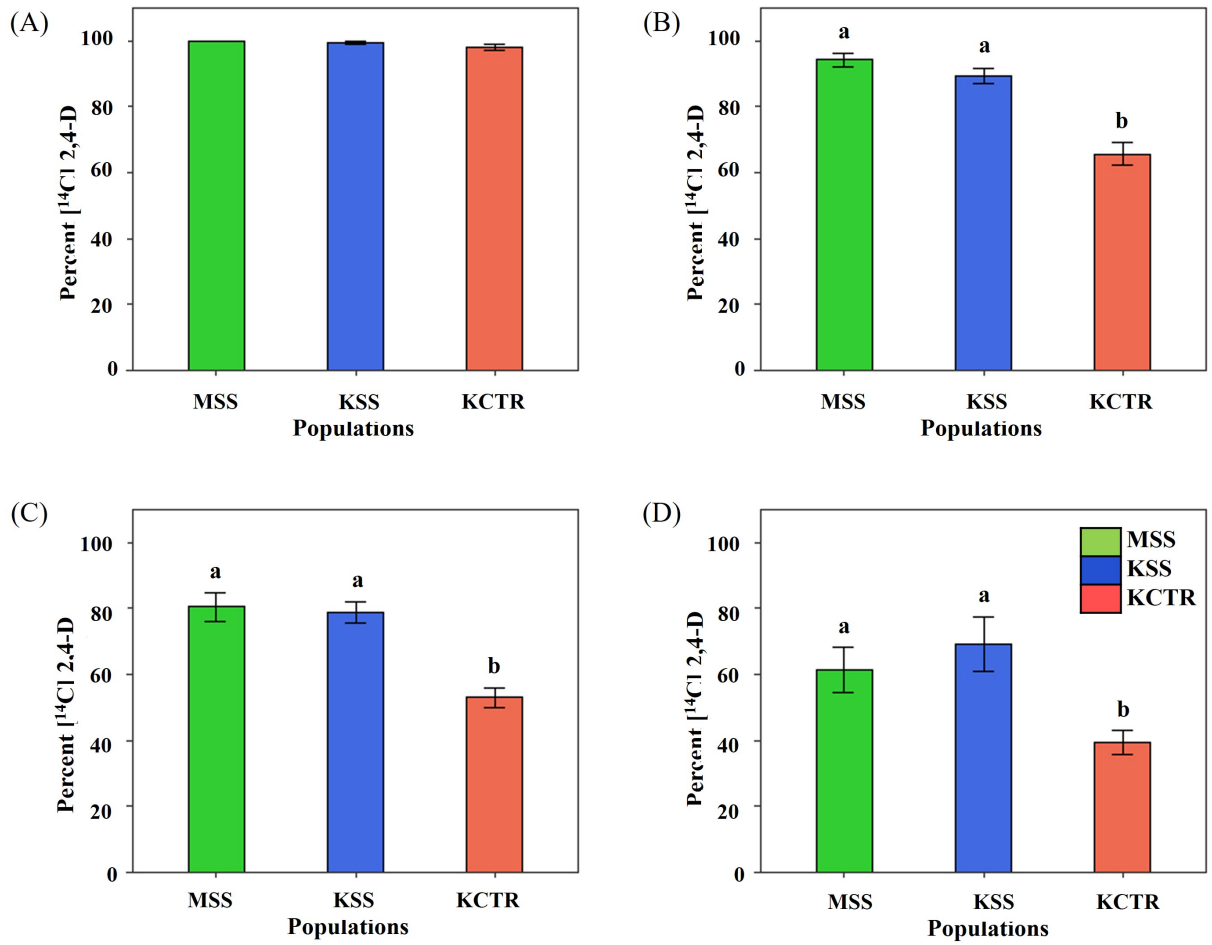

Figure 3.5 Percent of [^{14}C] 2,4-D in KSS (susceptible), MSS (susceptible), and KCTR (resistant) seedlings at (A) 6, (B) 24, (C) 48, and (D) 72 h after treatment (HAT) grown under growth chamber conditions. Error bars represent standard error of mean.

Effect of P450 inhibitors on KSS, and KCTR Palmer amaranth

For testing the involvement of P450s in mediating 2,4-D metabolism in KCTR Palmer amaranth, dose-response analyses were conducted with two known P450 inhibitors (malathion and PBO). The effect of malathion on the level of 2,4-D resistance in KSS and KCTR was assessed through '*drc*' plot (Figure 3.7). In the absence of malathion, the GR_{50} (the amount of 2,4-D required to cause 50% dry weight reduction) of KSS and KCTR were 187 and 1572 g ae

ha^{-1}, respectively (Figure 3.7, Table 3.4). However, when pre-treated with malathion followed by 2,4-D, the GR_{50} of KSS and KCTR were 197 and 627 g ae ha^{-1} respectively (Figure 3.7, Table 3.4). Thus, the addition of malathion resulted in a 60% reduction of GR_{50} in KCTR but failed to impact the response of KSS Palmer amaranth to 2,4-D application (Figure 3.7, Table 3.4). T-test analysis through '*comParm*' function in R program showed that there was a significant difference in estimated GR_{50} of KCTR with the application of malathion prior to 2,4-D treatment, while such difference was not present in the case of KSS (Figure 3.7, Table 3.4).

Table 3.4 Regression parameters (Equation 3.4) describing the response of KSS (susceptible), and KCTR (resistant) Palmer amaranth under growth chamber conditions, to 2, 4-D application with or without pre-treatment with malathion (cytochrome P450 inhibitor) (b: relative slope, d: upper limit; e: GR_{50} or dose required for 50% dry weight reduction in terms of g ae ha^{-1}; SE: standard error). Data combined over two runs. *P-value <0.1, **P-value <0.05, ***P-value <0.01, ^{NS}p-value= non-significant indicates the level of significance of difference means of GR_{50}).

Population	Treatment	b SE)	d (SE)	e (SE)	Reduction (%)	p-value
KSS	2,4-D	1.18 (0.26)	99.96 (4.49)	187.39 (41.77)	-11	0.8705^{NS}
	Malathion followed by 2,4-D	1.65 (0.55)	97.92 (4.82)	197.39 (44.80)		
KCTR	2,4-D	0.89 (0.23)	100.44 (5.99)	1572.47 (370.50)	60	0.0129**
	Malathion followed by 2,4-D	1.25 (0.18)	98.14 (4.36)	626.56 (74.67)		

Table 3.5 Regression parameters (Equation 3.4) describing the response of KSS (susceptible), and KCTR (resistant) Palmer amaranth to 2, 4-D with or without pre-treatment with piperonyl butoxide (PBO; cytochrome P450 inhibitor) under growth chamber conditions (b: relative slope, d: upper limit; e: GR_{50} or dose required for 50% dry weight reduction in terms of g ae ha^{-1}; SE: standard error). Data combined over two runs. *P-value <0.1, **P-value <0.05, ***P-value <0.01, ^{NS}p-value= non-significant indicates the level of significance of difference in means of GR_{50}).

Population	Treatment	b (SE)	d (SE)	e (SE)	Reduction (%)	p-value
KSS	2,4-D	1.68 (0.41)	100.06 (4.81)	257.25 (37.61)	-27	0.3216^{NS}
	PBO followed by 2,4-D	1.49 (0.39)	99.99 (4.99)	202.22 (40.71)		
KCTR	2,4-D	0.68 (0.19)	99.99 (4.64)	2244.12 (630.25)	16	0.7498^{NS}
	PBO followed by 2,4-D	0.67 (0.17)	100.08 (4.61)	2600.49 (921.62)		

The effect of PBO pre-treatment on dry weight accumulation of 2,4-D treated KSS and KCTR plants was also plotted (Figure 3.7). Unlike the treatment with malathion, the application of PBO did not have any effect on the level of 2,4-D resistance in KCTR (Figure 3.8, Table 3.4). The GR_{50} of KSS and KCTR after 2,4-D application were estimated as 257, and 2244 g ae ha^{-1}, respectively (Figure 3.8, Table 3.4). Upon pre-treatment with PBO, followed by 2,4-D application, the GR_{50} of KSS and KCTR were estimated at 202, and 2600 g ae ha^{-1}, respectively

(Figure 3.8, Table 3.4). Similar to malathion treatment, the addition of PBO did not significantly impact KSS response to 2,4-D treatment (Figure 3.8, Table 3.4). Overall, with the addition of PBO, there was only 16% reduction in GR_{50} of KCTR; however, it was not statistically significant compared to only 2,4-D treated plants (Figure 3.8, Table 3.4).

Discussion

In the US, Palmer amaranth is ranked as one of the top-most troublesome weeds by makes it even more challenging for its management WSSA (Van Wychen 2020). SAHs like 2,4-D have played a key role in managing herbicide-resistant Palmer amaranth populations across the US. However, in 2015 the first case of 2,4-D resistance in Palmer amaranth was documented in Kansas (Kumar et al. 2019b).

In this research, for the first time, enhanced metabolism of 2,4-D was found to confer 2,4-D resistance in KCTR Palmer amaranth. Besides 2,4-D, the evolution of resistance to dicamba has also been recently documented in a Palmer amaranth population from Tennessee (Heap 2021). Previously, several Palmer amaranth populations were found to have evolved metabolic resistance to ALS- (Nakka et al. 2017c, Shyam et al. 2021a), PS II- (Chahal et al. 2019b, Chaudhari et al. 2020, Nakka et al. 2017a, Shyam et al. 2021a), HPPD- (Küpper et al. 2018, Nakka et al. 2017b, Shyam et al. 2021a), and PPO- inhibitors (Shyam et al. 2021a, Varanasi et al. 2019). To understand the physiological basis of 2,4-D resistance in KCTR Palmer amaranth, first, the percent [^{14}C] 2,4-D absorption, and translocation after treatment was determined. Overall, ≥89% of [^{14}C] 2,4-D was absorbed in KSS, MSS, and KCTR. Similarly, high 2,4-D absorption has been reported in 2,4-D-resistant common waterhemp, a close relative of Palmer amaranth (Shergill et al. 2018b).

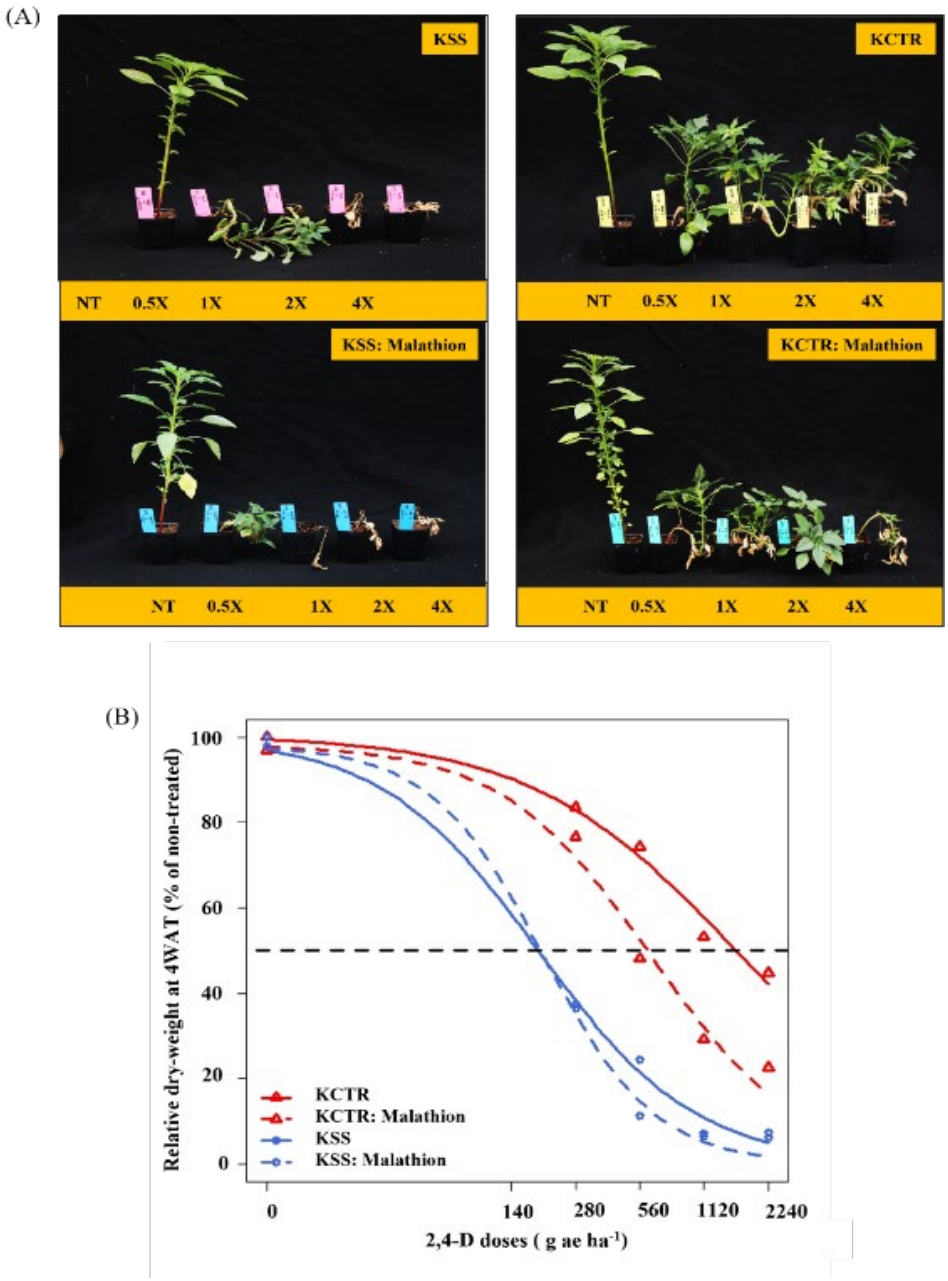

Figure 3.6 (A) Response of KSS (susceptible) and KCTR (resistant) with or without malathion (cytochrome P450 inhibitor) treatment to varying doses of 2,4-D ranging from non-treated (NT) to 4X, where 1X is the field recommended dose of 560 g ae ha^{-1} at 4 weeks after treatment (WAT). (B) Dose-response curves representing relative dry weight (% of non-treated) of KSS, and KCTR Palmer amaranth using the three-parameter log-logistic regression model (Equation 3.4) at 4 WAT. Dotted arrow at the center of the plot represents the 50% of relative dry weight.

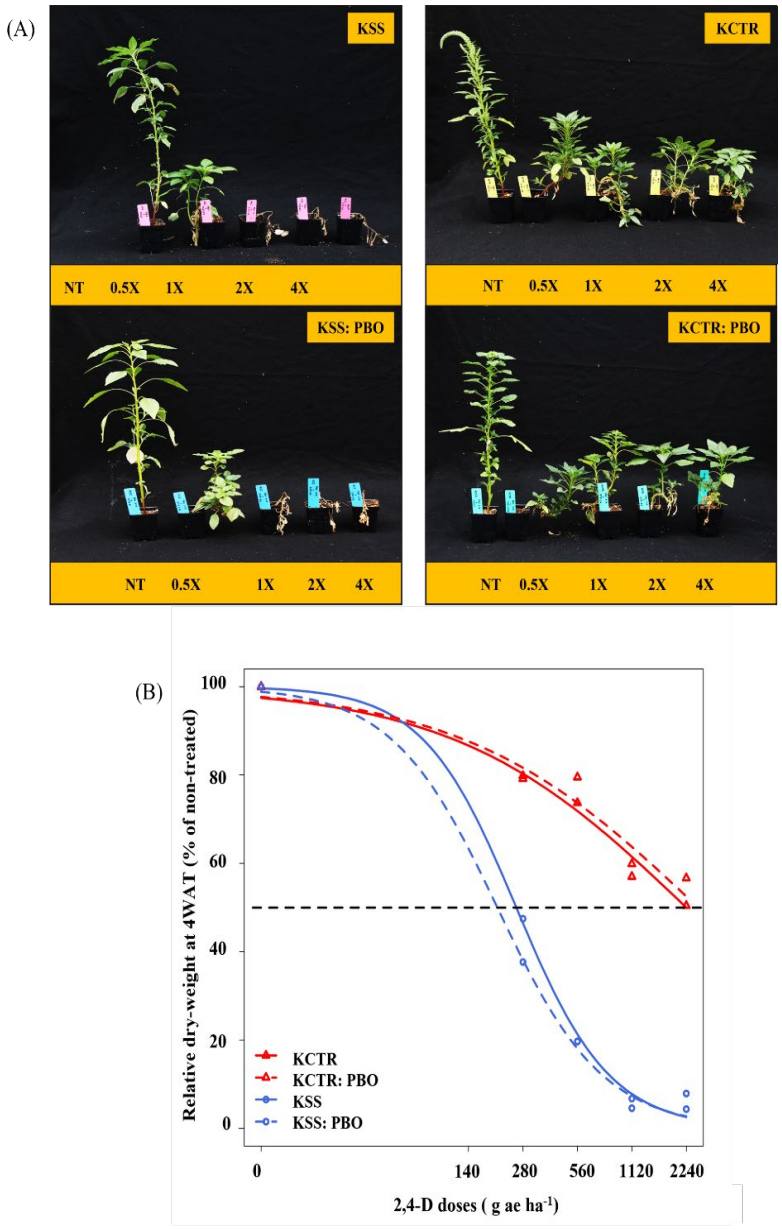

Figure 3.7 (A) Response of KSS (susceptible) and KCTR (resistant) with or without piperonyl butoxide (PBO; cytochrome P450 inhibitor) treatment to varying doses of 2,4-D ranging from non-treated (NT) to 4X, where 1X is the field recommended dose of 560 g ae ha^{-1} at 4 weeks after treatment. (B) Dose-response curves representing relative dry weight (% of non-treated) of KSS, and KCTR Palmer amaranth using the three-parameter log-logistic regression model (Equation 3.4) at 4 WAT. Dotted arrow at the center of the plot represents the 50% of relative dry weight.

Our results demonstrated that there is no significant difference in [^{14}C] 2,4-D absorption between resistant KCTR and the two susceptible populations, KSS, and MSS. This indicates that differential 2,4-D absorption is not contributing to 2,4-D resistance in KCTR Palmer amaranth. However, ~10% less and ~3 times slower [^{14}C] 2,4-D was found to translocate in KCTR compared to KSS and MSS plants (Figure 3.3, Table 3.2).

Considering that the three populations absorbed ≥89% of [^{14}C] 2,4-D applied and ≥84% of that was translocated, this difference in 2,4-D translocation is unlikely to significantly contribute to the resistance. Previously, no difference in [^{14}C] 2,4-D absorption or translocation was reported in 2,4-D-resistant common waterhemp populations from Nebraska and Missouri (Figueiredo et al. 2018, Shergill et al. 2018b). Nonetheless, differential 2,4-D uptake and/or translocation was reported to impart 2,4-D resistance in several weeds such as corn poppy, smooth pigweed, Parthenium (*Parthenium hysterophorus* L.), *Hirschfeldia incana* (L.), and wild radish (Goggin et al. 2016, Rey-Caballero et al. 2016). Influx (AUX1/LAX1) and efflux (PIN/ABCB) auxin transporters play a key role in directional transport of auxins (Cho and Cho 2013, Fischer et al. 1998, Petrášek and Friml 2009). Since SAHs mimic several physiological processes of natural plant hormones, therefore, alteration of auxin transporters can lead to decreased SAH transport resulting in reduced translocation of SAH. However, no field evolved resistance to SAH caused by alterations in the auxin transporters was reported. Interestingly, application of auxin transport inhibitors such as 2,3,5-triiodobenzoic acid and 1-N-naphthylphthalamic acid in 2,4-D- susceptible wild radish from Australia showed a reduced 2,4-D translocation mirroring the 2,4-D-resistant wild radish (Goggin et al. 2016).

Next, the metabolism of [^{14}C] 2,4-D in KSS, MSS, KCTR Palmer amaranth, and wheat (naturally tolerant) plants was determined *via* HPLC analysis. Our results confirmed that more

2,4-D is metabolized in KCTR Palmer amaranth compared to KSS and MSS populations. Even though there was no difference in the amount of parent [^{14}C] 2,4-D retained at 6 HAT in the three populations, overall, KCTR Palmer amaranth metabolized ~20-30% greater [^{14}C] 2,4-D at 24, 48, and 72 HAT compared to susceptible populations (Figure 3.6). Enhanced metabolism has been shown to impart 2,4-D resistance in common waterhemp populations in the US Midwest (Figueiredo et al. 2018, Shergill et al. 2018b). Also, metabolism of 2,4-D has been documented in corn poppy populations from France and smooth pigweed populations from Argentina (Dellaferrera et al. 2018, Rey-Caballero et al. 2016, Torra et al. 2017). Although limited information is available regarding how metabolites of 2,4-D translocate in resistant plants, reduced translocation (~10%) of 2,4-D in KCTR plants compared to KSS or MSS may be due to metabolites of 2,4-D, rather than the parental compound. This possibility needs to be investigated and confirmed.

The peak RT of polar metabolites of [^{14}C] 2,4-D in KCTR Palmer amaranth was compared with wheat (variety KS Western Star) at 24 HAT (Figure 3.5). No unique metabolites were found in wheat but not in KCTR Palmer amaranth (Figure 3.5). This indicates that possibly, KCTR Palmer amaranth can metabolize 2,4-D with the same biochemical process as wheat. 2,4-D metabolites in wheat (and other monocots) are usually considered to be stable sugar conjugates and non-toxic. On the other hand, amino acid conjugates are considered to be less stable than sugar conjugates and can reverse to an active form of the parent herbicide compound (2,4-D) which will be toxic (Sterling and Hall 1997). Comparison of 2,4-D metabolic pathways between tolerant and susceptible plants has been explored for a long time. For example, Hamburg et al. (2001) reported that both wheat and potato (*Solanum tuberosum* L.) metabolize 2,4-D *via* ester hydrolysis. In contrast, Jablonkai (2015) reviewed that in wheat, 2,4-D is primarily metabolized

through aromatic ring hydroxylation of 2,4-dichloro-phenyl moiety and subsequently an acidic aryl glycoside is formed by conjugation. Fang and Butts (1954) reported differential metabolism of 2,4-D in soybean (*Glycine max* L.) compared to corn and wheat. Future investigations will be directed towards identifying metabolites of 2,4-D in KCTR through the application of LC/MS techniques. This will shed light on the potential biochemical pathways through which 2,4-D is getting metabolized in KCTR Palmer amaranth. NTSR mechanisms such as metabolic resistance can be affected by different environmental conditions. Shyam et al. (2019) has demonstrated that 2,4-D resistant common waterhemp from Nebraska metabolized 2,4-D at a faster rate when grown at a higher temperature. Such a temperature effect in altering the rate of metabolism of 2,4-D, thereby, resistance, in KCTR Palmer amaranth needs to be tested.

The role of P450s in mediating 2,4-D metabolism in KCTR was tested using P450-inhibitors. Interestingly, the pre-treatment with malathion significantly reduced GR_{50} of KCTR by 60% compared to 2,4-D applied alone (Figure 3.7, Table 3.4). However, treatment with malathion followed by 2,4-D failed to affect the KSS Palmer amaranth's response to this herbicide. This suggests the potential involvement of P450s in mediating metabolic resistance to 2,4-D in KCTR. Malathion is commonly used to test the involvement of P450s in metabolic resistance to herbicides in weeds. P450 enzymes metabolize xenobiotics such as herbicides *via* oxygenation and NADPH-dependent monooxygenation reactions (Yuan et al. 2007). When organophosphate insecticides such as malathion react with P450s, they generate atomic sulfur and bind to P450 apoprotein, thereby inactivating it (Werk-Reichhart et al. 2000). Therefore, the application of malathion has been shown to inhibit P450-dependent metabolism and increase the metabolic half-life of herbicides (Kreuz and Fonné-Pfister 1992). Previously, the application of malathion has been shown to reverse metabolic resistance to 2,4-D in 2,4-D-resistant common

waterhemp (Figueiredo et al. 2018, Shergill et al. 2018b). In Chapter 2, it has been shown that malathion treatment reduced the level of mesotrione, 2,4-D, and lactofen resistance in KCTR (Figure 2.3). Similarly, in weeds with metabolic resistance to other herbicides such as mesotrione, lactofen, chlorsulfuron, *etc.* malathion is known to reverse the resistance. However, such impact on 2,4-D resistance in KCTR was not seen with PBO application as the GR_{50} of either KCTR or KSS with this treatment, was not significantly altered (Figure 3.8; Table 3.5). This indicates that P450s that are potentially not inhibited by PBO are involved in mediating 2,4-D resistance in KCTR. PBO is a known substrate, inhibitor, and inducer of P450 (Hodgson and Levi 1999), which is often used as a diagnostic tool to understand P450 based metabolism of pesticides. PBO hydroxylation *via* P450 generates a carbene molecule that forms a complex with the heme group of the P450 enzyme and causes inhibition (Hodgson and Levi 1999). The application of PBO has been shown to decrease the level of metabolic resistance to herbicides in other weeds, such as metribuzin resistance in rigid ryegrass (*Lolium rigidum* Gaudin) (Ma et al. 2020). Nonetheless, the selectivity of P450 inhibitors has been recorded in several metabolic-resistant weed populations. For instance, in chlorsulfuron-resistant rigid ryegrass, malathion treatment increased toxicity of chlorsulfuron but not with tetracylis (another P450 inhibitor) application (Christopher et al. 1992). Similarly, treatment of PBO reduced chlorotoluron and simazine resistance in rigid ryegrass; however, application of malathion did not affect resistance (Preston et al. 1996). Overall, this research strongly suggests that P450s are involved in metabolizing 2,4-D in KCTR Palmer amaranth. Dimaano and Iwakami (2021) have suggested that P450 mediated herbicide metabolism can be attributed to single nucleotide polymorphism, increased copy number, or even changes in gene regulation. However, despite the known involvement of P450s, it has been still a challenge to identify and isolate specific P450s that are

involved in herbicide metabolism. Recently, a P450, *CYP81A10v7* was confirmed to mediate metabolic resistance to ACCase-, ALS-, PS II-, HPPD-, and VLCFA-inhibitors in rigid ryegrass (Han et al. 2021). Another P450 cluster (*P450 81E8*) was found to be over-expressed in two different 2,4-D resistant common waterhemp populations from Nebraska and Illinois (Giacomini et al. 2020). Therefore, it will be important to test the expression of these P450 genes in KCTR before or after 2,4-D application.

Conclusion

In summary, the results of this Chapter provide strong evidence that metabolism of 2,4-D mediated by P450 activity bestows 2,4-D resistance in KCTR Palmer amaranth. Pre-treatment with P450-inhibitor, malathion prior to 2,4-D application, resulted in reversal of 2,4-D resistance and increase toxicity to 2,4-D in KCTR Palmer amaranth. However, no such response was found when another P450-inhibitor, PBO was found. This indicates that certain P450 enzymes that were inhibited by malathion probably facilitate 2,4-D metabolism in KCTR Palmer amaranth. The possible presence of TSR mechanisms leading to 2,4-D resistance in KCTR Palmer amaranth is yet to be deciphered. Metabolic herbicide resistance is currently one of the most complex challenges affecting the sustainability of crop production. Especially, the ability of metabolic resistance to confer cross-resistance to multiple herbicide MOAs as seen in the KCTR Palmer amaranth population is challenging. The dire lack of novel MOAs along with the widespread prevalence of metabolic resistance can reduce growers' option for weed control. Therefore, the implementation of integrated weed management techniques will be necessary for reducing dependability on herbicides for weed management.

Chapter 4 - Genetic Basis of 2,4-D resistance in Palmer amaranth

Abstract

A Palmer amaranth (*Amaranthus palmeri* S. Watson) population (KCTR: Kansas Conservation Tillage Resistant) in Kansas identified in a long-term conservation tillage field study was documented to have evolved metabolic resistance to at least five modes of action of herbicides, including 2,4-D, a synthetic auxinic herbicide. The KCTR Palmer amaranth rapidly metabolizes 2,4-D compared to two susceptible Palmer amaranth populations (KSS: Kansas Susceptible and MSS: Mississippi Susceptible) (Chapter 3). In this chapter, the genetic basis of 2,4-D resistance in KCTR was investigated. Direct and reciprocal crosses were performed using 2,4-D-resistant KCTR and susceptible KSS plants to generate three F_1 families, *i.e.*, F_1-1, F_1-2, and F_1-3. 2,4-D dose-response assays were conducted to evaluate the response of progenies from each F_1 family along with parental resistant (KCTR) and susceptible (KSS) plants in controlled environmental growth chambers. Plants were treated with varying doses of 2,4-D ranging from non-treated to 4X (where 1X is the field recommended dose of 2,4-D which is 560 g ae ha^{-1}) and was repeated once. Above-ground shoots were harvested at 4 weeks after treatment (WAT) and dry weight was measured. Additionally, 2,4-D-resistant male and female plants from each of the F_1 families were crossed to generate pseudo-F_2 families. In total, four F_2 families were generated *i.e.*, F_2-1, F_2-2, F_2-3-1, and F_2-3-2. Segregation (resistance or susceptibility) of progenies from the F_2 families in response to the field recommended dose of 2,4-D was evaluated. Analysis of dry weight data from the dose-response experiments of F_1 progenies derived from either direct or reciprocal crosses suggested that the F_1 progenies had an intermediate response to 2,4-D treatment relative to resistant and susceptible parents. Such response indicates that the 2,4-D resistance in KCTR is an incompletely dominant nuclear trait. Chi-square analyses of F_2

segregation data did not fit the Mendelian single gene segregation model (*i.e.,* 3:1 resistance:susceptibility), implying that 2,4-D resistance in KCTR is controlled by multiple gene(s). Moreover, significant variation in phenotypes among F_2 progenies treated with 2,4-D also corroborated the polygenic nature of 2,4-D resistance trait. Chapter 3 of this dissertation confirmed that 2,4-D resistance in KCTR is due to enhanced metabolism possibly mediated by cytochrome P450 enzyme activity. It is highly likely that either multiple P450 genes or P450 along with other genes may be governing the 2,4-D resistance in KCTR. Future efforts will be directed towards identifying the genes involved in mediating 2,4-D resistance in KCTR Palmer amaranth.

Introduction

The introduction of herbicides for crop production in the 1940s revolutionized global agriculture. However, over-reliance on herbicides has resulted in the evolution of herbicide resistance in many agriculturally important weeds. There are currently 521 unique cases of herbicide resistance across 263 weed species worldwide, including 152 dicots and 111 monocots (Heap 2021). A critical aspect of the evolution of herbicide resistance in weeds is understanding how the resistance is inherited and spread (Preston and Mallory-Smith 2001). Genetic factors, such as the degree of dominance of the trait and the number of alleles controlling the trait can impact how the trait will spread, which in turn will influence the weed management strategies (Maxwell et al. 1990). For instance, resistance conferred by dominant gene(s) is expressed in both homozygous and heterozygous states, and, therefore, can spread fast. In contrast, recessive traits can only express in a homozygous state and are slower to spread than dominant trait(s). If herbicide resistance is conferred by a single gene, it can spread faster compared to polygenic resistance traits, which require multiple recombination events during meiosis to accumulate multiple alleles (unless they inherit together). Knowledge of the inheritance of herbicide resistance in weeds can also help understand the evolution of resistance under various types of selection pressure (Menalled et al. 2016).

Herbicide resistance mechanisms in weeds/crops are broadly categorized into two types, *i.e.*, target-site (TSR) and non-target site (NTSR) based, which can influence the inheritance pattern of the trait. TSR mechanisms that involve modifications to herbicide target usually follow the Mendelian single gene inheritance model. However, TSR mechanism involving amplification of herbicide target gene, as reported for glyphosate resistance (*e.g.*, Palmer amaranth), does not follow such a pattern (Jugulam 2021, Koo et al. 2018). In contrast, the NTSR mechanisms do not

affect the herbicide target; instead, these alter the physiological processes involved in herbicide action (Jugulam and Shyam 2019). Metabolism of herbicides, a common NTSR mechanism, is reported to be conferred primarily by multiple genes in several weeds; albeit in rare cases by a single gene as well (Busi and Powles 2017, Kohlhase et al. 2018, Pandian et al. 2021, Shyam et al. 2021b).

2,4-dichlorophenoxyacetic acid (2,4-D) is a synthetic auxinic herbicide (SAH) commonly used for controlling dicot weeds in cereal crops such as corn (*Zea mays* L.), wheat (*Triticum aestivum* L.), and sorghum (*Sorghum bicolor* L.). Despite being used in agriculture for more than seven decades, the evolution of resistance to 2,4-D has been relatively slow. However, in the past decade, over-reliance on SAHs, like 2,4-D for controlling acetolactate synthase (ALS)- and 5-enol pyruvyl shikimate phosphate synthase (EPSPS)-inhibitor-resistant weeds, has led to an increase in weed resistance to SAHs. As of 2021, globally, 42 weeds are resistant to SAHs, and amongst them, 25 weeds have evolved resistance to 2,4-D (Heap 2021). This limits options for managing dicot weeds in cereal cropping systems. Moreover, the adoption of recently commercialized 2,4-D- tolerant crop technology poses the risk of increased 2,4-D selection pressure, which can ultimately lead to the evolution of 2,4-D resistance in several weeds. Therefore, a thorough understanding of 2,4-D resistance mechanisms can help develop tactics for minimizing the spread and further evolution of 2,4-D resistance.

Inheritance of 2,4-D resistance has been studied in several economically important weeds around the globe, such as common waterhemp, prickly lettuce (*Lactuca serriola* L.), wild radish (*Raphanus raphanistrum* L.), wild mustard (*Sinapis arvensis*), and oriental mustard (*Sisymbrium orientale* L.) (Busi and Powles 2017, Jugulam et al. 2005, Preston and Malone 2015, Riar et al. 2011, Sabaté et al. 2016). So far, the inheritance pattern of resistance to SAHs such as 2,4-D has

varied depending on the resistance mechanism and the weed species involved. For instance, 2,4-D resistance in prickly lettuce was governed by a single co-dominant allele (Riar et al. 2011). Similarly, in wild mustard (*Brassica kaber* L.) a single gene was found to mediate 2,4-D resistance (Jugulam et al. 2005). However, polygenic inheritance was documented for 2,4-D resistance in common waterhemp and 2-methyl-4-chlorophenoxyacetic acid (MCPA) resistance in hemp-nettle (*Galeopsis tetrahit* L.) (Sabaté et al. 2016, Weinberg et al. 2006).

Palmer amaranth (*Amaranthus palmeri* S. Watson), a native of Mexico and the southern US, is one of the most troublesome weeds in the US (Van Wychen 2020). So far, Palmer amaranth has evolved resistance to 8 herbicide modes of action (MOAs) (Heap 2021). The reproductive biology of Palmer amaranth plays a crucial role in its ability to evolve resistance to herbicides. For instance, Palmer amaranth is a dioecious plant, making cross-pollination a necessity to produce seeds, which also creates the potential of the flow of herbicide-resistant genes *via* both intraspecific and interspecific hybridization (Gaines et al. 2012, Sosnoskie et al. 2012). Female Palmer amaranth plants are also characterized by high fecundity resulting in the production of abundant seeds per plant (Keeley et al. 1987, Webster and Grey 2015). These reproductive traits make Palmer amaranth a highly persistent weed and allow further spread of herbicide resistance genes.

We recently documented a population of Palmer amaranth (KCTR: Kansas Conservation Tillage resistant) from Kansas which has evolved resistance to six herbicides MOAs including SAH 2,4-D. Dose-response assays have confirmed that KCTR is 6- to11- fold resistant to 2,4-D compared to two susceptible Palmer amaranth populations (KSS: Kansas Susceptible and MSS: Mississippi Susceptible). Enhanced metabolism of 2,4-D was found to confer resistance in KCTR Palmer amaranth (Chapter 3). Further, application of cytochrome P450 (P450) inhibitor,

malathion, has increased efficacy of 2,4-D in KCTR plants, indicating the involvement of P450(s) in mediating 2,4-D metabolism (Chapter 3). Although resistance to 2,4-D in Palmer amaranth has been documented in two populations in Kansas (Heap 2021, Kumar et al. 2019b), the information on the genetic basis of 2,4-D resistance is lacking. This study was conducted to investigate the genetic basis of 2,4-D resistance in KCTR Palmer amaranth. The specific objectives were: i) generate F_1 progenies and evaluate their response to 2,4-D application to determine the dominant or recessive nature of resistance allele(s) in KCTR, and ii) generate F_2 progenies and assess their response to 2,4-D treatment to determine the number of allele(s) conferring the resistance in KCTR.

Materials and Methods

Plant Materials and Growth Conditions

2,4-D-resistant (KCTR) and susceptible (KSS) Palmer amaranth populations were used in this study. KCTR population was originally collected in 2018 from a long-term conservation tilled field with sorghum production in Riley County, KS. The KSS population is known to be susceptible to 2,4-D and was also collected from a nearby field in Riley County, Kansas. Experiments to generate F_1 and F_2 progenies and for characterizing their response to 2,4-D were conducted either in greenhouse or controlled environmental growth chambers. The following greenhouse conditions were maintained: 30/24 ºC (d/n) temperature and 16/8 h (d/n) photoperiod with natural light supplemented with 250 µmol m^{-2} s^{-1} illumination by sodium vapor lamps. Growth chambers were maintained at 32.5/22.5 ºC (d/n) with a photoperiod of 15/9 h (d/n) with 60±10% relative humidity and a light intensity of 750 µmol m^{-2} s^{-1} provided by incandescent and fluorescent lamps. Seeds of KCTR and KSS Palmer amaranth populations were sown in small

plastic trays (21 × 6 × 4 cm) with commercial potting mix (Pro-Mix® premium potting mix, Premier Tech Home and Garden Inc., Ontario, Canada) in the greenhouse and allowed to emerge. Palmer amaranth seedlings with 3-4 leaves were transplanted into small pots (6 × 6 × 6.5 cm) for herbicide treatment. Plants were watered as needed (procedure for herbicide treatment is given later).

Generation of F_1 and F_2 Families of KCTR Palmer amaranth

Ten-12 cm tall KCTR seedlings (n = 20) were treated with 2,4-D (2,4-D 4L Amine, Winfield Solutions, LLC., St. Paul, Minnesota, US) at 1120 g ae ha^{-1} and allowed to grow in the greenhouse. At 4 weeks after treatment (WAT) the survivors were identified and transferred to new pots (15 × 10 × 15 cm) to initiate flowering. Once flowered, healthy male and female KCTR plants were selected for performing direct and reciprocal crosses (Figure 4.1).

Direct crosses were performed by enclosing female (♀) KCTR (survivors of 2,4-D application) individually with male (♂) KSS (non-treated with 2,4-D) in a pollination bag (Figure 4.1). Reciprocal crosses were performed with female (♀) KSS plants (non-treated with 2,4-D) paired with male (♂) KCTR (survivors of 2,4-D application) plants (Figure 4.1). Before enclosing the plants, existing flower parts were removed to avoid any contamination and ensure crossing between selected plants. Once both plants flowered, male Palmer amaranth plants were gently shaken periodically to disperse pollen and pollinate the stigmas of female plants. At maturity, seeds were collected from each female plant separately, cleaned, and stored at 32 ºC in greenhouse to allow over-ripening of the seeds necessary for good germination. In total, seeds of three F_1 families were generated from these crosses, *i.e.*, F_1-1, F_1-2, and F_1-3 (Figure 4.1). Palmer amaranth is a dioecious plant therefore true-F_2 families cannot be generated by selfing. Hence, some plants from each F_1 family were further screened with 2,4-D (1120 g ae ha^{-1}) to

identify female and male F_1 plants resistant to 2,4-D to be used to generate pseudo-F_2 families (Figure 4.1). In total four F_2 families were generated upon crossing male and female F_1 survivors (F_2-1, F_2-2, F_2-3-1, F_2-3-2; Figure 4.1). These progenies raised from the F_1 and F_2 families were also used in further studies.

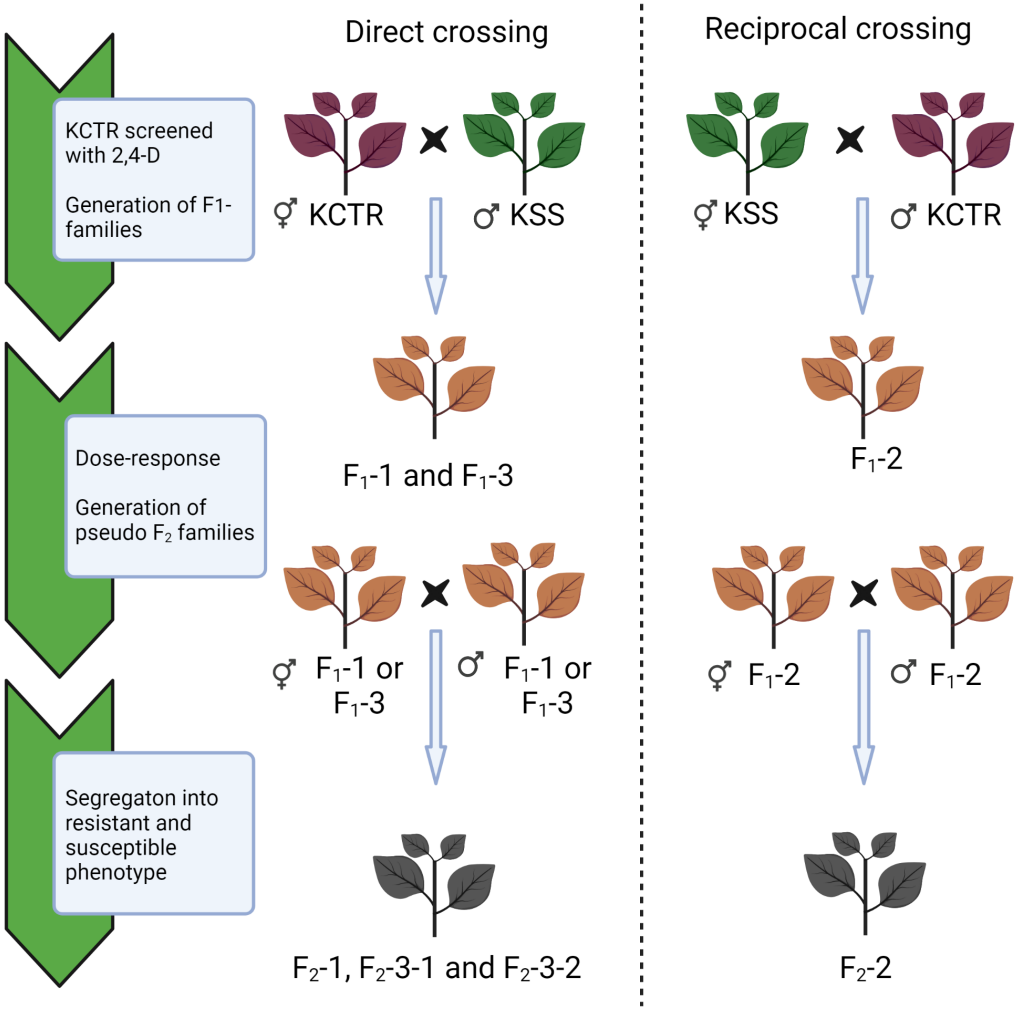

Figure 4.1 Schematic diagram showing the generation of F_1 and F_2 families of KCTR (resistant) and KSS (susceptible) Palmer amaranth and the experimental steps in the study [Created with BioRender.com].

2,4-D Dose-response study using KCTR, KSS, and F_1 Progenies

Whole-plant dose-response experiments were conducted with parental KCTR, KSS, and F_1 Palmer amaranth progenies generated from three F_1 families. The experiments were conducted in a completely randomized design in growth chambers. KCTR, KSS, and progenies from individual F_1 families were germinated in small trays and transplanted into small pots as described previously. One week after transplanting, the seedlings were transferred to growth chambers (maintained at the same conditions as described in 'Plant Materials and Growth Conditions'). Palmer amaranth seedlings (10-12 cm tall) were treated with 2,4-D at the following doses *i.e.,* 0 (non-treated), 140 (0.25X), 280 (0.5X), 560 (1X; field recommended dose), 1120 (2X; discriminatory dose under growth chamber conditions), and 2240 (4X) g ae ha^{-1}. After 30 minutes of herbicide treatment, plants were returned to the growth chamber. 2,4-D was applied using a bench-track sprayer (Generation III, DeVries Manufacturing, Hollandale, Minnesota, US) using a flat-fan nozzle tip (8002 Teejet, Spraying Systems Co., Wheaton, Illinois, US) and was calibrated to spray equivalent to 187 L ha^{-1} or 20 gallons per acre (GPA). At 4 WAT, plant survival (dead or alive) was assessed at each dose of 2,4-D treatment. Above-ground plant parts were harvested at 4 WAT and oven-dried at 65 ºC for at least 72 h. Dose-response experiments with each F_1 family were repeated at least once, and five replications were maintained for each dose of 2,4-D. Dry weight was measured and converted into relative dry weight (% of non-treated) as described below (Equation 4.1):

$$RDW\ (\%) = [(DW \times 100)/ADW]$$

In equation 4.1, RDW is relative dry weight (% of non-treated control), DW is dry weight of the sample and ADW is the average dry weight of non-treated control.

Evaluation of Segregation (2,4-D resistance or susceptibility) of F_2 Progenies

Seeds of F_2-families (F_2-1, F_2-2, F_2-3-1, F_2-3-2) were grown in the greenhouse as described above. Based on the response of parental populations, *i.e.,* KCTR and KSS in greenhouse conditions, the field recommended dose of 2,4-D (560 g ae ha^{-1}) was identified to discriminate resistant (alive) or susceptible (dead) plants. The same dose was used to determine the segregation of F_2 progenies into resistant or susceptible plants. Approximately 20-120 progenies (10-12 cm tall; Table 4.4) representing each F_2 family were treated with 560 g ae ha^{-1} of 2,4-D in each run. Around 20-30 plants of KSS Palmer amaranth were also treated (in each run) along with the F_2 progenies as a negative control. At 4 WAT, live or dead (resistance or susceptible) phenotype of the 2,4-D-treated plants was documented (Figure 4.1). The experiment was repeated. For the second run of the experiment, at 4 WAT, the above-ground dry weight of F_2 plants was harvested, dried, and measured (as described above).

Data Analysis

Relative dry weight data from two runs of 2,4-D dose-response experiments of each F_1 family were tested using Levene's test ($\alpha = 0.05$) in R (version 4.0.3, R Core Team, 2020) for homogeneity of variance. Since the output was non-significant, the relative dry weight data were pooled across two runs. Relative dry weight datasets for each F_1 family were analyzed using three-parameter log-logistic '*drc*' models in R (Knezevic et al. 2007, Ritz et al. 2015). The model had the following formula (Equation 4.2):

$$Y = d + \exp [b (\log x - \log e)]$$

where Y is the response variable, x is the herbicide dose, d is the upper limit, b is the relative slope around e and e is GR_{50} (amount of 2,4-D required to reduce plant dry weight by 50%). The GR_{50} values of different F_1 families and populations were further tested *via* t-test using the

function '*compParm*' in '*drc*' package to determine differences across the doses. In-built '*plot*' function in the '*drc*' package was used to visualize the dose-response curves. Resistance index (RI) was calculated as a ratio of GR_{50} of KCTR or F_1 families and GR_{50} of KSS population. Cumulative dry weight distribution of the F_1 progenies along with the parental populations (KSS and KCTR) at each dose of 2,4-D was illustrated (as density plot; Figure 4.5) using '*ggplot2*' package in R (Wickham 2016).

The number of F_2 progenies observed to have survived after 2,4-D treatment was compared with the expected number of survivors using chi-square analyses. The expected segregation of 2,4-D resistant (alive) or susceptible (dead) phenotypes was determined based on the assumption of the Mendelian single locus segregation model, which suggests that the 2,4-D resistance in KCTR is controlled by a single gene. The following equation (4.3) was used:

$$\text{Exp. } F_2 = (1 \times \text{Obs. KCTR} + 2 \times \text{Obs. } F_1 + 1 \times \text{Obs. KSS})$$

In this equation Exp. F_2 is the expected segregation frequency of the F_2 progenies and Obs. is the observed segregation frequency of KCTR, F_1, and KSS phenotypes. Based on previous experiments conducted in greenhouse conditions, the KCTR plants and F_1 progenies were found resistant to 560 g ae ha^{-1} of 2,4-D, while KSS plants were susceptible. This dose was used to document resistant or susceptible plants among F_2 progenies. Chi-square goodness of fit test ($\chi 2$) was used to analyze the data of the plant segregation as resistance or susceptibility to 2,4-D by comparing the observed segregation frequency with the expected. The null-hypothesis (H_0) for the test was that segregation of 2,4-D resistance in F_2 progenies will follow the 3:1 ratio (resistant: susceptible) and p-value from each F_2 family was compared with the significance level of $\alpha = 0.05$. The formula used for the test is (Equation 4.4):

$$\chi 2 = (E\text{-}O)^2 / E$$

where $\chi 2$ is the chi-square goodness of fit-value, O and E are the observed and expected segregation frequencies, respectively, of progenies in each F_2 family. H_0 was rejected if the obtained p-value was < 0.05. Dry weight data of the F_2 progenies were also visualized as density plot (described previously; Figure 4.6).

Results

2,4-D Dose-response with KCTR, KSS, and F_1 Progenies of Palmer amaranth

Response of progenies from F_1 families and parental populations KCTR and KSS Palmer amaranth to 2,4-D was assessed *via* dose-responses experiments. Averaged across dose-response experiments, 90% of KCTR and 15% of KSS plants survived the discriminatory dose of 2,4-D. The KSS plants that survived 2,4-D treatment, showed high levels of epinasty (downward curling of plant parts, a typical symptom of SAH injury) and stayed stunted, and did not show any new growth for up to 4 WAT. Nonetheless, the KCTR Palmer amaranth plants survived even the highest dose used in the study (2240 g ae ha^{-1}). Compared to that, 87% of F_1-1, 92% of F_1-2, and 89% of F_1-3 survived application of 2,4-D at 1120 g ae ha^{-1}. The lack of 100% survival of F_1 plants in response to 2,4-D (1120 g ae ha^{-1}) suggests that the parent KCTR plants used for crossing were heterozygous for 2,4-D resistance. The majority of progenies of F_1 plants in all the three families, generated by both direct or reciprocal crosses, were resistant to 2,4-D, implying that the resistance to 2,4-D in KCTR is a nuclear trait. In the F_1-1 dose-response experiments, the amount of 2,4-D required to cause a 50% reduction in growth (GR_{50}) of KCTR, F_1-1 and KSS were 1800, 837, and 194 g ae ha^{-1}, respectively (Table 4.1; Figure 4.2), suggesting that the KCTR and F_1-1 are 9- and 4-fold resistant to 2,4-D compared to KSS (Table 4.1). Similar results were obtained from F_1-2 and F_1-3 dose-response experiments (Table 4.2, 4.3; Figure 4.3, 4.4). The GR_{50} of KCTR, KSS, and F_1-2 plants were 1188, 501, and 200 g ae ha^{-1}, respectively (Table

4.2; Figure 4.3), indicating that the KCTR and F_1-2 plants were 6- and 3-fold resistant, respectively, to 2,4-D treatment, compared to KSS. Likewise, the GR_{50} of KCTR, KSS, and F_1-3 were 1317, 500, and 167 g ae ha^{-1}, respectively (Table 4.3; Figure 4.4), with RI of 7 and 3, compared to KSS (Table 4.3). Overall, the results of these experiments suggest an intermediate response of F_1 progenies to 2,4-D compared to KCTR and KSS plants. This implies that 2,4-D resistance in KCTR Palmer amaranth is an incompletely dominant trait.

Further, cumulative dry weight data from the F_1 dose-response assays were plotted for KSS, KCTR, and F_1 plants at each 2,4-D dose (Figure 4.5). A minimal difference was found in the spread and distribution of dry weight of KSS, KCTR, and the F_1 plants in those that were not treated with 2,4-D (NT; 0 g ae ha^{-1}) (Figure 4.5A). However, when treated with up to 2X dose of 2,4-D (1120 g ae ha^{-1}), F_1 and KCTR plants had similar distribution (Figure 4.5B, C, D, E). At a high dose of 2,4-D (4X; 2240 g ae ha^{-1}), KCTR plants had a wider spread than F_1 plants (Figure 4.5F), indicating higher resistance in KCTR compared to F_1 plants. Such behavior of F_1 progenies supports the incompletely dominant nature of 2,4-D resistance trait in KCTR.

Table 4.1 Regression parameters (Equation 4.2) describing the response of KSS (susceptible), KCTR (resistant) Palmer amaranth, and F_1-1 progenies to 2, 4-D under growth chamber conditions (b: relative slope, d: upper limit; e: GR_{50} or dose required for 50% dry weight reduction; SE: standard error; RI: resistance index).

F_1-family/ Population	b (SE)	d (SE)	e (SE)	RI
F_1-1	0.76 (0.12)	99.60 (4.57)	837.31 (162.98)	4.3
KCTR	0.65 (0.13)	100.74 (5.32)	1800 (566.45)	9.3
KSS	0.81 (0.16)	99.68 (5.52)	194 (46.96)	-

Table 4.2 Regression parameters (Equation 4.2) describing the response of KSS (susceptible), KCTR (resistant) Palmer amaranth, and F_1-2 progenies to 2, 4-D under growth chamber conditions (b: relative slope, d: upper limit; e or GR_{50}: dose required for 50% dry weight reduction; SE: standard error; RI: resistance index).

F_1-family/ Population	b (SE)	d (SE)	e (SE)	RI
F_1-2	1.13 (0.23)	100.80 (6.71)	501.18 (106.62)	2.5
KCTR	0.99 (0.23)	102.11 (6.69)	1188.02 (288.70)	5.9
KSS	1.09 (0.29)	107.87 (8.83)	200.02 (53.59)	-

Table 4.3 Regression parameters (Equation 4.2) describing the response of KSS (susceptible), KCTR (resistant) Palmer amaranth, and F_1-3 progenies to 2, 4-D under growth chamber conditions (b: relative slope, d: upper limit; e or GR_{50}: dose required for 50% dry weight reduction; SE: standard error; RI: resistance index).

F_1-family/ Population	b (SE)	d (SE)	e (SE)	RI
F_1-3	1.27 (0.24)	100.61 (6.07)	500.2 (90.43)	3.0
KCTR	0.80 (0.17)	102.74 (5.61)	1316.97 (343.25)	7.9
KSS	0.93 (0.22)	100.31 (6.61)	166.50 (43.50)	-

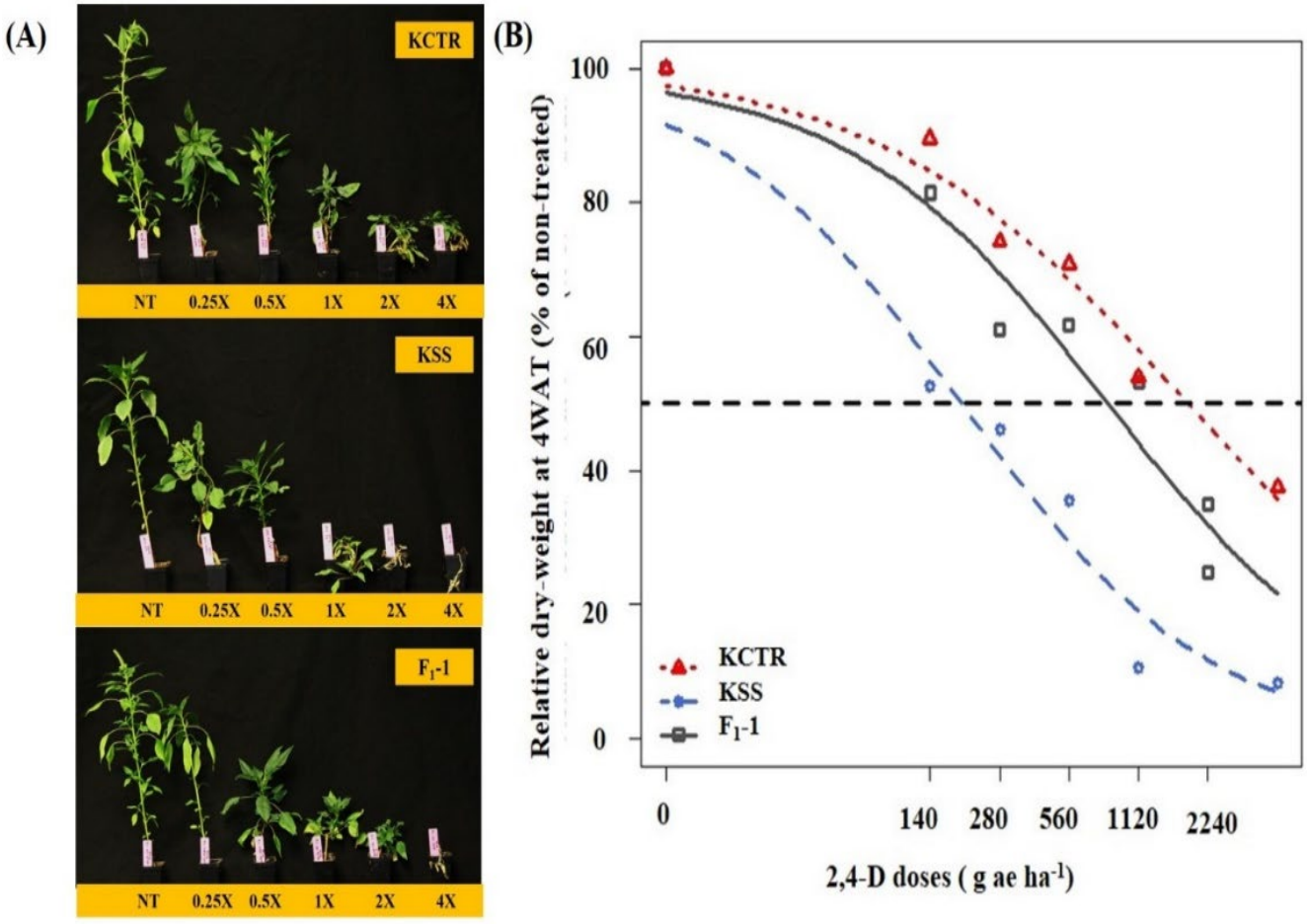

Figure 4.2 (A) Response of KSS (susceptible), KCTR (resistant), and F_1-1 progenies to 2,4-D at varying doses of 2,4-D ranging from non-treated (NT) to 4X; where1X represents the field recommended dose of 2,4-D at 560 g ae ha^{-1} at 4 weeks after treatment (WAT). (B) Dose-response curves representing the relative dry weight (% of non-treated) of KSS, KCTR, and F_1-1 progenies using the three-parameter log-logistic regression model (Equation 4.2) at 4 WAT. Dotted arrow at the center of the plot represents the 50% of relative dry weight.

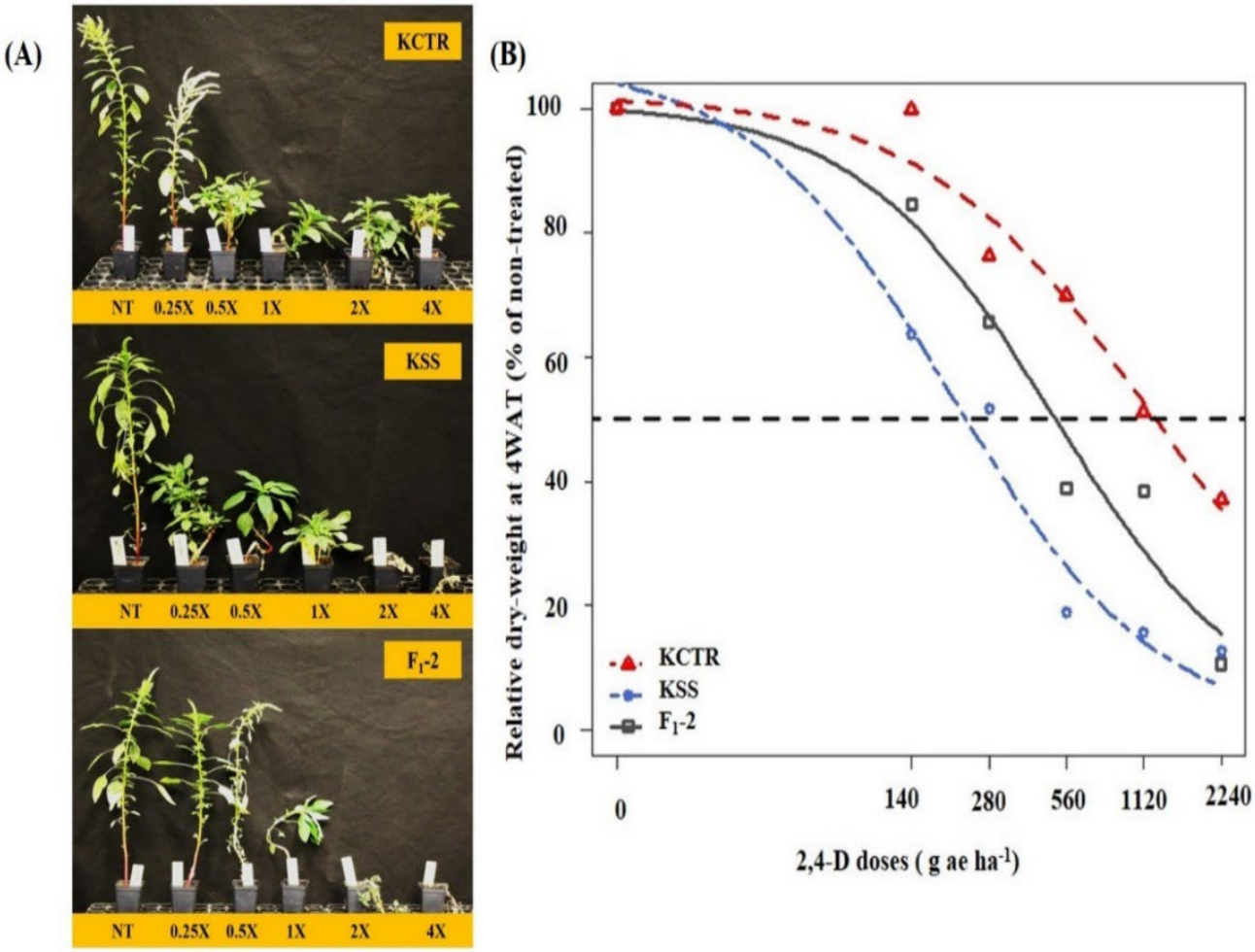

Figure 4.3 (A) Response of KSS (susceptible), KCTR (resistant), and F_1-2 progenies to 2,4-D at varying doses of 2,4-D ranging from non-treated (NT) to 4X; where1X represents the field recommended dose of 2,4-D at 560 g ae ha^{-1} at 4 weeks after treatment (WAT). (B) Dose-response curves representing the relative dry weight (% of non-treated) of KSS, KCTR, and F_1-2 progenies using the three-parameter log-logistic regression model (Equation 4.2) at 4 WAT. Dotted arrow at the center of the plot represents the 50% of relative dry weight.

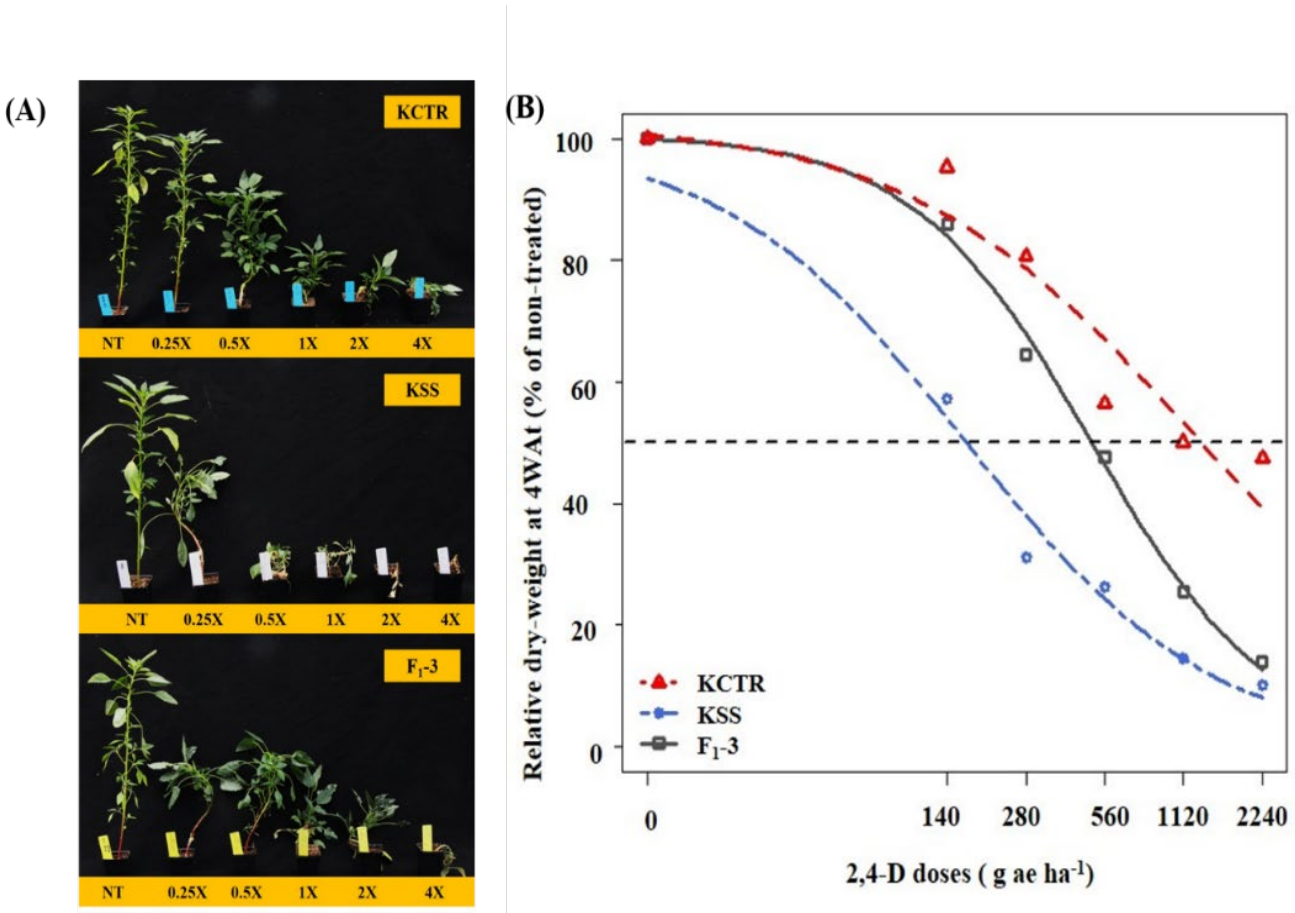

Figure 4.4 (A) Response of KSS (susceptible), KCTR (resistant), and F_1-3 progenies to 2,4-D at varying doses of 2,4-D ranging from non-treated (NT) to 4X; where1X represents the field recommended dose of 2,4-D at 560 g ae ha^{-1} at 4 weeks after treatment (WAT). (B) Dose-response curves representing the relative dry weight (% of non-treated) of KSS, KCTR, and F_1-3 progenies using the three-parameter log-logistic regression model (Equation 4.2) at 4 WAT. Dotted arrow at the center of the plot represents the 50% of relative dry weight.

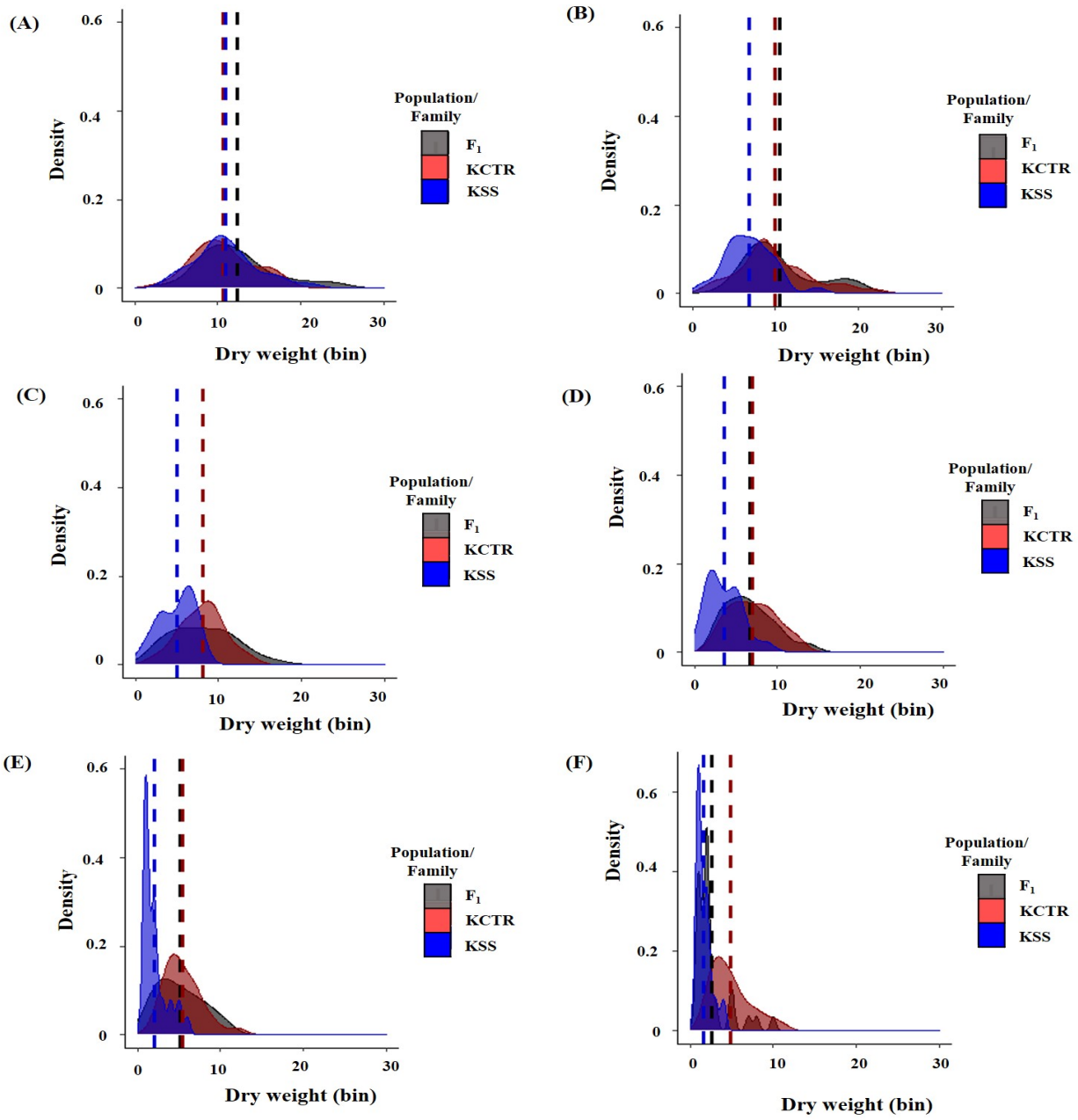

Figure 4.5 Visualization of cumulative dry weight distribution of KSS (susceptible), KCTR (resistant), and F_1 progenies in response to 2,4-D at (A) 0 (Non-treated) (B) 140, (C) 280, (D) 560 (field recommended dose), (E) 1120, and (F) 2240 g ae ha^{-1} *via* density plots. Dry weight of samples was converted into bins and plotted in the X-axis. Y-axis was plotted by calculating density which is the proportion of total plants in each bin. Dotted lines represent mean dry weight of KSS, KCTR, and F_1 plants.

Segregation (2,4-D resistance or susceptibility) of F_2 Progenies

Response of progenies from F_2 families and KSS population (as control) were evaluated by treating with 2,4-D at the field recommended dose of 560 g ae ha^{-1}. As expected, upon treatment with 560 g ae ha^{-1} of 2,4-D, all KSS plants (n = ~50), either completely died or had dried leaf and stunted growth at 4 WAT (data not shown). Interestingly, the percent survival of F_2 progenies highly varied among families.

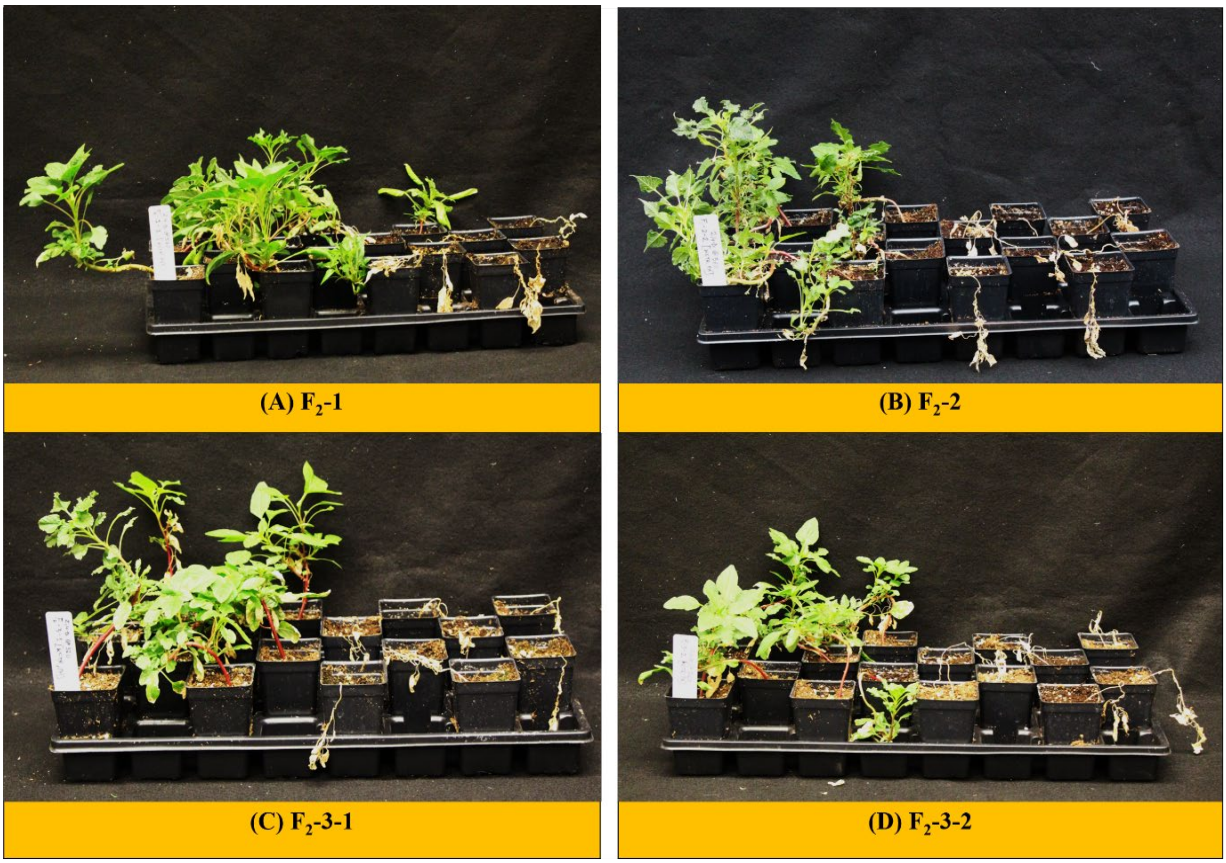

Figure 4.6 Response of progenies from four F_2 families *i.e.*, (A) F_2-1, (B) F_2-2, (C) F_2-3-1, and (D) F_2-3-2 to treatment with 2,4-D at 560 g ae ha^{-1} (field recommended dose).

Varied phenotypes of F_2 progenies ranging from high resistance (plants showing low epinasty and high dry weight accumulation) to very low-level resistance (plants showing high epinasty and low dry weight accumulation) were found (Figure 4.6). In the first run of this assay, all F_2 families deviated from the expected 3:1 (resistance: susceptibility) segregation ratio (Table 4.4). In the second run, however, except for the F_2-1 family, all other families did not follow the 3:1 segregation ratio (Table 4.4). Overall, when combined (run 1 and 2; Table 4.4) all F_2- families failed to segregate as 3:1 (resistance and susceptible) ratio, indicating that more than one gene(s) is involved in mediating 2,4-D resistance in KCTR Palmer amaranth.

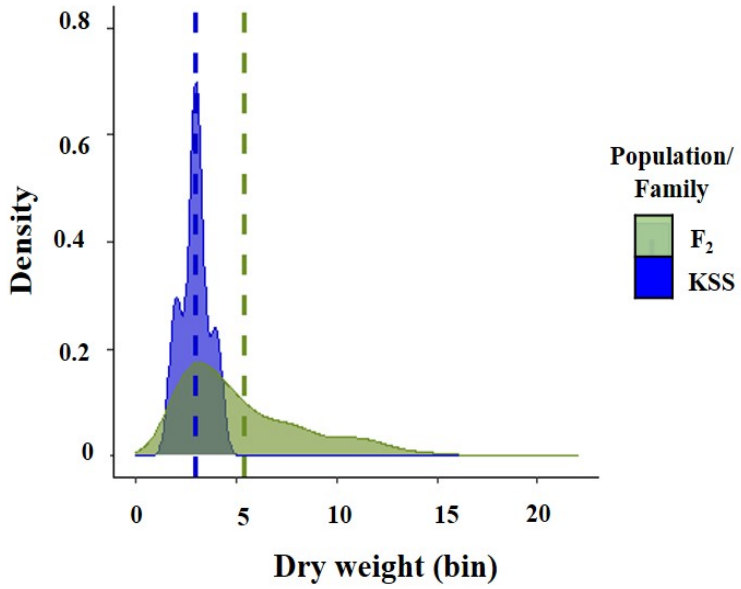

Figure 4.7 Visualization of cumulative dry weight data distribution of KSS (susceptible; n = 21) and F_2 progenies (n = 332) in response to 2,4-D at 560 g ae ha^{-1} (field recommended dose) *via* density plot. Dry weight of samples was converted into bins and plotted on the X-axis. Y-axis was plotted by calculating density which is the proportion of total plants in each bin. Dotted lines represent mean dry weight of KSS and F_2 plants.

Table 4.4 Chi-square test for goodness of fit of the observed segregation of plants as resistance or susceptible to 2,4-D to the expected frequency for a single-locus model in pseudo-F_2 families of Palmer amaranth. The expected survival frequency for each F_2 family was calculated (Equation 4.3) and compared with the observed frequency of survival (Equation 4.4).

Experimental Run	F_2 family/ Population	Plants treated	Observed survival		Expected survival		p-value
			Alive	Dead	Alive	Dead	
1	F_2-1	72	30	42	54	18	< .00001
	F_2-2	101	56	45	75.75	25.25	< .00001
	F_2-3-1	74	46	28	55.5	18.5	0.01076
	F_2-3-2	53	25	28	39.75	13.25	< .00001
	Total	300	157	143	225	75	< .00001
2	F_2-1	92	63	29	69	23	0.14856
	F_2-2	20	9	11	15	5	0.00195
	F_2-3-1	124	79	45	93	31	0.00369
	F_2-3-2	96	46	50	72	24	< .00001
	Total	332	197	135	249	83	< .00001
1 & 2 Combined	F_2-1	164	93	71	123	41	< .00001
	F_2-2	121	65	56	90.75	30.25	< .00001
	F_2-3-1	198	125	73	148.5	49.5	0.00011
	F_2-3-2	149	71	78	111.75	37.25	< .00001
	Total	632	354	278	474	158	< .00001

The distribution of shoot dry weight per plant (g) of the F_2 progenies (n = 332) and KSS plants (n = 21) was illustrated through a density plot (Figure 4.7). The distribution showed a

widespread of dry weight bins representing plant to plant variation in response to 2,4-D treatment (Figure 4.7).

Discussion

The evolution of 2,4-D resistance in Palmer amaranth has been reported only in two populations from Kansas, including, KCTR (Heap 2021, Kumar et al. 2019b). KCTR was also documented to exhibit predominantly metabolic resistance to at least 5 herbicide MOAs including ALS-, photosystem II (PS II)-, protoporphyrinogen oxidase (PPO)-, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitors and SAHs (Chapter 2). Moreover, physiological studies have confirmed enhanced metabolism of 2,4-D in KCTR compared to susceptible populations KSS and MSS (Chapter 3). Information on the genetic basis of 2,4-D resistance in Palmer amaranth is lacking. Therefore, in the current study, we investigated the inheritance of 2,4-D resistance in KCTR. Three F_1 families (F_1-1, F_1-2, F_1-3) were generated, which were further used to generate four pseudo-F_2 families (F_2-1, F_2-2, F_2-3-1, F_2-3-2).

In response to the discriminatory (dead or alive) dose of 1120 g ae ha^{-1} of 2,4-D treatment, the lack of 100% survival of progenies in all F_1 families suggested that the 2,4-D resistant KCTR plants used as parents in crossing program to generate F_1 seed were heterozygous. Even though the KCTR plants used in this study were obtained after two rounds of screening with 2,4-D at a dose higher than the field recommended dose, there is a high possibility of progenies being heterozygous, because of the dioecious nature of Palmer amaranth. In the dose-response assays, KCTR plants, as expected, showed a high level of 2,4-D resistance (6-9 fold) compared to KSS plants (Figure 4.2, 4.3, 4.4; Table 4.1, 4.2, 4.3). Progenies generated by both direct and reciprocal crosses were found resistant to 2,4-D, indicating that the 2,4-D resistance in KCTR is

a nuclear trait (Figure 4.2, 4.3, 4.4; Table 4.1, 4.2, 4.3). In general, resistance to majority of herbicides is a nuclear trait. One exception is the TSR to PS II-inhibitors in weeds, which is inherited maternally/cytoplasmically (MacHado and Bandeen 1982). Nuclear inheritance of 2,4-D inheritance has been reported in several weeds, including wild radish, wild mustard, oriental mustard, common waterhemp, and prickly lettuce (Busi and Powles 2017, Jugulam et al. 2005, Preston and Malone 2015, Riar et al. 2011, Sabaté et al. 2016). Nuclear traits are transferred by both seed and pollen of resistant plants. Previously, pollen-mediated intra- and interspecific transfer of TSR and NTSR traits have been documented in *Amaranthus sp*. (Gaines et al. 2012, Jhala et al. 2021, Nakka et al. 2017a, Nandula et al. 2014, Oliveira et al. 2018b, Sosnoskie et al. 2012, Shyam et al. 2021b). Therefore, there is a potential of inter-or intraspecific transfer of 2,4-D resistance *via* pollen-mediated gene flow.

The results of F_1 dose-response assay showed an intermediate level of 2,4-D resistance in all F_1 families compared to the parental resistant (KCTR) and susceptible (KSS) plants (Figure 4.2, 4.3, 4.4; Table 4.1, 4.2, 4.3). Moreover, from cumulative dry weight distribution, it was found that F_1 progenies had narrower dry weight distribution at 4X dose *i.e.*, 2240 g ae ha^{-1}, whereas the distribution was found similar at lower doses of 2,4-D (Figure 4.5). Both chi-square analysis and distribution plotting confirmed that 2,4-D resistance in KCTR Palmer amaranth is an incompletely dominant trait. Most cases of herbicide resistance, including metabolic resistance, have been reported to be controlled by either completely or incompletely dominant genes (Busi and Powles 2017, Huffman et al. 2015, Preston and Malone 2015, Shyam et al. 2021b). Recessive allele(s) govern very few instances of herbicide resistance. For example, clopyralid and picloram resistance in yellow starthistle (*Centaurea solstitialis* L.) and quinclorac resistance in false cleavers (*Galium spurium* L.) were found to be conferred by recessive alleles (Sabba et

al. 2003, Van Eerd et al. 2004). Similar to the findings of the current study, 2,4-D resistance in common waterhemp and wild radish was found to be incompletely dominant (Busi and Powles 2017, Sabaté et al. 2016). In contrast, inheritance of 2,4-D resistance in wild radish and dicamba resistance in kochia (*Bassia scoparia* L.) were found to be controlled by dominant alleles (Busi and Powles 2017, Jasieniuk et al. 1995, Preston et al. 2009). An incompletely dominant trait is expressed in both homozygous and heterozygous state; therefore, the spread of 2,4-D resistance will be faster than a recessive trait.

Segregation of F_2 progenies in KCTR Palmer amaranth at the field recommended dose of 2,4-D (560 g ae ha^{-1}) did not follow the Mendelian 3:1 (resistance: susceptible) ratio, as expected for a single gene inheritance (Table 4.4). Only one F_2-family (F_2-1) in the second run of the study segregated in the 3:1 ratio (Table 4.4). However, in the first run and the combined analysis of two runs, the F_2-1 segregation did not follow the 3:1 (resistance: susceptible) ratio (Table 4.4). This indicates that the 2,4-D resistance in KCTR Palmer amaranth is not inherited by a single gene, but a polygenic trait. Furthermore, analysis of the chi-square goodness of fit test data suggests variability in the phenotypes among the F_2 progenies resistant to 2,4-D, which cannot be explained by a single major gene-mediated resistance (Figure 4.6, 4.7). Although a majority of TSR is controlled by a single gene (Patzoldt et al. 2003, Tardif et al. 1996), the inheritance of NTSR was reported to be conferred by both single and multiple genes (Huffman et al. 2015, Kohlhase et al. 2018, Oliveira et al. 2018a, Pandian et al. 2021, Shyam et al. 2021b). For instance, monogenic metabolic resistance to chlorsulfuron and atrazine was documented in common waterhemp (*Amaranthus tuberculatus* Moq. Sauer.), Palmer amaranth, and sorghum (Huffman et al. 2015, Pandian et al. 2021, Shyam et al. 2021b). Polygenic inheritance was reported for metabolic resistance to mesotrione in common waterhemp and Palmer amaranth

(Huffman et al. 2015, Kohlhase et al. 2018, Oliveira et al. 2018a, Shyam et al. 2021b). Similar to our findings, 2,4-D resistance in common waterhemp and MCPA resistance in hemp-nettle was found to be polygenic (Sabaté et al. 2016, Weinberg et al. 2006). On the other hand, single-gene inherited 2,4-D resistance was reported in wild mustard, oriental mustard, prickly lettuce (Jugulam et al. 2005, Preston and Malone 2015, Riar et al. 2011). Traits inherited by multiple genes spread slower than those with single alleles. Therefore, the spread of 2,4-D resistance in Palmer amaranth could be slower than that inherited by a single dominant allele.

The results of Chapter 3, indicate that KCTR Palmer amaranth evolved metabolic resistance to 2,4-D, mediated by cytochrome P450 monooxygenases (P450s) activity. Similarly, in other 2,4-D-resistant weed species, such as common waterhemp metabolic resistance mediated by P450 has been reported (Figueiredo et al. 2018, Shergill et al. 2018b). Increased expression of a P450 cluster (*P450- 81E8*) was recently reported in 2,4-D-resistant common waterhemp populations from both NE and IL (Giacomini et al. 2020). Based on the data from this chapter, multiple genes confer metabolic resistance to 2,4-D in KCTR Palmer amaranth. Multiple genes, possibly either a combination of P450s or other closely-linked genes that can metabolize 2,4-D may be involved in 2,4-D resistance in KCTR Palmer amaranth. Metabolic resistance to herbicides usually follows a three-step detoxification process, including several genes facilitating herbicide conversion, degradation, and transporting or compartmentalizing of degraded molecules into the vacuole of a plant cell. Metabolic resistance might also involve genes that help in plant recovery after herbicide treatment. Therefore, a more in-depth investigation is needed to decipher the genes involved in the metabolic resistance of 2,4-D in KCTR Palmer amaranth.

Conclusion

In summary, the data from this study suggest that 2,4-D resistance in KCTR Palmer amaranth is an incompletely dominant nuclear trait, governed by multiple alleles. The possible presence of any TSR to 2,4-D in KCTR has not been investigated yet and needs to be investigated. In case any TSR mechanism is present, that will likely complicate the inheritance pattern. Future research shall also be aimed at discovering metabolic genes that mediate 2,4-D resistance in KCTR. Availability of the Palmer amaranth genome (Montgomery et al. 2020) and application of transcriptomic approach can enable identifying specific P450s involved in metabolic resistance in weeds. For example, a P450 gene (P450-81A10v7) was documented to confer metabolic resistance to at least 5 MOAs of action in rigid ryegrass (*Lolium rigidum* L.) (Han et al. 2021). Studies to understand if metabolic resistance to 2,4-D can be transferred *via* pollen to other *Amaranthus sp*. are also important. This will help in formulating management practices specific to *Amaranthus sp*. Overall, 2,4-D has been a viable option to manage ALS- and EPSPS-inhibitor resistant Palmer amaranth; however, the evolution of resistance to this herbicide restricts this option to growers. Therefore, the incorporation of integrated weed management techniques will be necessary for slowing the evolution and spread of 2,4-D resistance.

Chapter 5 - Management of Glyphosate-Resistant Palmer amaranth in 2,4-D Tolerant Soybean

Manuscript Information

Manuscript Title: Management of glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) in 2,4-D-, glufosinate-, and glyphosate-resistant soybean

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Abstract

Glyphosate-resistant (GR) Palmer amaranth is a problematic, annual broadleaf weed in soybean production fields in Nebraska and many other states in the US. Soybean tolerant to 2,4-D, glyphosate, and glufosinate (Enlist E3^{TM}) has been developed and was first grown commercially in 2019. The objectives of this research were to evaluate the effect of herbicide programs applied pre-emergence (PRE), PRE followed by (fb) late post-emergence (LPOST), and early post-emergence (EPOST) fb LPOST on GR Palmer amaranth control, density, and biomass reduction, soybean injury, and yield. Field experiments were conducted near Carleton, Nebraska, in 2018, and 2019 in a grower's field infested with GR Palmer amaranth in 2,4-D-, glyphosate-, and glufosinate-tolerant soybean. Sulfentrazone + cloransulam-methyl, imazethapyr + saflufenacil + pyroxasulfone, and chlorimuron ethyl + flumioxazin + metribuzin applied PRE provided 84% to 97% control of GR Palmer amaranth compared with the non-treated control 14 d after PRE. Averaged across herbicide programs, PRE fb 2,4-D and/or glufosinate, and sequential application of 2,4-D or glufosinate applied EPOST fb LPOST resulted in 92% and 88% control of GR Palmer amaranth, respectively, compared with 62% control with PRE-only programs 14 d after LPOST. Reductions in Palmer amaranth biomass followed the same trend; however, Palmer amaranth density was reduced 98% in EPOST fb LPOST programs compared with 91% reduction in PRE fb LPOST and 76% reduction in PRE-only programs. PRE fb LPOST and EPOST fb LPOST programs resulted in an average soybean yield of 4,478 and 4,706 kg ha^{-1}, respectively, compared with 3,043 kg ha^{-1} in PRE-only programs. Herbicide programs evaluated in this study resulted in no soybean injury. The results of this research illustrate that herbicide programs are available for the management of GR Palmer amaranth in 2,4-D-, glyphosate-, and glufosinate-tolerant soybean.

Introduction

Commercialization of herbicide-resistant (HR) crop technology in the late 1990s led to a turning point in the history of weed management (Reddy and Nandula 2012). Glyphosate-resistant (GR) soybean and corn (*Zea mays* L.) were rapidly adopted by growers in the US because of their ease of use, cost-effectiveness, and broad-spectrum weed control (Green and Owen 2011). Currently, HR soybean constitutes 90% of the total soybean planted in the US (USDA 2021). HR soybean including a single trait such as glyphosate resistance or glufosinate resistance, or multiple HR traits such as glyphosate and dicamba resistance are grown in the US. The cultivation and widespread adoption of GR corn and soybean after their commercialization reduced the use of residual herbicides because of flexibility in application timing, excellent weed control, and a wide margin of crop safety with glyphosate (Green 2012); however, repeated application of glyphosate resulted in the evolution of GR weeds (Heap and Duke 2018). Currently, 52 weed species have been reported to have evolved resistance to glyphosate globally, including 17 species in the US (Heap 2021).

Native to the southwestern US, Palmer amaranth has been ranked as one of the most troublesome weeds in agronomic cropping systems in the US in a survey conducted by the Weed Science Society of America (Van Wychen 2020). It is also one of the most economically important weeds in agronomic crops in the US (Beckie 2011, Chahal et al. 2018). Palmer amaranth is a prolific seed producer (Keeley et al. 1987) and can survive and produce a lot of seeds even under moisture-stressed situations (Chahal et al. 2018). It has a high photosynthetic rate, resulting in a fast growth rate and considerable biomass accumulation compared with other Amaranthus species (Ehleringer 1983, Jha and Norsworthy 2009), along with continuous emergence throughout the growing season, leading to season-long crop interference (Jha and

Norsworthy 2009). In addition to the aforementioned weedy characteristics, the evolution of herbicide resistance in Palmer amaranth has made control of this weed especially challenging in cotton (*Gossypium sp*. L.) and soybean. GR Palmer amaranth was first reported in Georgia (Culpepper et al. 2006), and since then, 27 other U.S. states have documented the presence of GR Palmer amaranth (Heap 2021).

Glyphosate is a systemic, nonselective POST herbicide that targets 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the chloroplast of sensitive plants, causing inhibition of aromatic amino acid production (Bentley 1990). Current mechanisms of target-site resistance to glyphosate in Palmer amaranth have been due to either mutation in the *EPSPS* gene, the molecular target of glyphosate (Dominguez-Valenzuela et al. 2017), or overamplification and expression of the *EPSPS* gene (Chahal et al. 2017, Gaines et al. 2010, Koo et al. 2018). Reduced glyphosate absorption and translocation have also been reported to impart nontarget-site resistance to glyphosate in Palmer amaranth (Dominguez-Valenzuela et al. 2017, Nandula et al. 2012). Considering the extent of GR Palmer amaranth populations, effective management programs in soybean should focus on methods that reduce survival, seed production, and transfer of herbicide-resistance alleles.

Palmer amaranth interference can cause substantial yield losses in agronomic crops. For example, a Palmer amaranth density of 3 plants m^{-2} caused 60% yield loss in soybean in Arkansas. Klingaman and Oliver (1994), and Bensch et al. (2003) reported 79% soybean yield loss at a density of 8 plants m^{-2} in Kansas. Early emergence (0-1 week after crop emergence) and establishment of Palmer amaranth reduce soybean yield compared with late emergence (2-8 weeks after crop emergence) (Korres et al. 2019). Acker et al. (1993) reported the critical period of weed control in soybean ranged from the second soybean-node growth stage (V2) to the

beginning pod growth stage (R3). However, in a recent multilocation and multiyear study conducted in Nebraska, the critical period of weed removal in soybean was delayed from V1–V2 to V4–R5 using pre-emergence (PRE) herbicides such as saflufenacil + imazethapyr + pyroxasulfone and saflufenacil + imazethapyr (Knezevic et al. 2019).

Herbicide applied PRE is a foundation for the management of GR weeds such as Palmer amaranth and waterhemp (*Amaranthus tuberculatus* Moq. Sauer) followed by (fb) post-emergence (POST) herbicides to control late-emerged weeds (Jhala et al. 2017). Sarangi and Jhala (2019) reported 97% and 86% control of Palmer amaranth with the application of PRE fb POST herbicide with and without layered residual activity, respectively. This finding suggests that POST herbicide mixed with an additional foliar, active herbicide such as glufosinate + 2,4-D or a residual herbicide such as acetochlor can lead to better weed control compared with POST herbicide applied alone for control of Palmer amaranth (Aulakh and Jhala 2015).

A GR Palmer amaranth population was confirmed in a grower's field in a continuous GR corn-soybean rotation located in Thayer County, Nebraska (Chahal et al. 2017). A subsequent greenhouse dose-response bioassay confirmed that Palmer amaranth was 37- to 40-fold resistant to glyphosate compared with a glyphosate-susceptible population. Moreover, the mechanism of resistance was found to be amplification of *EPSPS* gene (32- to 105-fold) compared with a glyphosate-susceptible biotype (Chahal et al. 2017). Interestingly, the GR Palmer amaranth population was less sensitive to POST herbicides such as atrazine, mesotrione, and halosulfuron-methyl, but was effectively controlled (≥ 95%) with glufosinate at 593 g ai ha^{-1} (Chahal et al. 2017).

A new, multiple HR-soybean trait that exhibits tolerance to 2,4-D, glufosinate, and glyphosate has been developed by Corteva™ Agriscience and was commercialized in 2019 in the

US. However, little scientific literature exists on the most effective herbicide programs for managing GR Palmer amaranth in this new soybean production system. Therefore, the objectives of this research were to investigate and compare the effect of three preformulated herbicide mixtures applied PRE alone or fb a late-POST (LPOST) application of either 2,4-D or glufosinate or both and sequential applications (early-POST [EPOST] fb LPOST) of 2,4-D or glufosinate on Palmer amaranth density, biomass, crop injury, and yield in a multiyear field study in NE.

Material and Methods

Description of Field-trials

Field experiments were conducted in 2018 and 2019 in a grower's field infested with GR Palmer amaranth in Thayer County, Carleton, Nebraska (40.30°N, 97.67°E) (Chahal et al. 2017). The field was rain-fed without supplementary irrigation. Palmer amaranth was the predominant weed species at the research site. The soil at the experimental site was silt loam with 63% silt, 19% sand, 18% clay, 2.63% organic matter, and 4.8 pH. The previous crop at the site was no-till soybean. The experiment was arranged in a randomized complete block design with four replications. Individual plots were 3 m wide (four soybean rows spaced 0.76 m apart) and 9 m long. Glyphosate-, 2,4-D-, and glufosinate-tolerant soybean with 2.5 maturity group was no-till planted at a rate of 322,000 seeds ha^{-1} at a depth of 3 cm on May 10, 2018, and May 6, 2019.

Herbicide Treatments

Herbicide programs included PRE, PRE fb LPOST, and EPOST fb LPOST applications with a total of 15 treatments, including a non-treated control (Table 5.1). Herbicides were applied using a CO_2-pressurized backpack sprayer calibrated to deliver 200 L ha^{-1} at 210 kPa equipped with a 2-m wide spray boom equipped with AIXR 110015 flat-fan nozzles (TeeJet,

Spraying Systems Co., Wheaton, IL) spaced 50 cm apart for PRE herbicides and AIXR11004 for 2,4-D and XR11005 nozzles for glufosinate application. The PRE herbicides were applied the same day after planting soybean; EPOST herbicides were applied at 39 d after planting (DAP) on June 18, 2018, and 35 DAP on June 10, 2019; and LPOST herbicides were applied at 18 d after EPOST (DA-EPOST) on July 6, 2018, and 14 DA-EPOST herbicides on June 24, 2019. Soybean stages corresponding to application timings were V2-V3 soybean growth stage for EPOST and V4-V5 for LPOST application. Palmer amaranth height corresponding to the EPOST applications was 8 to 12 cm and for LPOST applications was 10 to 15 cm.

Data Collection

Palmer amaranth control and soybean injury were visually assessed at 14 d after PRE (DA-PRE), 14 DA-EPOST, and 14 d after late POST (DA-LPOST) on a scale of 0% to 100%, where 0% was equivalent to no Palmer amaranth control or soybean injury and 100% was equivalent to complete control or soybean-plant death. Palmer amaranth density was recorded from two randomly placed,0.5 m^2 quadrats $plot^{-1}$ at 14 DA-EPOST and 14 DA-LPOST. Likewise, aboveground Palmer amaranth biomass was collected from two randomly placed 0.5 m^2 quadrats $plot^{-1}$ at 14 DA-EPOST and 14 DA-LPOST. At each of these intervals, Palmer amaranth plants were placed in paper bags, oven-dried, and weighed. At 14 DA-PRE, data were collected from the plots treated with PRE herbicides and the non-treated control; at 14 DA-EPOST and14 DA-LPOST, Palmer amaranth control, density, and biomass data were collected from all plots. Palmer amaranth density and above-ground biomass data were converted into percent density and biomass reduction compared with the non-treated control, using equation 5.1:

$$\text{Density or biomass reduction (\%)} = \frac{(B-C) \times 100}{B}$$

Table 5.1 Details of herbicide programs, application timing, and doses used for control of glyphosate-resistant Palmer amaranth in 2,4-D-, glyphosate, and glufosinate- tolerant soybean in field experiments conducted at Carleton, Nebraska during 2018 and 2019 growing seasons. Ammonium sulphate at 2.5% v/v was mixed with glufosinate treatments.

Herbicide program and dose		Trade name		Manufacturer
PRE/EPOST	LPOST	PRE/EPOST	LPOST	
Sulfentrazone + cloransulam-methyl (PRE) (220 g ai ha^{-1})	-	Sonic®	-	Corteva Agriscience, Wilmington, DE 19880
	2,4-D choline (2,080 g ae ha^{-1})		Enlist One®	Corteva Agriscience
	Glufosinate (656 g ai ha^{-1})		Liberty® 280 SL	BASF Corporation, Research Triangle Park, NC 27709
	2,4-D choline + glufosinate (2,080 g ae ha^{-1} + 656 g ai ha^{-1})		Enlist One® + Liberty® 280 SL	Corteva Agriscience + BASF
Imazethapyr + saflufenacil + pyroxasulfone (PRE) (215 g ai ha^{-1})	-	Zidua® Pro	-	BASF Corporation
	2,4-D choline (2,080 g ae ha^{-1})		Enlist One®	Corteva Agriscience
	Glufosinate (656 g ai ha^{-1})		Liberty® 280 SL	BASF Corporation
	2,4-D choline + glufosinate (2,080 g ae ha^{-1} + 656 g ai ha^{-1})		Enlist One® + Liberty® 280 SL	Corteva Agriscience + BASF Corporation
Chlorimuron ethyl + flumioxazin + metribuzin (PRE)	-	Trivence®	-	Corteva Agriscience
	2,4-D choline (2,080 g ae ha^{-1})		Enlist One®	Corteva Agriscience
	Glufosinate (656 g ai ha^{-1})		Liberty® 280 SL	BASF Corporation

(344 g ai ha^{-1})	2,4-D choline + glufosinate (2,080 g ae ha^{-1}+ 656 g ai ha^{-1})		Enlist One® + Liberty® 280 SL	Corteva Agriscience + BASF Corporation
Glufosinate (EPOST) (656 g ai ha^{-1})	Glufosinate (656 g ai ha^{-1})	Liberty® 280 SL	Liberty® 280 SL	BASF Corporation
2,4-D choline (EPOST) (2,080 g ae ha^{-1})	2,4-D choline (2,080 g ae ha^{-1})	Enlist One®	Enlist One®	Corteva Agriscience

Table 5.2 Average air temperature and total precipitation during the 2018 and 2019 growing seasons and the 30-yr average at the Hebron, Nebraska weather station near Carleton, Nebraska. Air temperature and precipitation data were obtained from High Plains Regional Climate Center (HPRCC 2020).

Timing	Average temperature (C)			Average precipitation (mm)		
	2018	2019	30-yr average	2018	2019	30-yr average
May	20.6	14.6	16.5	78	172.7	122
June	25	21.8	22.2	96	153.2	121.4
July	24.7	25.1	25.1	95.5	137.2	105
August	23.3	23.1	24	92.2	155	94.7
September	20.6	22.6	18.8	153.4	120.4	74.2
October	10.6	9.6	11.8	99.8	118.1	56.1
Annual	10.6	10.3	10.9	614.9	856.5	797.3

where B is Palmer amaranth density or aboveground biomass of the non-treated control plot and C is the Palmer amaranth density or aboveground biomass collected from an individual experimental plot. At maturity, soybean was harvested from the middle two rows using a plot combine, weighed, and soybean yield was adjusted to 13.5% moisture content. However, due to pending export approval of 2,4-D-, glyphosate-, and glufosinate- tolerant soybean in few countries, the field experiment was destroyed in 2018, so yield data are available only for 2019.

Data Analysis

ANOVA was performed on Palmer amaranth control, density, aboveground biomass, and soybean yield data using PROC GLIMMIX in SAS, version 9.4 (SAS Institute, Cary, NC). Before analysis, data were subjected to PROC UNIVARIATE analysis for testing normality and homogeneity of variance. Visual estimates of Palmer amaranth control and percent density and bio-mass reduction data were arcsine square-root transformed and were back-transformed for presentation.

Results

Year-by-treatment interactions for Palmer amaranth control, density, and aboveground biomass at 14 DA-PRE, 14 DA-EPOST, and14 DA-LPOST were not significant ($P \geq 0.05$); therefore, data from both years were pooled. The average monthly temperature during May 2018 was higher than May 2019 and the 30-yr average (Table 5.2). Apart from that, monthly temperatures during the crop season in both years were similar to the 30-yr average. More precipitation fell in 2019 compared with 2018 and the 30-yr average (Table 5.2). There was no soybean injury from the herbicides evaluated (data not presented).

Glyphosate-Resistant Palmer amaranth Control

Herbicides applied PRE in this study provided 84% to 97% control of GR Palmer amaranth at 14 DA-PRE, and no differences in control were observed among the PRE herbicides (Table 5.3). Although statistically similar, sulfentrazone + cloransulam-methyl provided 84% to 87% control, whereas imazethapyr + saflufenacil + pyroxasulfone, and chlorimuron ethyl + flumioxazin + metribuzin provided 87% to 97% control at 14 DA-PRE (Table 5.3). The residual activity of herbicides applied PRE declined as the season progressed. For example, sulfentrazone + cloransulam-methyl provided 27% control of Palmer amaranth 14 DA-LPOST compared with 75% control with imazethapyr + saflufenacil + pyroxasulfone and chlorimuron ethyl + flumioxazin + metribuzin. In 2018, greater Palmer amaranth control (86%) was achieved at 14 DA-EPOST compared with 2019 (74%). At 14 DA-EPOST, glufosinate or 2,4-D provided 88% and 65% control of Palmer amaranth, respectively (Table 5.3). At 14 DA-LPOST, herbicides applied PRE without a follow-up POST herbicide did not maintain Palmer amaranth control compared with PRE fb POST or EPOST fb LPOST herbicide programs (Table 5.3). Among the PRE fb LPOST programs, sulfentrazone + cloransulam-methyl fb 2,4-D provided the lowest (70%) Palmer amaranth control (Table 5.3). GR Palmer amaranth control provided by the remaining PRE fb LPOST programs ranged from 88% to 100% (Table 5.3). Interestingly, chlorimuron ethyl + flumioxazin + metribuzin applied PRE alone provided statistically similar control (85%) as sequential application of 2,4-D (93%) or glufosinate (92%) (Table 5.3). The sequential application of 2,4-D or glufosinate provided 85% to 92% control of Palmer amaranth 14 DA-LPOST (Table 5.3). Contrast analysis showed that PRE fb LPOST programs resulted in 92% Palmer amaranth control compared with 62% control with PRE-only programs (Table 5.3).

Table 5.3 Glyphosate-resistant Palmer amaranth control as affected by herbicide programs in 2,4-D-, glyphosate-, and glufosinate- tolerant soybean in field experiments conducted at Carleton, Nebraska in 2018 and 2019 growing seasons. Means presented within each column with no common letter(s) are significantly different based on Fisher's protected LSD test, where $\alpha = 0.05$.

Herbicide programs		Palmer amaranth control (%)		
PRE/EPOST	LPOST	14 DA-PRE	14 DA-EPOST	14 DA-LPOST
Non-treated control	-	0	0	0
Sulfentrazone + cloransulam-methyl (PRE)	-	85 a*	49 f	27 e
	2,4-D choline	87 a	71 de	70 d
	Glufosinate	85 a	81 b-d	88 bc
	2,4-D choline + glufosinate	84 a	75 c-e	96 ab
Imazethapyr + saflufenacil + pyroxasulfone (PRE)	-	97 a	81 b-d	75 d
	2,4-D choline	95 a	89 ab	95 ab
	Glufosinate	95 a	92 ab	99 a
	2,4-D choline + glufosinate	87 a	94 a	96 ab
Chlorimuron ethyl + flumioxazin + metribuzin (PRE)	-	94 a	75 c-e	85 c
	2,4-D choline	93 a	90 ab	93 a-c
	Glufosinate	89 a	87 a-c	92 a-c
	2,4-D choline + glufosinate	97 a	92 ab	100 a
Glufosinate (EPOST)	Glufosinate	-	88 ab	85 c
2,4-D choline (EPOST)	2,4-D choline	-	65 e	92 a-c
p-value		0.2076	<0.0001	<0.0001
Contrast Analysis				
PRE vs. PRE fb LPOST		-	-	62 vs. 92***
PRE fb LPOST vs. EPOST fb LPOST		-	-	92 vs. 88^{NS}

Glyphosate-Resistant Palmer amaranth Density Reduction

Palmer amaranth emergence was greater in 2019 compared with 2018, leading to greater Palmer amaranth density. For example, Palmer amaranth density in the non-treated control ranged from100 to 200 plants m^{-2} in 2018 compared with 300 to 500 plants m^{-2} in 2019 (data not shown). At 14 DA-EPOST, greater density reduction was obtained in 2018 (89%) compared with 71% in 2019, which can be attributed to greater emergence in 2019. At 14 DA-EPOST, glufosinate provided 86% Palmer amaranth density reduction compared with a 60% reduction with 2,4-D (Table 5.4). At 14 DA-LPOST, all PRE fb LPOST herbicide programs except sulfentrazone + cloransulam-methyl fb 2,4-D provided 89% to 100% reduction in Palmer amaranth density compared with the non-treated control (Table 5.4). Sulfentrazone + cloransulam-methyl fb 2,4-D resulted in 42% Palmer amaranth density reduction. When averaged across herbicide programs, PRE fb LPOST pro-grams (91%) resulted in a statistically higher density reduction of GR Palmer amaranth compared with PRE-only programs (76%). Thus, the application of an LPOST herbicide caused a 20% increase in density reduction compared with PRE-only herbicide programs alone (Table 5.4). Overall, Palmer amaranth density reduction ranged from 89% to 100% without a difference in the most PRE fb POST herbicide programs at 14 DA-LPOST (Table 5.4). EPOST fb LPOST programs (sequential application of glufosinate or 2,4-D) resulted in a 98% density reduction of Palmer amaranth compared with the non-treated control at 14 DA-LPOST (Table 5.4). Contrast analysis revealed that EPOST fb LPOST programs resulted in a 98% reduction in Palmer amaranth density compared with a 76% reduction from PRE fb LPOST programs (Table 5.4). Except for sulfentrazone + cloransulam-methyl fb 2,4-D, all other PRE fb LPOST programs were comparable to EPOST fb LPOST programs.

Glyphosate-Resistant Palmer amaranth Biomass Reduction

Aboveground biomass reduction of Palmer amaranth followed the same trend as density reduction (Table 5.4). In 2019, a lower biomass reduction (60%) was obtained compared with 2018 (78%) at14 DA-EPOST. Glufosinate or 2,4-D resulted in 69% to 71% reduction in GR Palmer amaranth biomass 14 DA-EPOST. The PRE fb LPOST programs resulted in 91% to 100% biomass reduction, except sulfentrazone + cloransulam-methyl fb 2,4-D (32%) and were comparable to EPOST fb LPOST herbicide programs (99%) at 14 DA-LPOST (Table 5.4). Averaged across herbicide programs, PRE fb LPOST programs resulted in 89% reduction of Palmer amaranth biomass compared with 58% with PRE-only programs 14 DA-LPOST (Table 5.4). EPOST fb LPOST programs (99%) provided a comparable reduction in biomass compared with PRE fb LPOST programs (89%) (Table 5.4).

2,4-D-Tolerant Soybean Yield

Averaged across treatments, PRE fb LPOST herbicide programs resulted in greater yield (4,478 kg ha^{-1}) compared with PRE-only programs (3,043 kg ha^{-1}) (Table 5.5). Therefore, the PRE fb LPOST programs prevented a 32% soybean yield loss that would have occurred with PRE-only herbicide programs (Table 5.5). The lowest yield (2,398 kg ha^{-1}) was obtained in the non-treated control, which was comparable to the PRE-only herbicide programs of sulfentrazone + cloransulam-methyl (2,835 kg ha^{-1}) and chlorimuron ethyl + flumioxazin (2,832 kg ha^{-1}) (Table 5.5). There was no difference in yield obtained from PRE fb LPOST programs (4,478 kg ha^{-1}) and sequential application of 2,4-D or glufosinate (EPOST fb LPOST) programs (4,706 kg ha^{-1}) (Table 5.5).

Table 5.4 Glyphosate-resistant Palmer amaranth density and above-ground biomass as affected by the herbicide programs in 2,4-D-, glyphosate-, and glufosinate- tolerant soybean in field experiments conducted in Carleton, Nebraska during 2018 and 2019 growing seasons. Means presented within each column with no common letter(s) are significantly different based on Fisher's protected LSD, where $\alpha = 0.05$.

Herbicide programs		Reduction in Palmer amaranth density (%)		Reduction in Palmer amaranth biomass (%)	
PRE/EPOST	LPOST	14 DA-EPOST	14 DA-LPOST	14 DA-EPOST	14 DA-LPOST
Non-treated control	-	-	-	-	-
Sulfentrazone + cloransulam-methyl (PRE)	-	67 bc	47 b	30 d	29 d
	2,4-D choline	69 bc	42 b	56 bc	32 d
	Glufosinate	80 ab	93 a	54 cd	93 ab
	2,4-D choline + glufosinate	69 bc	99 a	64 a-c	98 ab
Imazethapyr + saflufenacil + pyroxasulfone (PRE)	-	75 a-c	90 a	72 a-c	81 b
	2,4-D choline	88 a	96 a	81 ab	91 ab
	Glufosinate	92 a	100 a	76 a-c	100 a
	2,4-D choline + glufosinate	93 a	100 a	82 a	100 a
Chlorimuron ethyl/flumioxazin/metribuzin (PRE)	-	81 ab	93 a	64 a-c	63 c
	2,4-D choline	92 a	96 a	71 a-c	92 ab
	Glufosinate	86 ab	89 a	79 a-c	94 ab
	2,4-D choline + glufosinate	92 a	100 a	88 a	100 a
Glufosinate (EPOST)	Glufosinate	86 ab	98 a	69 a-c	99a
2,4-D choline (EPOST)	2,4-D choline	60 c	98 a	71 a-c	99 a
p-value		<0.0001	<0.0001	<0.0001	<0.0001
Contrast Analysis					
PRE vs. PRE fb LPOST		-	76 vs. 91***	-	58 vs. 99***
PRE fb LPOST vs. EPOST fb LPOST		-	91 vs. 98*	-	89 vs. 99*

Figure 5.1 Glyphosate-resistant Palmer amaranth control in 2,4-D-tolerant soybean in response to (A) Non-treated, (B) Imazethapyr + saflufenacil + pyroxasulfone (PRE) fb glufosinate (LPOST), (C) Imazethapyr + saflufenacil + pyroxasulfone (PRE) fb 2,4-D choline + glufosinate (LPOST), (D) Chlorimuron ethyl + flumioxazin + metribuzin fb 2,4-D choline + glufosinate, (E) Glufosinate (EPOST) fb glufosinate (LPOST), (F) 2,4-D choline (EPOST) fb 2,4-D choline (LPOST) at 14 DA-LPOST.

Table 5.5 Soybean yield affected by herbicide programs in 2,4-D-, glyphosate-, and glufosinate-tolerant soybean in a field experiment conducted at Carleton, Nebraska in 2019. Means presented within each column with no common letter(s) are significantly different based on Fisher's protected LSD test, where $\alpha = 0.05$.

Herbicide programs		Soybean yield
PRE/EPOST	LPOST	(kg ha^{-1})
Non-treated control	-	2,398 f
Sulfentrazone + cloransulam-methyl (PRE)	-	2,835 f
	2,4-D choline	4,394 abcd
	Glufosinate	4,068 d
	2,4-D choline + glufosinate	4,561 abcd
Imazethapyr + saflufenacil + pyroxasulfone (PRE)	-	3,462 e
	2,4-D choline	4,752 ab
	Glufosinate	4,325 bcd
	2,4-D choline + glufosinate	4,657 abc
Chlorimuron ethyl + flumioxazin + metribuzin (PRE)	-	2,832 f
	2,4-D choline	4,712 ab
	Glufosinate	4,162 cd
	2,4-D choline + glufosinate	4,671 ab
Glufosinate (EPOST)	Glufosinate	4,574 abc
2,4-D choline (EPOST)	2,4-D choline	4,837 a
p-value		<0.001
Contrast Analysis		
PRE vs. PRE fb LPOST		3,043 vs. 4,478***
PRE fb LPOST vs. EPOST fb LPOST		4,478 vs. 4,706^{NS}

Discussion

GR Palmer amaranth is an important troublesome weed in soybean production in the Midwest US. This current study aimed to evaluate effective herbicide programs to manage GR

Palmer amaranth in 2,4-D- tolerant soybean. All the PRE herbicides evaluated in this study provided good control of GR Palmer amaranth at 14 DA-PRE (Table 5.3). Similar to this findings, Sarangi and Jhala (2019) reported 97% to 100% control of Palmer amaranth with chlorimuron ethyl + flumioxazin + metribuzin, saflufenacil + imazethapyr + dimethenamid-P, and sulfentrazone + metribuzin at 14 DA-PRE in soybean. In another study, Aulakh and Jhala (2015) reported 95% control of waterhemp with sulfentrazone + cloransulam-methyl at 15 DA-PRE in glufosinate- tolerant soybean in Nebraska. Among the EPOST treatments, at 14 DA-EPOST, 2,4-D application provided less control of GR Palmer amaranth than glufosinate (Table 5.3). Less control by 2,4-D could be attributed to the variable Palmer amaranth height at the time of application. For instance, Craigmyle et al. (2013) and Everitt and Keeling (2007) reported that weed height at the time of 2,4-D application can affect the level of broadleaf weed control achieved. The residual activity of herbicides applied PRE declined as the season progressed (Table 5.3). A similar decline in residual activity of soil-applied PRE herbicides has been reported in no-till soybean in Nebraska where PRE-only herbicides resulted in 66% control of Palmer amaranth compared with 86% control by PRE fb POST herbicide programs at 28 d after POST (Sarangi and Jhala 2019). Statistically similar control by chlorimuron ethyl + flumioxazin + metribuzin applied PRE alone and sequential application of 2,4-D or glufosinate (Table 5.3) might be attributed to a high level of GR Palmer amaranth control by the residual activity of this premix. Palmer amaranth is known for its extended emergence pattern (Jha and Norsworthy 2009); however, emergence is reported to be higher from early May to mid-July, which is before soybean canopy closure (Jha and Norsworthy 2009). Thus, PRE herbicide would not only provide emerging soybean seedlings a weed-free start but also result in reduced reliance on POST herbicides (Norsworthy et al. 2012). Sequential application (EPOST fb LOST) of 2,4-D or

glufosinate provided 85% to 92% control of GR Palmer amaranth (Table 5.3). Similarly, Chahal and Jhala (2015) reported 86% to 98% waterhemp control 75 DA-LPOST with single as well as sequential application of glufosinate in glufosinate- tolerant soybean. Meyer et al. (2015) showed that synthetic auxin herbicide-based LPOST programs can be options to control GR Palmer amaranth and waterhemp in soybean traits resistant to 2,4-D or dicamba. Overall, this study showed that PRE fb LPOST programs managed GR Palmer amaranth more effectively than PRE alone but provided similar control as sequential POSTs (Table 5.3). Similarly, Sarangi et al. (2017) reported 90% control of GR waterhemp in soybean with PRE fb LPOST herbicide programs. Contrary to these findings, authors of several studies have found greater control of GR waterhemp with PRE fb POST herbicide programs compared with POST-only programs in soybean (Aulakh and Jhala 2015, Johnson et al. 2012, Sarangi et al. 2017).

Higher Palmer amaranth emergence in 2019 could be attributed to adequate rainfall in 2019 compared with 2018 (Table 5.2) and a substantial Palmer amaranth seed bank at this location. Adequate soil moisture favors the germination of Palmer amaranth seeds (Hartzler et al. 1999). Following the trend of GR Palmer amaranth control, percent density reduction at 14 DA-EPOST was higher with glufosinate than 2,4-D application (Table 5.4). However, Palmer amaranth was at a variable height when EPOST herbicides were applied and it is well known that the efficacy of auxinic herbicides can vary with weed height and density (Barnett et al. 2013, Craigmyle et al. 2013, Everitt and Keeling 2007, Jhala et al. 2017, Steckel et al. 1997). Poor density reduction of GR Palmer amaranth by sulfentrazone + cloransulam-methyl fb 2,4-D (42%) was most likely due to declining residual activity which led to uneven Palmer amaranth size when LPOST herbicides were applied. Overall, contrast analysis showed that sequential POST application resulted in a higher reduction of GR Palmer amaranth density compared to

PRE fb LPOST (Table 5.4), However, this statistical difference can be attributed to 42% density reduction by sulfentrazone + cloransulam-methyl fb 2,4-D, which influenced the estimated mean of the PRE fb LPOST programs (Table 5.4). Norsworthy et al. (2012) and Aulakh and Jhala (2015) have stressed the importance of PRE fb POST with residual herbicide programs as important in-season measures to reduce GR Palmer amaranth density and seed production in soybean. Moreover, PRE fb LPOST programs will be more sustainable than the sequential application of 2,4-D or glufosinate in EPOST fb LPOST pro-grams, due to the integration of herbicides with multiple sites of action. Miller and Norsworthy (2016) reported a lower density of GR Palmer amaranth plants with herbicide programs involving multiple sites of action compared with a single EPOST application of 2,4-D + glyphosate in 2,4-D-, glyphosate-, and glufosinate- tolerant soybean. More importantly, repeated use of herbicides with the same site of action (*e.g.*, 2,4-D or glufosinate) would select for HR-weed biotypes. It is important to note that 2,4-D resistance has been already confirmed in waterhemp populations from Nebraska, Illinois, and Missouri (Bernards et al. 2012, Evans et al. 2019, Figueiredo et al. 2018, Shergill et al. 2018a, Shyam et al. 2019) and Palmer amaranth population from Kansas (Kumar et al. 2019b, Shyam et al. 2021a).

Aboveground biomass reduction of Palmer amaranth followed the same trend as density reduction (Table 5.4). Low reduction of biomass was found with glufosinate or 2,4-D application. This can be attributed to taller Palmer amaranth plants, which reduced the efficacy of glufosinate or 2,4-D. Overall, the PRE fb LPOST programs resulted in good biomass reduction (except sulfentrazone + cloransulam-methyl fb 2,4-D) and were comparable to EPOST fb LPOST herbicide programs at 14 DA-LPOST (Table 5.4). Averaged across herbicide programs, PRE fb LPOST programs resulted in a higher reduction of Palmer amaranth biomass

compared with PRE-only programs at 14 DALPOST (Table 5.4). EPOST fb LPOST and PRE fb LPOST programs provided comparable reduction in biomass (Table 5.4). This was due to the poor efficacy of sulfentrazone + cloransulam-methyl fb 2,4-D, which failed to reduce Palmer amaranth biomass. Chahal and Jhala (2015) reported a higher reduction in GR volunteer corn biomass with sequential application of glufosinate in glufosinate- tolerant soybean. Sarangi and Jhala (2019) showed a high biomass reduction of Palmer amaranth in soybean with PRE fb POST herbicides with residual activity ranging from 96% to 100%. PRE herbicides in this study were fb a mixture of 2,4-D + glufosinate; however, Palmer amaranth biomass reduction was similar to PRE fb 2,4-D or glufosinate. Tank mixing herbicides such as glufosinate + 2,4-D can further improve control of GR Palmer amaranth if applied with PRE herbicide application at planting. For example, Ganie and Jhala (2017) reported that tank mixing 2,4-D with glufosinate provided 80% to 92% control of GR giant ragweed. In this study, preformulated mixtures of imazethapyr + saflufenacil + pyroxasulfone, and chlorimuron ethyl + flumioxazin + metribuzin applied PRE fb 2,4-D or glufosinate, controlled GR Palmer amaranth 92% to 99% and reduced density and biomass by 89% to 100% and 91% to 100%, respectively; therefore, a tank mixture of 2,4-D + glufosinate is not needed to achieve optimum Palmer amaranth control if PRE herbicide is applied; however, if grass weeds are present in the field, tank mixing 2,4-D with glyphosate or glufosinate will be needed. To maintain the effectiveness of this herbicide program, however, it will be crucial to follow labeled application timings with appropriate soybean and Palmer amaranth growth stages, because 2,4-D can be applied up to the R2 soybean growth stage whereas glufosinate cannot be applied after soybean starts flowering.

Averaged across treatments, PRE fb LPOST herbicide programs resulted in greater yield (4,478 kg ha^{-1}) compared with PRE-only programs (3,043 kg ha^{-1}) (Table 5.5). This indicates that if Palmer amaranth is the predominant weed in the soybean production field, using PRE-only program will not provide optimum yield. Similarly, Whitaker et al. (2010) reported greater soybean yield with PRE herbicide fb fomesafen compared with PRE-only programs. Even though yield obtained from PRE fb LPOST programs and sequential application of 2,4-D or glufosinate (EPOST fb LPOST) programs were comparable (Table 5.5), it is advisable to use PRE fb POST herbicides because it will provide more opportunity to use an herbicide program with multiple sites of action and reduce the exposure of a substantial number of Palmer amaranth plants to POST herbicides. Sarangi and Jhala (2019) reported that PRE fb POST herbicide programs were effective for controlling Palmer amaranth and obtaining greater soybean yield, whereas Grey et al. (2013) showed that combinations of multiple herbicide sites of action applied PRE and POST would be necessary to manage multiple HR Palmer amaranth. Butts et al. (2016) stated the importance of PRE fb POST herbicide programs as a component of an integrated weed management program to effectively control *Amaranthus* species as well as increase soybean yield. Similarly, multiyear field experiments conducted in Nebraska have shown that PRE fb POST herbicides with or without residual activity can effectively control weeds such as GR waterhemp and prevent yield loss of GR and glufosinate tolerant soybean (Jhala et al. 2017, Sarangi et al. 2017).

Conclusion

Overall, this study showed that 2,4-D- tolerant soybean will provide additional POST herbicide options such as 2,4-D or glufosinate to soybean growers for managing not only GR but also multiple HR Palmer amaranth. Adoption of integrated weed management practice is

necessary to maintain the effectiveness of 2,4-D-, glyphosate-, and glufosinate-resistant crop technology (Miller and Norsworthy 2016). For example, Enlist 3^{TM} corn, resistant to 2,4-D, glufosinate, glyphosate, and aryloxyphenoxypropionates is available commercially, but it should not be planted in rotation with 2,4-D-, glyphosate-, and glufosinate- tolerant soybean to avoid the use of the same POST herbicides in the same field.

Chapter 6 - General Summary and Conclusions

Palmer amaranth (*Amaranthus palmeri* S. Watson) is a driver weed in various cropping systems throughout the US (Van Wychen 2020). Rapid biomass accumulation, high adaptability, fecundity, and ability to evolve resistance to herbicides are some of the factors contributing to rapid adaptation of this weed across the US (Ehleringer 1985, Heap 2021, Horak and Loughin 2000, Keeley et al. 1987). In Palmer amaranth evolution of resistance to 8 modes of action (MOA), *i.e.*, acetolactate synthase (ALS)-, photosystem II (PS II)-, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-, hydroxyphenylpyruvate dioxygenase (HPPD)-, protoporphyrinogen oxidase (PPO)-, very long-chain fatty acid (VLCFA)-inhibitors and synthetic auxinic herbicides (SAH) has been documented (Heap 2021).

2,4-dichlorophenoxyacetic acid (2,4-D), a SAH is widely used to control broadleaf weeds (Peterson et al. 2016). In recent times, 2,4-D usage has increased primarily to control ALS- and EPSPS- inhibitor-resistant weeds. However, so far only two populations of Palmer amaranth, including KCTR (Kansas Conservation Tillage Resistant; the focus of this dissertation) have been documented to evolve resistance to 2,4-D (Kumar et al. 2019b, Heap 2021). Moreover, 2,4-D-resistant cotton, soybean, and corn technologies have been commercialized, which can increase the use of this herbicide thereby increasing selection pressure. Characterization and investigation of mechanism(s) and genetic basis of resistance to 2,4-D in resistant weeds, such as KCTR Palmer amaranth, will help understand the possible spread and further evolution of such resistance. Further, it is also important to evaluate herbicide programs that can increase the longevity of 2,4-D resistant crop technology and offer growers additional options in controlling glyphosate-resistant (GR) Palmer amaranth.

In this dissertation, characterization of multiple herbicide resistance (Chapter 2), as well as physiological (Chapter 3) and genetic aspects (Chapter 4) of 2,4-D resistance in KCTR, were investigated. Additionally, effective herbicide programs to manage and control GR Palmer amaranth in 2,4-D- tolerant soybean were evaluated (Chapter 5). KCTR was found to be multiple herbicide-resistant to at least 6 herbicide MOAs including ALS- (imazethapyr, imazamox, and thifensulfuron), PS II- (atrazine, and metribuzin), EPSPS- (glyphosate), HPPD- (mesotrione, and tembotrione), PPO-inhibitors (lactofen, and fomesafen) and SAH (2,4-D). Complete control of KCTR plants was achieved only with the application of either PS I- (paraquat)- or glutamine synthase-(glufosinate) inhibitors (Shyam et al. 2021). *ALS* (target site of ALS-inhibitors) and *psbA* (target site of PS II-inhibitors) genes of KCTR plants that survived imazethapyr and chlorsulfuron (ALS-inhibitors) and atrazine (PS II-inhibitors), respectively, were sequenced and analyzed. But none of the known mutations which confer resistance to ALS or PS II-inhibitors were found (Chapter 2). Up to 88 copies of *EPSPS* gene (target site of glyphosate) were found in GR KCTR plants (Chapter 2) indicating that amplification of *EPSPS* gene is the primary mechanism of glyphosate resistance, as reported in other GR Palmer amaranth populations (Gaines et al. 2010, Koo et al. 2018). Moreover, KCTR plants treated with cytochrome P450 (P450)-inhibitor, malathion, prior to application of lactofen, 2,4-D, and mesotrione or glutathione *S*-transferase (GST)-inhibitor, 4-chloro-7-nitrobenzofurazan (NBD-Cl) before atrazine application exhibited decreased resistance levels indicating the predominance of metabolic resistance, mediated by P450s or GSTs in this population (Chapter 2). Previously, three other populations of Palmer amaranth from Kansas with multiple herbicide resistance have been documented (Chaudhuri et al. 2020, Kumar et al. 2019b, Nakka et al. 2017a, 2017b, 2017c).

Investigation of the physiological basis of 2,4-D resistance confirmed that enhanced metabolism of 2,4-D bestows resistance to this herbicide in KCTR Palmer amaranth (Chapter 3). KCTR plants metabolized ~20-30% more [^{14}C] 2,4-D than the susceptible populations KSS (Kansas Susceptible) and MSS (Mississippi Susceptible) at 24, 48, and 72 hours after treatment (HAT). In addition, a comparison of percent parent [^{14}C] 2,4-D retained and the retention time (RT) of polar metabolites in wheat (KS Western Star) and KCTR plants revealed that even though wheat plants metabolized [^{14}C] 2,4-D at a much faster rate than KCTR, no unique metabolites were found in wheat. No significant difference was found in the amount of [^{14}C] 2,4-D absorption between KCTR, KSS, and MSS indicating that differential absorption is not involved in 2,4-D resistance. However, ~10% less [^{14}C] 2,4-D translocated in KCTR, and the rate of translocation was also found to be ~3 times slower (Chapter 3). Considering the high rate of [^{14}C] 2,4-D absorption and translocation, such a difference in percent translocation is less likely to contribute to 2,4-D resistance in KCTR. Moreover, slower translocation might involve slower movement of [^{14}C] 2,4-D polar metabolites. Whole plant dose-response assays were conducted to evaluate the level of 2,4-D resistance in KCTR and KSS plants pretreated with P450- inhibitors, malathion, or PBO before 2,4-D application. Interestingly, application of malathion prior to 2,4-D treatment significantly increased susceptibility to 2,4-D in KCTR indicating the involvement of P450s in metabolizing 2,4-D. Overall, the results of Chapter 3 confirmed that 2,4-D is being metabolized rapidly by P450s in KCTR compared to KSS and MSS populations. Similar to our findings, enhanced metabolism of 2,4-D and reversal of resistance by malathion pre-treatment was documented in common waterhemp (*Amaranthus tuberculatus* Moq. Sauer) populations from Nebraska and Missouri (Figueredo et al. 2018, Shergill et al. 2018b), a close relative of Palmer amaranth.

Metabolic resistance to herbicides in weeds has been documented to be inherited either by a single or multiple genes (Huffman et al. 2015, Oliveira et al. 2018a, Pandian et al. 2021, Shyam et al. 2021). In Chapter 4 of this dissertation, the genetic basis of 2,4-D resistance was investigated. F_1 and F_2 families were generated by crossing 2,4-D-resistant, KCTR, and 2,4-D-susceptible, KSS Palmer amaranth. The genetic analyses of the data from these experiments revealed that 2,4-D resistance is a nuclear incompletely dominant polygenic trait in KCTR. Similarly, incompletely dominant 2,4-D resistance was reported in wild radish (*Raphanus raphanistrum* L.) (Busi and Powles 2017). Also, polygenic resistance to 2-methyl-4-chlorophenoxyacetic acid (MCPA) was documented in hemp-nettle (*Galeopsis tetrahit* L.) (Weinberg et al. 2006). On the other hand, single gene-mediated 2,4-D resistance was found in wild mustard (*Brassica kaber* L.), and a completely dominant gene controlling 2,4-D resistance was also found in wild radish and wild mustard (Busi and Powles 2017, Jugulam et al. 2005).

In Chapter 5, herbicide programs involving pre-emergence (PRE), PRE followed by (fb) late post-emergence (LPOST) and early post-emergence (EPOST) fb LPOST applications were evaluated to control GR Palmer amaranth in 2,4-D-tolerant soybean. Application of PRE effectively controlled GR Palmer amaranth effectively at 14 days after PRE (DA-PRE) but over time as the season progressed their residual efficacy declined. A similar decline in efficacy of PRE herbicides in the cropping season was documented by Sarangi and Jhala (2019). Overall, PRE fb 2,4-D and/or glufosinate, and sequential application of either 2,4-D or glufosinate significantly controlled GR Palmer amaranth, caused a reduction of biomass and density, resulting in high soybean yield. No soybean injury was observed from any of the herbicide programs evaluated. Previously, Sarangi and Jhala (2019) reported that PRE fb LPOST programs provided better control of Palmer amaranth compared to PRE-only applications later in the

season. Even though sequential application of either 2,4-D or glufosinate as EPOST fb LPOST effectively controlled GR palmer amaranth, it is not advised to practice such programs, since these involve a single herbicide MOA and can encourage the selection of herbicide resistance. Instead, PRE fb LPOST programs incorporating multiple herbicide MOAs are more effective long-term in controlling GR Palmer amaranth in 2,4-D-tolerant soybean and reducing selection pressure.

In conclusion, the KCTR population was found to have predominantly metabolic resistance to at least 5 herbicide MOAs including ALS-, PS II-, HPPD-, PPO-inhibitors, and SAH (Shyam et al. 2021a). Enhanced metabolism possibly mediated by P450(s) is the primary mechanism of 2,4-D resistance in KCTR Palmer amaranth. Genetic studies confirmed that 2,4-D resistance in KCTR is an incompletely dominant polygenic trait. Lastly, PRE fb LPOST programs involving multiple herbicide MOAs should be followed for effective control of GR Palmer amaranth in 2,4-D-tolerant soybean.

Future research can be focused on identifying the polar metabolites of 2,4-D *via* LC/MS techniques to better characterize the biochemical pathway of 2,4-D degradation in KCTR in comparison to tolerant cereals such as wheat. Specific P450 genes or other genes involved in physiological processes that facilitate 2,4-D resistance in KCTR need to be identified. With the availability of good quality Palmer amaranth genome (Montgomery et al. 2020), an RNA-sequencing approach can be employed to further identify differentially expressed genes in 2,4-D-resistant KCTR and susceptible KSS populations. It will also be important to understand whether the identified P450s are involved in mediating multiple herbicide resistance in KCTR. The possible presence of target-site resistance (TSR) to auxins in receptor or transporter proteins also needs to be investigated. Recently, two instances of field evolved TSR to SAH have been

documented in the AUX/IAA repressor in kochia and Indian hedge mustard (Figueredo et al. 2021, Le Clere et al. 2018). The possible co-existence of TSR along with non-target site resistance in the KCTR population for mediating 2,4-D resistance needs to be investigated. Investigation of aspects such as intra-specific movement of 2,4-D resistance to other *Amaranthus sp*. and the effect of temperature stress on 2,4-D resistance is also valuable. Overall, the findings of this research significantly improve our understanding of 2,4-D resistance in Palmer amaranth and will help in answering more in-depth questions in the future. As the adoption of 2,4-D-tolerant crop technology is on the rise, the possibility of increased evolution of 2,4-D resistance is also on the rise. Therefore, information obtained from the current and future research on this population can help us formulate better sustainable management practices and reduce the selection of 2,4-D resistance.

References

Abas L, Kolb M, Stadlmann J, Janacek DP, Lukic K, Schwechheimer C, Sazanov LA, Mach L, Friml J, Hammes UZ (2021) Naphthylphthalamic acid associates with and inhibits PIN auxin transporters. Proc Natl Acad Sci USA 118: e2020857118

Abendroth JA, Martin AR, Roeth FW (2006) Plant response to combinations of mesotrione and photosystem II inhibitors. Weed Technol 20:267–274

Acker RCV, Swanton CJ, Weise SF (1993) The critical period of weed control in soybean [*Glycine max* (L.) Merr.]. Weed Sci 41:194–200

Aulakh JS, Jhala AJ (2015) Comparison of glufosinate-based herbicide programs for broad-spectrum weed control in glufosinate-resistant soybean. Weed Technol 29:419–430

Aulakh JS, Price AJ, Enloe SF, Santen EV, Wehtje G, Patterson MG (2010) Integrated Palmer amaranth management in glufosinate-resistant cotton: I. Soil-inversion, high-residue cover crops and herbicide regimes. Agronomy 24:295-311

Ballas N, Wong LM, Theologis A (1993) Identification of the auxin-responsive element, AuxRE, in the primary indoleacetic acid-inducible gene, *PS-IAA4/5*, of pea (*Pisum sativum*). J Mol Biol 233:580–596

Barnett KA, Culpepper AS, York AC, Steckel LE (2013) Palmer amaranth (*Amaranthus palmeri*) Control by glufosinate plus fluometuron applied postemergence to WideStrike® cotton. Weed Technol 27:291–297

Basinger NT, Jennings KM, Monks DW, Jordan DL, Everman WJ, Hestir EL, Bertucci MB, Brownie C (2019a) Large crabgrass (*Digitaria sanguinalis*) and Palmer amaranth (*Amaranthus palmeri*) intraspecific and interspecific interference in soybean. Weed Sci 67:649–656

Basinger NT, Jennings KM, Monks DW, Jordan DL, Everman WJ, Hestir EL, Waldschmidt MD, Smith SC, Brownie C (2019b) Interspecific and intraspecific interference of Palmer amaranth (*Amaranthus palmeri*) and large crabgrass (*Digitaria sanguinalis*) in sweet potato. Weed Sci 67:426–432

Beckie HJ (2011) Herbicide-resistant weed management: Focus on glyphosate. Pest Manag Sci 67:1037–1048

Beckie HJ, Hall LM, Shirriff SW, Martin E, Leeson JY (2019) Triple-resistant kochia [*Kochia scoparia* (L.) Schrad.] in Alberta. Can J Plant Sci 99:281–285

Behrens MR, Mutlu N, Chakraborty S, Dumitru R, Jiang WZ, Lavallee BJ, Herman PL, Clemente TE, Weeks DP (2007) Dicamba resistance: Enlarging and preserving biotechnology-based weed management strategies. Science 316:1185–1188

Bensch CN, Horak MJ, Peterson D (2003) Interference of redroot pigweed (*Amaranthus retroflexus),* Palmer amaranth (*A. palmeri*), and common waterhemp (*A. rudis*) in soybean. Weed Sci 51:37–43

Bentley R (1990) The shikimate pathway--A metabolic tree with many branches. Crit Rev Biochem Mol Biol 25:307–384

Berger ST, Ferrell JA, Rowland DL, Webster TM (2015) Palmer amaranth (*Amaranthus palmeri)* competition for water in cotton. Weed Sci 63:928–935

Bernards ML, Crespo RJ, Kruger GR, Gaussoin R, Tranel PJ (2012) A waterhemp (*Amaranthus tuberculatus*) population resistant to 2,4-D. Weed Sci 60:379–384

Bertucci MB, Jennings KM, Monks DW, Schultheis JR, Louws FJ, Jordan DL (2019) Interference of Palmer amaranth (*Amaranthus palmeri*) density in grafted and nongrafted watermelon. Weed Sci 67:229–238

Boller T, Herner RC, Kende H (1979) Assay for and enzymatic formation of an ethylene precursor, 1-aminocyclopropane-1-carboxylic acid. Planta 145:293–303

Bourdot GW, Saville DJ, Hurrell GA (1996) Ecological fitness and the decline of resistance to the herbicide MCPA in a population of *Ranunculus acris*. J Appl Ecol 33:151–160

Brabham C, Norsworthy JK, Houston MM, Varanasi VK, Barber T (2019) Confirmation of S-metolachlor resistance in Palmer amaranth (*Amaranthus palmeri*). Weed Technol 33:720–726

Bristol DW, Ghanuni AM, Oleson AE (1977) Metabolism of 2,4-dichlorophenoxyacetic acid by wheat cell suspension cultures. J Agric Food Chem 25:1308–1314

Burke IC, Schroeder M, Thomas WE, Wilcut JW (2007) Palmer amaranth interference and seed production in peanut. Weed Technol 21:367–371

Busi R, Goggin DE, Heap IM, Horak MJ, Jugulam M, Masters RA, Napier RM, Riar DS, Satchivi NM, Torra J, Westra P, Wright TR (2018) Weed resistance to synthetic auxin herbicides. Pest Manag Sci 74:2265–2276

Busi R, Powles SB (2017) Inheritance of 2,4-D resistance traits in multiple herbicide- resistant *Raphanus raphanistrum* populations. Plant Sci Int J Exp Plant Biol 257:1–8

Butts TR, Norsworthy JK, Kruger GR, Sandell LD, Young BG, Steckel LE, Loux MM, Bradley KW, Conley SP, Stoltenberg DE, Arriaga FJ, Davis VM (2016) Management of pigweed (*Amaranthus spp.)* in glufosinate-resistant soybean in the Midwest and Mid-south. Weed Technol 30:355–365

Calderón Villalobos LIA, Lee S, De Oliveira C, Ivetac A, Brandt W, Armitage L, Sheard LB, Tan X, Parry G, Mao H, Zheng N, Napier R, Kepinski S, Estelle M (2012) A combinatorial TIR1/AFB-Aux/IAA co-receptor system for differential sensing of auxin. Nat Chem Biol 8:477–485

Chahal PS, Irmak S, Jugulam M, Jhala AJ (2018) Evaluating effect of degree of water stress on growth and fecundity of Palmer amaranth (*Amaranthus palmeri*) using soil moisture sensors. Weed Sci 66:738–745

Chahal PS, Jhala AJ (2015) Herbicide programs for control of glyphosate-resistant volunteer corn in glufosinate-resistant soybean. Weed Technol 29:431–443

Chahal PS, Jhala AJ (2018) Economics of management of photosystem II- and HPPD-inhibitor-resistant palmer amaranth in corn. Agron J 110:1905–1914

Chahal PS, Jugulam M, Jhala AJ (2019a) Basis of atrazine and mesotrione synergism for controlling atrazine- and HPPD inhibitor-resistant Palmer amaranth. Agron J 111:3265–3273

Chahal PS, Jugulam M, Jhala AJ (2019b) Mechanism of atrazine resistance in atrazine- and HPPD inhibitor-resistant Palmer amaranth (*Amaranthus palmeri* S. Wats.) from Nebraska. Can J Plant Sci 99:815–823

Chahal PS, Varanasi VK, Jugulam M, Jhala AJ (2017) Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) in Nebraska: Confirmation, *EPSPS* gene amplification, and response to post corn and soybean herbicides. Weed Technol 31:80–93

Chandi A, Jordan DL, York AC, Milla-Lewis SR, Burton JD, Culpepper AS, Whitaker JR (2012) Interference of selected palmer amaranth (*Amaranthus palmeri*) biotypes in soybean (*Glycine max*). Int J Agron 2012:e168267

Chaudhari S, Varanasi VK, Nakka S, Bhowmik PC, Thompson CR, Peterson DE, Currie RS, Jugulam M (2020) Evolution of target and non-target based multiple herbicide resistance in a single Palmer amaranth (*Amaranthus palmeri*) population from Kansas. Weed Technol 34:447–453

Cho M, Cho H-T (2013) The function of ABCB transporters in auxin transport. Plant Signal Behav 8

Christopher JT, Preston C, Powles SB (1992) Malathion antagonizes metabolism-based chlorsulfuron resistance in *Lolium rigidum*. Pestic Biochem Phys 49:172–182

Copeland JD, Giacomini DA, Tranel PJ, Montgomery GB, Steckel LE (2018) Distribution of *PPX2* mutations conferring PPO-inhibitor resistance in Palmer amaranth populations of Tennessee. Weed Technol 32:592–596

Craigmyle BD, Ellis JM, Bradley KW (2013) Influence of herbicide programs on weed management in soybean with resistance to glufosinate and 2,4-D. Weed Technol 27:78–84

Cranston HJ, Kern AJ, Hackett JL, Miller EK, Maxwell BD, Dyer WE (2001) Dicamba resistance in kochia. Weed Sci 49:164–170

Crespo RJ, Wingeyer AB, Kruger GR, Riggins CW, Tranel PJ, Bernards ML (2017) Multiple-herbicide resistance in a 2,4-D–resistant waterhemp (*Amaranthus tuberculatus*) population from Nebraska. Weed Sci 65:743–754

Culpepper AS, Grey TL, Vencill WK, Kichler JM, Webster TM, Brown SM, York AC, Davis JW, Hanna WW (2006) Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) confirmed in Georgia. Weed Sci 54:620–626

Cummins I, Wortley DJ, Sabbadin F, He Z, Coxon CR, Straker HE, Sellars JD, Knight K, Edwards L, Hughes D, Kaundun SS, Hutchings S-J, Steel PG, Edwards R (2013) Key role for a glutathione transferase in multiple-herbicide resistance in grass weeds. Proc Natl Acad Sci USA 110:5812–5817

Dang HT, Malone JM, Boutsalis P, Krishnan M, Gill G, Preston C (2018) Reduced translocation in 2,4-D-resistant oriental mustard populations (*Sisymbrium orientale* L.) from Australia. Pest Manag Sci 74:1524–1532

Davidonis GH, Hamilton RH, Mumma RO (1982) Metabolism of 2,4-dichlorophenoxyacetic acid in 2,4-dichlorophenoxyacetic acid-resistant soybean callus tissue. Plant Physiol 70:104–107

deBoer GJ, Thornburgh S, Ehr RJ (2006) Uptake, translocation and metabolism of the herbicide florasulam in wheat and broadleaf weeds. Pest Manag Sci 62:316–324

deBoer GJ, Thornburgh S, Gilbert J, Gast RE (2011) The impact of uptake, translocation and metabolism on the differential selectivity between blackgrass and wheat for the herbicide pyroxsulam. Pest Manag Sci 67:279–286

Debreuil DJ, Friesen LF, Morrison IN (1996) Growth and seed return of auxin-type herbicide resistant wild mustard (*Brassica kaber*) in wheat. Weed Sci 44:871–878

Dellaferrera I, Cortés E, Panigo E, De Prado R, Christoffoleti P, Perreta M (2018) First report of *Amaranthus hybridus* with multiple resistance to 2,4-D, dicamba, and glyphosate. Agronomy 8:140

Dharmasiri N, Dharmasiri S, Estelle M (2005a) The F-box protein TIR1 is an auxin receptor. Nature 435:441–445

Dharmasiri N, Dharmasiri S, Weijers D, Lechner E, Yamada M, Hobbie L, Ehrismann JS, Jürgens G, Estelle M (2005b) Plant development is regulated by a family of auxin receptor F box proteins. Dev Cell 9:109–119

Dille JA, Stahlman PW, Thompson CR, Bean BW, Soltani N, Sikkema PH (2020) Potential yield loss in grain sorghum (*Sorghum bicolor*) with weed interference in the United States. Weed Technol 34:624–629

Dimaano NG, Iwakami S (2021) Cytochrome P450-mediated herbicide metabolism in plants: Current understanding and prospects. Pest Manag Sci 77:22–32

Dimaano NG, Yamaguchi T, Fukunishi K, Tominaga T, Iwakami S (2020) Functional characterization of cytochrome P450 *CYP81A* subfamily to disclose the pattern of cross-resistance in *Echinochloa phyllopogon*. Plant Mol Biol 102:403–416

Dixon DP, Skipsey M, Edwards R (2010) Roles for glutathione transferases in plant secondary metabolism. Phytochemistry 71:338–350

Dominguez-Valenzuela JA, Gherekhloo J, Fernández-Moreno PT, Cruz-Hipolito HE, Alcántara-de la Cruz R, Sánchez-González E, De Prado R (2017) First confirmation and characterization of target and non-target site resistance to glyphosate in Palmer amaranth (*Amaranthus palmeri*) from Mexico. Plant Physiol Biochem PPB 115:212–218

Dumont M, Letarte J, Tardif FJ (2016) Identification of a *psbA* mutation (Valine219 to Isoleucine) in Powell amaranth (*Amaranthus powellii*) conferring resistance to linuron. Weed Sci 64:6–11

Egan JF, Maxwell BD, Mortensen DA, Ryan MR, Smith RG (2011) 2,4-dichlorophenoxyacetic acid (2,4-D)-resistant crops and the potential for evolution of 2,4-D-resistant weeds. Proc Natl Acad Sci USA108:E37–E37

Ehleringer J (1983) Ecophysiology of *Amaranthus palmeri*, a Sonoran Desert summer annual. Oecologia 57:107–112

Ehleringer J (1985) Annuals and perennials of warm deserts. Pages 162–180 *in* BF Chabot, HA Mooney, eds. Physiological ecology of north american plant communities. Dordrecht: Springer Netherlands

Epp JB, Alexander AL, Balko TW, Buysse AM, Brewster WK, Bryan K, Daeuble JF, Fields SC, Gast RE, Green RA, Irvine NM, Lo WC, Lowe CT, Renga JM, Richburg JS, Ruiz JM, Satchivi NM, Schmitzer PR, Siddall TL, Webster JD, Weimer MR, Whiteker GT, Yerkes CN (2016) The discovery of Arylex™ active and Rinskor™ active: Two novel auxin herbicides. Bioorg Med Chem 24:362–371

Evans CM, Strom SA, Riechers DE, Davis AS, Tranel PJ, Hager AG (2019) Characterization of a waterhemp (*Amaranthus tuberculatus*) population from Illinois resistant to herbicides from five site-of-action groups. Weed Technol 33:400–410

Everitt JD, Keeling JW (2007) Weed Control and Cotton (*Gossypium Hirsutum*) Response to preplant applications of dicamba, 2,4-D, and diflufenzopyr plus dicamba. Weed Technol 21:506–510

Fang SC, Butts JS (1954) Studies in plant metabolism. III. Absorption, translocation and metabolism of radioactive 2,4-D in corn and wheat plants. 123. Plant Physiol 29:56–60

Figueiredo MRA de, Küpper A, Malone JM, Petrovic T, Figueiredo ABTB de, Campagnola G, Peersen OB, Prasad KVSK, Patterson EL, Reddy ASN, Kubeš MF, Napier R, Preston C, Gaines TA (2021) An in-frame deletion mutation in the degron tail of auxin co-receptor *IAA2* confers resistance to the herbicide 2,4-D in *Sisymbrium orientale*. bioRxiv:2021.03.04.433944

Figueiredo MR, Leibhart LJ, Reicher ZJ, Tranel PJ, Nissen SJ, Westra P, Bernards ML, Kruger GR, Gaines TA, Jugulam M (2018) Metabolism of 2,4-dichlorophenoxyacetic acid contributes to resistance in a common waterhemp (*Amaranthus tuberculatus*) population. Pest Manag Sci 74:2356–2362

Fischer W-N, André B, Rentsch D, Krolkiewicz S, Tegeder M, Breitkreuz K, Frommer WB (1998) Amino acid transport in plants. Trends Plant Sci 3:188–195

Fox J, Weisberg S (2019) An {R} Companion to applied regression. 3rd edition. Thousand Oaks CA: Sage. Available at socialsciences.mcmaster.ca/jfox/Books/Companion/

Franssen AS, Skinner DZ, Al-Khatib K, Horak MJ, Kulakow PA (2001) Interspecific hybridization and gene flow of ALS resistance in *Amaranthus* species. Weed Sci 49:598–606

Frear DS (1995) Wheat microsomal cytochrome P450 monooxygenases: Characterization and importance in the metabolic detoxification and selectivity of wheat herbicides. Drug Metabol Drug Interact 12:329–357

Friesen LF, Beckie HJ, Warwick SI, Acker RCV (2011) The biology of Canadian weeds. 138. *Kochia scoparia* (L.) Schrad. Can J Plant Sci

Gaines TA, Shaner DL, Ward SM, Leach JE, Preston C, Westra P (2011) Mechanism of resistance of evolved glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*). J Agric Food Chem 59:5886–5889

Gaines TA, Ward SM, Bukun B, Preston C, Leach JE, Westra P (2012) Interspecific hybridization transfers a previously unknown glyphosate resistance mechanism in *Amaranthus* species. Evol Appl 5:29–38

Gaines TA, Zhang W, Wang D, Bukun B, Chisholm ST, Shaner DL, Nissen SJ, Patzoldt WL, Tranel PJ, Culpepper AS, Grey TL, Webster TM, Vencill WK, Sammons RD, Jiang J, Preston C, Leach JE, Westra P (2010) Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. Proc Natl Acad Sci USA107:1029–1034

Gaines TA (2020) The quick and the dead: a new model for the essential role of ABA accumulation in synthetic auxin herbicide mode of action. J Exp Bot 71:3383–3385

Ganie, ZA, Jhala, AJ (2017) Interaction of 2,4-D or dicamba with glufosinate for control of glyphosate-resistant giant ragweed (*Ambrosia trifida* L.) in glufosinate-resistant maize (*Zea mays* L.). Front Plant Sci 8:1207

Geisler M, Aryal B, di Donato M, Hao P (2017) A critical view on ABC transporters and their interacting partners in auxin transport. Plant Cell Physiol 58:1601–1614

Ghanizadeh H, Harrington KC (2017) Cross-resistance to auxinic herbicides in dicamba-resistant *Chenopodium album*. N Z J Agric Res 60:45–53

Ghanizadeh H, Harrington KC, James TK (2018) A comparison of dicamba absorption, translocation and metabolism in *Chenopodium album* populations resistant and susceptible to dicamba. Crop Prot 110:112–116

Gharde Y, Singh PK, Dubey RP, Gupta PK (2018) Assessment of yield and economic losses in agriculture due to weeds in India. Crop Prot 107:12–18

Giacomini DA, Patterson EL, Küpper A, Beffa R, Gaines TA, Tranel PJ (2020) Coexpression clusters and allele-specific expression in metabolism-based herbicide resistance. Genome Biol Evol 12:2267–2278

Giacomini DA, Umphres AM, Nie H, Mueller TC, Steckel LE, Young BG, Scott RC, Tranel PJ (2017) Two new *PPX2* mutations associated with resistance to PPO-inhibiting herbicides in *Amaranthus palmeri*. Pest Manag Sci 73:1559–1563

Giacomini DA, Westra P, Ward SM (2014) Impact of genetic background in fitness cost studies: An example from glyphosate-resistant Palmer amaranth. Weed Sci 62:29–37

Godar AS, Stahlman PW, Jugulam M, Dille JA (2015) Glyphosate-resistant kochia (*Kochia scoparia)* in Kansas: *EPSPS* gene copy number in relation to resistance levels. Weed Sci 63:587–595

Goggin DE, Beckie HJ, Sayer C, Powles SB (2019) No auxinic herbicide–resistance cost in wild radish (*Raphanus raphanistrum*). Weed Sci 67:539–545

Goggin DE, Bringans S, Ito J, Powles SB (2020) Plasma membrane receptor-like kinases and transporters are associated with 2,4-D resistance in wild radish. Ann Bot 125:821–832

Goggin DE, Cawthray GR, Powles SB (2016) 2,4-D resistance in wild radish: reduced herbicide translocation via inhibition of cellular transport. J Exp Bot 67:3223–3235

Goggin DE, Kaur P, Owen MJ, Powles SB (2018) 2,4-D and dicamba resistance mechanisms in wild radish: subtle, complex and population specific? Ann Bot 122:627–640

Goss GA, Dyer WE (2003) Physiological characterization of auxinic herbicide-resistant biotypes of kochia (*Kochia scoparia*). Weed Sci 51:839–844

Graves S, Piepho H, Selzer L, Dorai-Raj S (2019) *multcompView*: Visualizations of paired comparisons. R package version 0.1-8. Available at CRAN.R-project.org/package=multcompView

Green JM (2012) The benefits of herbicide-resistant crops. Pest Manag Sci 68:1323–1331

Green JM, Owen MDK (2011) Herbicide-resistant crops: Utilities and limitations for herbicide-resistant weed management. J Agric Food Chem 59:5819–5829

Gressel J, Segel LA (1982) Interrelating factors controlling the rate of appearance of resistance: the outlook for the future. future. Pages 325-348 *In* LeBaron HM, Gressel J, ed. Herbicide Resistance in Plants. New York: John Wiley and Sons

Grey TL, Cutts GS, Newsome LJ, Newell SH (2013) Comparison of pyroxasulfone to soil residual herbicides for glyphosate resistant Palmer amaranth control in glyphosate resistant soybean. Crop Manag 12:CM-2013-0032-RS

Grossmann K (2003) Mediation of herbicide effects by hormone interactions. J Plant Growth Regul 22:109–122

Grossmann K, Kwiatkowski J (1995) Evidence for a causative role of cyanide, derived from ethylene biosynthesis, in the herbicidal mode of action of quinclorac in barnyard grass. Pestic Biochem Physiol 51:150–160

Grossmann K, Scheltrup F (1998) Studies on the mechanism of selectivity of the auxin herbicide quinmerac. Pestic Sci 52:111–118

Hall JC, Alam SMM, Murr DP (1993) Ethylene biosynthesis following foliar application of picloram to biotypes of wild mustard (*Sinapis arvensis* L.) susceptible or resistant to auxinic herbicides. Pestic Biochem Physiol 47:36–43

Hall JC, Romano M (1995) Morphological and physiological differences between the auxinic herbicide-susceptible (S) and -resistant (R) wild mustard (*Sinapis arvensis* L.) biotypes. Pestic Biochem Phys 52:149–155

Hamburg A, Puvanesarajah V, Burnett TJ, Barnekow DE, Premkumar ND, Smith GA (2001) Comparative degradation of [^{14}C]-2,4-dichlorophenoxyacetic acid in wheat and potato

after foliar application and in wheat, radish, lettuce, and apple after soil application. J Agric Food Chem 49:146–155

Han H, Yu Q, Beffa R, González S, Maiwald F, Wang J, Powles SB (2021) Cytochrome P450 *CYP81A10v7* in *Lolium rigidum* confers metabolic resistance to herbicides across at least five modes of action. Plant J Cell Mol Biol 105:79–92

Hansen H, Grossmann K (2000) Auxin-induced ethylene triggers abscisic acid biosynthesis and growth inhibition. Plant Physiol 124:1437–1448

Hartzler RG, Buhler DD, Stoltenberg DE (1999) Emergence characteristics of four annual weed species. Weed Sci 47:578–584

Heap I (2021) The International Herbicide-Resistant Weed Database. Online. Thursday, August 26, 2021. Available at www.weedscience.org

Heap I, Duke SO (2018) Overview of glyphosate-resistant weeds worldwide. Pest Manag Sci 74:1040–1049

Hodgson E, Levi PE (1999) 3 - Interactions of piperonyl butoxide with cytochrome P450. Pages 41–54 *in* DG Jones, ed. Piperonyl butoxide: the insecticide synergist. London: Academic Press

Horak MJ, Loughin TM (2000) Growth analysis of four *Amaranthus* species. Weed Sci 48:347–355

Howatt KA, Westra P, Nissen SJ (2006) Ethylene effect on kochia (*Kochia scoparia*) and emission following dicamba application. Weed Sci 54:31–37

Hoyerova K, Hosek P, Quareshy M, Li J, Klima P, Kubes M, Yemm AA, Neve P, Tripathi A, Bennett MJ, Napier RM (2018) Auxin molecular field maps define *AUX1* selectivity: many auxin herbicides are not substrates. New Phytol 217:1625–1639

HPRCC: High Plains Regional Climate Center (2020) Available at hprcc.unl.edu/. Accessed: June 27, 2020

Huffman J, Hausman NE, Hager AG, Riechers DE, Tranel PJ (2015) Genetics and inheritance of nontarget-site resistances to atrazine and mesotrione in a waterhemp (*Amaranthus tuberculatus*) population from Illinois. Weed Sci 63:799–809

Ito H, Gray WM (2006) A gain-of-function mutation in the *Arabidopsis* pleiotropic drug resistance transporter *PDR9* confers resistance to auxinic herbicides. Plant Physiol 142:63–74

Jablonkai I (2015) Herbicide metabolism in weeds - Selectivity and herbicide resistance., *In* Price A, Kelton J, Sarunaite L, ed. Herbicides, physiology of action, and safety. IntechOpen. Available from: www.intechopen.com/chapters/49345

Jasieniuk M, Maxwell B (1994) Population genetics and the evolution of herbicide resistance in weeds. Phytoprotection 75:25

Jasieniuk M, Morrison IN, Brûlé-Babel AL (1995) Inheritance of dicamba resistance in wild mustard (*Brassica Kaber*). Weed Sci 43:192–195

Jha P, Norsworthy JK (2009) Soybean canopy and tillage effects on emergence of Palmer amaranth (*Amaranthus palmeri*) from a natural seed bank. Weed Sci 57:644–651

Jhala AJ, Norsworthy JK, Ganie ZA, Sosnoskie LM, Beckie HJ, Mallory-Smith CA, Liu J, Wei W, Wang J, Stoltenberg DE (2021) Pollen-mediated gene flow and transfer of resistance alleles from herbicide-resistant broadleaf weeds. Weed Technol. 35: 173–187.

Jhala AJ, Sandell LD, Sarangi D, Kruger GR, Knezevic SZ (2017) Control of glyphosate-resistant common waterhemp (*Amaranthus rudis*) in glufosinate-resistant soybean. Weed Technol 31:32–45

Johnson G, Breitenbach F, Behnken L, Miller R, Hoverstad T, Gunsolus J (2012) Comparison of herbicide tactics to minimize species shifts and selection pressure in glyphosate-resistant soybean. Weed Technol 26:189–194

Johnston CR, Malladi A, Vencill WK, Grey TL, Culpepper AS, Henry G, Czarnota MA, Randell TM (2020) Investigation of physiological and molecular mechanisms conferring diurnal variation in auxinic herbicide efficacy. PLOS ONE 15:e0238144

Jugulam M (2021) Can non-Mendelian inheritance of extrachromosomal circular DNA-mediated *EPSPS* gene amplification provide an opportunity to reverse resistance to glyphosate? Weed Res 61:100–105

Jugulam M, McLean MD, Hall JC (2005) Inheritance of picloram and 2,4-D resistance in wild mustard (*Brassica kaber*). Weed Sci 53:417–423

Jugulam M, Shyam C (2019) Non-target-site resistance to herbicides: Recent developments. Plants 8

Kathare PK, Dharmasiri S, Vincill ED, Routray P, Ahmad I, Roberts DM, Dharmasiri N (2020) *Arabidopsis PIC30* encodes a major facilitator superfamily transporter responsible for the uptake of picolinate herbicides. Plant J 102:18–33

Kati V, Kioleoglou V, Scarabel L, Délye C (2016) Evidence for co-occurrence of resistance to ALS inhibitors and 2,4-D in corn poppy (*Papaver rhoeas*). International Weed Science Congress, IWSC 2016, IWSC., Prague, Czech Republic. pp.345.

Kaundun SS, Jackson LV, Hutchings S-J, Galloway J, Marchegiani E, Howell A, Carlin R, Mcindoe E, Tuesca D, Moreno R (2019) Evolution of target-site resistance to glyphosate in an *Amaranthus palmeri* population from Argentina and its expression at different plant growth temperatures. Plants 8

Keeley PE, Carter CH, Thullen RJ (1987) Influence of planting date on growth of Palmer amaranth (*Amaranthus palmeri*). Weed Sci 35:199–204

Kelley KB, Riechers DE (2007) Recent developments in auxin biology and new opportunities for auxinic herbicide research. Pestic Biochem Physiol 89:1–11

Kelley KB, Zhang Q, Lambert KN, Riechers DE (2006) Evaluation of auxin-responsive genes in soybean for detection of off-target plant growth regulator herbicides. Weed Sci 54:220–229

Kim J, Harter K, Theologis A (1997) Protein-protein interactions among the Aux/IAA proteins. Proc Natl Acad Sci USA 94:11786–11791

Klingaman TE, Oliver LR (1994) Palmer Amaranth (*Amaranthus palmeri*) Interference in Soybeans (*Glycine max*). Weed Sci 42:523–527

Knezevic SZ, Pavlovic P, Osipitan OA, Barnes ER, Beiermann C, Oliveira MC, Lawrence N, Scott JE, Jhala A (2019) Critical time for weed removal in glyphosate-resistant soybean as influenced by preemergence herbicides. Weed Technol 33:393–399

Knezevic SZ, Streibig JC, Ritz C (2007) Utilizing R software package for dose-response studies: The concept and data analysis. Weed Technol 21:840–848

Kniss AR, Vassios JD, Nissen SJ, Ritz C (2011) Nonlinear regression analysis of herbicide absorption studies. Weed Sci 59:601–610

Kohler EA, Throssell CS, Reicher ZJ (2004) 2,4-D rate response, absorption, and translocation of two ground ivy (*Glechoma hederacea*) populations. Weed Technol 18:917–923

Kohlhase DR, Edwards JW, Owen MDK (2018) Inheritance of 4-hydroxyphenylpyruvate dioxygenase inhibitor herbicide resistance in an *Amaranthus tuberculatus* population from Iowa, USA. Plant Sci Int J Exp Plant Biol 274:360–368

Koo D-H, Molin WT, Saski CA, Jiang J, Putta K, Jugulam M, Friebe B, Gill BS (2018) Extrachromosomal circular DNA-based amplification and transmission of herbicide resistance in crop weed *Amaranthus palmeri*. Proc Natl Acad Sci USA 115:3332–3337

Koo SJ, Neal JC, DiTomaso JM (1997) Mechanism of action and selectivity of quinclorac in grass roots. Pestic Biochem Physiol 57:44–53

Korres NE, Norsworthy JK, Mauromoustakos A (2019) Effects of Palmer amaranth (*Amaranthus palmeri)* establishment time and distance from the crop row on biological and phenological characteristics of the weed: Implications on soybean yield. Weed Sci 67:126–135

Kraft M, Kuglitsch R, Kwiatkowski J, Frank M, Grossmann K (2007) Indole-3-acetic acid and auxin herbicides up-regulate *9-cis-epoxycarotenoid dioxygenase* gene expression and abscisic acid accumulation in cleavers (*Galium aparine*): Interaction with ethylene. J Exp Bot 58:1497–1503

Křeček P, Skůpa P, Libus J, Naramoto S, Tejos R, Friml J, Zažímalová E (2009) The PIN-FORMED (PIN) protein family of auxin transporters. Genome Biol 10:249

Kreuz K, Fonné-Pfister R (1992) Herbicide-insecticide interaction in maize: Malathion inhibits cytochrome P450-dependent primisulfuron metabolism. Pestic Biochem Physiol 43:232–240

Kubeš M, Yang H, Richter GL, Cheng Y, Młodzińska E, Wang X, Blakeslee JJ, Carraro N, Petrášek J, Zažímalová E, Hoyerová K, Peer WA, Murphy AS (2012) The *Arabidopsis* concentration-dependent influx/efflux transporter *ABCB4* regulates cellular auxin levels in the root epidermis. Plant J Cell Mol Biol 69:640–654

Kumar V, Engel RP, Currie R, Jha P, Stahlman PW, Thompson C (2019a) Dicamba-resistant kochia (*Bassia scoparia*) in Kansas: Characterization and management with fall- or spring-applied PRE herbicides. Weed Technol 33:342–348

Kumar V, Liu R, Boyer G, Stahlman PW (2019b) Confirmation of 2,4-D resistance and identification of multiple resistance in a Kansas Palmer amaranth (*Amaranthus palmeri*) population. Pest Manag Sci 75:2925–2933

Küpper A, Borgato EA, Patterson EL, Netto AG, Nicolai M, Carvalho SJP de, Nissen SJ, Gaines TA, Christoffoleti PJ (2017) Multiple resistance to glyphosate and acetolactate synthase inhibitors in Palmer amaranth (*Amaranthus palmeri*) identified in Brazil. Weed Sci 65:317–326

Küpper A, Peter F, Zöllner P, Lorentz L, Tranel PJ, Beffa R, Gaines TA (2018) Tembotrione detoxification in 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitor-resistant Palmer amaranth (*Amaranthus palmeri* S. Wats.). Pest Manag Sci 74:2325–2334

Lavy M, Estelle M (2016) Mechanisms of auxin signaling. Development 143:3226–3229

LeClere S, Wu C, Westra P, Sammons RD (2018) Cross-resistance to dicamba, 2,4-D, and fluroxypyr in *Kochia scoparia* is endowed by a mutation in an *AUX/IAA* gene. Proc Natl Acad Sci USA 115:E2911–E2920

Length RV (2016) Least-Squares Means: The R Package *lsmeans.* J Stat Softw 69:1–33

Lu H, Yu Q, Han H, Owen MJ, Powles SB (2019) Metribuzin resistance in a wild radish (*Raphanus raphanistrum*) population *via* both *psbA* gene mutation and enhanced metabolism. J Agric Food Chem 67:1353–1359

Luo J, Zhou J-J, Zhang J-Z (2018) *Aux/IAA* gene family in plants: Molecular structure, regulation, and function. Int J Mol Sci 19

Ma H, Lu H, Han H, Yu Q, Powles S (2020) Metribuzin resistance *via* enhanced metabolism in a multiple herbicide resistant *Lolium rigidum* population. Pest Manag Sci 76:3785–3791

Ma R, Kaundun SS, Tranel PJ, Riggins CW, McGinness DL, Hager AG, Hawkes T, McIndoe E, Riechers DE (2013) Distinct detoxification mechanisms confer resistance to mesotrione and atrazine in a population of waterhemp. Plant Physiol 163:363–377

MacHado VS, Bandeen JD (1982) Genetic analysis of chloroplast atrazine resistance in *Brassica campestris*: Cytoplasmic inheritance. Weed Sci 30:281–285

MacRae AW, Webster TM, Sosnoskie LM, Culpepper AS, Kichler JM (2013) Cotton yield loss potential in response to length of Palmer amaranth (*Amaranthus palmeri*) interference 17:6

Maher EP, Martindale SJB (1980) Mutants of *Arabidopsis thaliana* with altered responses to auxins and gravity. Biochem Genet 18:1041–1053

Massinga RA, Currie RS, Horak MJ, John Boyer Jr (2001) Interference of Palmer amaranth in Corn. Weed Sci 49:202–208

Maxwell BD, Roush ML, Radosevich SR (1990) Predicting the evolution and dynamics of herbicide resistance in weed populations. Weed Technol 4:2–13

McCauley CL, McAdam SAM, Bhide K, Thimmapuram J, Banks JA, Young BG (2020) Transcriptomics in *Erigeron canadensis* reveals rapid photosynthetic and hormonal responses to auxin-herbicide application. J Exp Bot 71:3701–3709

McFadden JJ, Frear DS, Mansager ER (1989) Aryl hydroxylation of diclofop by a cytochrome P450 dependent monooxygenase from wheat. Pestic Biochem Physiol 34:92–100

Menalled FD, Peterson RKD, Smith RG, Curran WS, Páez DJ, Maxwell BD (2016) The Eco-evolutionary imperative: revisiting weed management in the midst of an herbicide resistance crisis. Sustainability 8:1297

Menges RM (1987) Allelopathic effects of Palmer amaranth (*Amaranthus palmeri*) and other plant residues in soil. Weed Sci 35:339–347

Mengistu LW, Christoffers MJ, Lym RG (2005) A *psbA* mutation in *Kochia scoparia* (L) Schrad from railroad rights-of-way with resistance to diuron, tebuthiuron and metribuzin. Pest Manag Sci 61:1035–1042

Meyer CJ, Norsworthy JK, Young BG, Steckel LE, Bradley KW, Johnson WG, Loux MM, Davis VM, Kruger GR, Bararpour MT, Ikley JT, Spaunhorst DJ, Butts TR (2015) Herbicide program approaches for managing glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) and waterhemp (*Amaranthus tuberculatus* and *Amaranthus rudis*) in future soybean-trait technologies. Weed Technol 29:716–729

Meyers SL, Jennings KM, Schultheis JR, Monks DW (2010) Interference of Palmer amaranth (*Amaranthus palmeri*) in sweetpotato. Weed Sci 58:199–203

Miller MR, Norsworthy JK (2016) Evaluation of herbicide programs for use in a 2,4-D–resistant soybean technology for control of glyphosate-resistant palmer amaranth (*Amaranthus palmeri*). Weed Technol 30:366–376

Miller MR, Norsworthy JK (2018) Florpyrauxifen-benzyl weed control spectrum and tank-mix compatibility with other commonly applied herbicides in rice. Weed Technol 32:319–325

Mithila J, Hall JC, Johnson WG, Kelley KB, Riechers DE (2011) Evolution of resistance to auxinic herbicides: Historical perspectives, mechanisms of resistance, and implications for broadleaf weed management in agronomic crops. Weed Sci 59:445–457

Molin WT, Nandula VK, Wright AA, Bond JA (2021) Transfer and Expression of ALS Inhibitor Resistance from Palmer Amaranth (*Amaranthus palmeri*) to an *A. spinosus* x *A. palmeri* Hybrid. Weed Sci 64:240–247

Molin WT, Wright AA, VanGessel MJ, McCloskey WB, Jugulam M, Hoagland RE (2018) Survey of the genomic landscape surrounding the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene in glyphosate-resistant *Amaranthus palmeri* from geographically distant populations in the USA. Pest Manag Sci 74:1109–1117

Molin WT, Yaguchi A, Blenner M, Saski CA (2020) The EccDNA replicon: A heritable, extranuclear vehicle that enables gene amplification and glyphosate resistance in *Amaranthus palmeri*. Plant Cell 32:2132–2140

Montgomery JS, Giacomini D, Waithaka B, Lanz C, Murphy BP, Campe R, Lerchl J, Landes A, Gatzmann F, Janssen A, Antonise R, Patterson E, Weigel D, Tranel PJ (2020) Draft genomes of *Amaranthus tuberculatus*, *Amaranthus hybridus*, and *Amaranthus palmeri*. Genome Biol Evol 12:1988–1993

Moore JW, Murray DS, Westerman RB (2004) Palmer amaranth (*Amaranthus palmeri*) effects on the harvest and yield of grain sorghum (*Sorghum bicolor*). Weed Technol 18:23–29

Mora AD, Rosario J, Rojano-Delgado AM, Palma-Bautista C, Torra J, Alcántara-de la Cruz R, De Prado R (2019) Multiple resistance to synthetic auxin herbicides and glyphosate in *Parthenium hysterophorus* occurring in citrus orchards. J Agric Food Chem 67:10010–10017

Morgan GD, Baumann PA, Chandler JM (2001) Competitive impact of palmer Amaranth (*Amaranthus palmeri*) on cotton (*Gossypium hirsutum*) development and yield. Weed Technol 15:408–412

Morgan PW, Hall WC (1963) Metabolism of 2,4-D by cotton and grain sorghum. Weeds 11:130–135

Mortensen DA, Egan JF, Maxwell BD, Ryan MR, Smith RG (2012) Navigating a critical juncture for sustainable weed management. BioScience 62:75–84

Nakka S, Godar AS, Thompson CR, Peterson DE, Jugulam M (2017a) Rapid detoxification *via* glutathione S-transferase (GST) conjugation confers a high level of atrazine resistance in Palmer amaranth (*Amaranthus palmeri*). Pest Manag Sci 73:2236–2243

Nakka S, Godar AS, Wani PS, Thompson CR, Peterson DE, Roelofs J, Jugulam M (2017b) Physiological and molecular characterization of hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor resistance in Palmer amaranth (*Amaranthus palmeri* S.Wats.). Front Plant Sci 8

Nakka S, Thompson CR, Peterson DE, Jugulam M (2017c) Target site–based and non–target site based resistance to ALS inhibitors in Palmer amaranth (*Amaranthus palmeri*). Weed Sci 65:681–689

Nandula VK (2019) Herbicide resistance traits in maize and soybean: Current status and future outlook. Plants 8:337

Nandula VK, Reddy KN, Koger CH, Poston DH, Rimando AM, Duke SO, Bond JA, Ribeiro DN (2012) Multiple resistance to glyphosate and pyrithiobac in Palmer amaranth (*Amaranthus palmeri*) from Mississippi and response to flumiclorac. Weed Sci 60:179–188

Nandula VK, Wright AA, Bond JA, Ray JD, Eubank TW, Molin WT (2014) *EPSPS* amplification in glyphosate-resistant spiny amaranth (*Amaranthus spinosus*): A case of gene transfer *via* interspecific hybridization from glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*). Pest Manag Sci 70:1902–1909

Norsworthy JK, Ward SM, Shaw DR, Llewellyn RS, Nichols RL, Webster TM, Bradley KW, Frisvold G, Powles SB, Burgos NR, Witt WW, Barrett M (2012) Reducing the risks of herbicide resistance: best management practices and recommendations. Weed Sci 60:31–62

Obenland OA, Ma R, O'Brien SR, Lygin AV, Riechers DE (2019) Carfentrazone-ethyl resistance in an *Amaranthus tuberculatus* population is not mediated by amino acid alterations in the PPO2 protein. PloS One 14:e0215431

Oerke E -C (2006) Crop losses to pests. J Agric Sci 144:31–43

Oliveira MC, Gaines TA, Jhala AJ, Knezevic SZ (2018a) Inheritance of mesotrione resistance in an *Amaranthus tuberculatus* (var. *rudis*) population from Nebraska, USA. Front Plant Sci 9

Oliveira MC, Gaines TA, Patterson EL, Jhala AJ, Irmak S, Amundsen K, Knezevic SZ (2018b) Interspecific and intraspecific transference of metabolism-based mesotrione resistance in dioecious weedy *Amaranthus*. Plant J 96:1051–1063

Ou J (2018) The mechanism(s) and management of dicamba resistance in kochia (*Kochia scoparia*). Ph.D. dissertation. Manhattan, KS: Kansas State University

Ou J, Gaines TA, Fritz AK, Stahlman PW, Jugulam M (2021) Dicamba resistance in kochia from Kansas and Nebraska evolved independently. Pest Manag Sci 77:126–130

Overvoorde PJ, Okushima Y, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Liu A, Onodera C, Quach H, Smith A, Yu G, Theologis A (2005) Functional genomic analysis of the AUXIN/INDOLE-3-ACETIC ACID gene family members in *Arabidopsis thaliana*. Plant Cell 17:3282–3300

Owen MJ, Martinez NJ, Powles SB (2015) Multiple herbicide-resistant wild radish (*Raphanus raphanistrum*) populations dominate Western Australian cropping fields. Crop Pasture Sci 66:1079–1085

Palhano MG, Norsworthy JK, Barber T (2018) Cover crops suppression of Palmer amaranth (*Amaranthus palmeri*) in Cotton. Weed Technol 32:60–65

Palma-Bautista C, Hoyos V, Plaza G, Vázquez-García JG, Rosario J, Rojano-Delgado AM, De Prado R (2020a) Evolving multiple resistance to EPSPS, GS, ALS, PSI, PPO, and synthetic auxin herbicides in Dominican Republic *Parthenium hysterophorus* populations. A physiological and biochemical study. Agronomy 10:554

Palma-Bautista C, Rojano-Delgado AM, Dellaferrera I, Rosario JM, Vigna MR, Torra J, de Prado R (2020b) Resistance mechanisms to 2,4-D in six different dicotyledonous weeds around the world. Agronomy 10:566

Palma-Bautista C, Torra J, Garcia MJ, Bracamonte E, Rojano-Delgado AM, Alcántara-de la Cruz R, De Prado R (2019) Reduced absorption and impaired translocation endows glyphosate resistance in *Amaranthus palmeri* harvested in glyphosate-resistant soybean from Argentina. J Agric Food Chem 67:1052–1060

Pandian BA, Sathishraj R, Djanaguiraman M, Prasad PVV, Jugulam M (2020) Role of cytochrome P450 enzymes in plant stress response. Antioxidants 9:454

Pandian BA, Sathishraj R, Prasad PVV, Jugulam M (2021) A single gene inherited trait confers metabolic resistance to chlorsulfuron in grain sorghum (*Sorghum bicolor*). Planta 253:48

Patzoldt WL, Dixon BS, Tranel PJ (2003) Triazine resistance in *Amaranthus tuberculatus* (Moq) Sauer that is not site-of-action mediated. Pest Manag Sci 59:1134–1142

Peterson MA, McMaster SA, Riechers DE, Skelton J, Stahlman PW (2016) 2,4-D past, present, and future: A review. Weed Technol 30:303–345

Petrášek J, Friml J (2009) Auxin transport routes in plant development. Development 136:2675–2688

Pettinga DJ, Ou J, Patterson EL, Jugulam M, Westra P, Gaines TA (2018) Increased chalcone synthase (*CHS*) expression is associated with dicamba resistance in *Kochia scoparia*. Pest Manag Sci 74:2306–2315

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45

Pickett FB, Wilson AK, Estelle M (1990) The *AUX1* mutation of *Arabidopsis* confers both auxin and ethylene resistance. Plant Physiol 94:1462–1466

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team (2021) *nlme*: Linear and nonlinear mixed effects models. R package version 3.1-152, Available at CRAN.R-project.org/package=nlme>

Preston C, Belles DS, Westra PH, Nissen SJ, Ward SM (2009) Inheritance of resistance to the auxinic herbicide dicamba in kochia (*Kochia scoparia*). Weed Sci 57:43–47

Preston C, Dolman FC, Boutsalis P (2013) Multiple resistance to acetohydroxyacid synthase–inhibiting and auxinic herbicides in a population of oriental mustard (*Sisymbrium orientale*). Weed Sci 61:185–192

Preston C, Mallory-Smith CA (2001) Biochemical Mechanisms, inheritance, and molecular genetics of herbicide resistance in weeds. Page 38 *in* Powles SB, Shaner DL, ed. Herbicide resistance and world grains. Boca Raton: CRC Press

Preston C, Malone JM (2015) Inheritance of resistance to 2,4-D and chlorsulfuron in a multiple-resistant population of *Sisymbrium orientale*. Pest Manag Sci 71:1523–1528

Preston C, Tardif FJ, Powles SB (1996) Multiple mechanisms and multiple herbicide resistance in *Lolium rigidum*. Pages 117–129 *in* Brown TM, ed. Molecular genetics and evolution of pesticide resistance. American Chemical Society symposium series.

Prigge MJ, Greenham K, Zhang Y, Santner A, Castillejo C, Mutka AM, O'Malley RC, Ecker JR, Kunkel BN, Estelle M (2016) The *Arabidopsis* auxin receptor F-Box Proteins AFB4 and AFB5 are required for response to the synthetic auxin picloram. G3-Genes Genom Genet 6:1383–1390

Qin X, Zeevaart JAD (1999) The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. Proc Natl Acad Sci 96:15354–15361

Queiroz ARS de, Delatorre CA, Lucio FR, Rossi CVS, Zobiole LHS, Merotto A (2020) Rapid necrosis: A novel plant resistance mechanism to 2,4-D. Weed Sci 68:6–18

Raghavan C, Ong EK, Dalling MJ, Stevenson TW (2006) Regulation of genes associated with auxin, ethylene and ABA pathways by 2,4-dichlorophenoxyacetic acid in *Arabidopsis*. Funct Integr Genomics 6:60–70

Rangani G, Salas-Perez RA, Aponte RA, Knapp M, Craig IR, Mietzner T, Langaro AC, Noguera MM, Porri A, Roma-Burgos N (2019) A novel single-site mutation in the catalytic domain of protoporphyrinogen oxidase IX (PPO) confers resistance to PPO-inhibiting herbicides. Front Plant Sci 10

Reddy KN, Nandula V (2012) Herbicide resistant crops: History, development and current technologies. Ind J Agron 57:1–7

Remy E, Cabrito TR, Baster P, Batista RA, Teixeira MC, Friml J, Sá-Correia I, Duque P (2013) A major facilitator superfamily transporter plays a dual role in polar auxin transport and drought stress tolerance in *Arabidopsis*. Plant Cell 25:901–926

Rey-Caballero J, Menéndez J, Giné-Bordonaba J, Salas M, Alcántara R, Torra J (2016) Unravelling the resistance mechanisms to 2,4-D (2,4-dichlorophenoxyacetic acid) in corn poppy (*Papaver rhoeas*). Pestic Biochem Physiol 133:67–72

Riar DS, Burke IC, Yenish JP, Bell J, Gill K (2011) Inheritance and physiological basis for 2,4-D resistance in prickly lettuce (*Lactuca serriola* L.). J Agric Food Chem 59:9417–9423

Ricci G, De Maria F, Antonini G, Turella P, Bullo A, Stella L, Filomeni G, Federici G, Caccuri AM (2005) 7-Nitro-2,1,3-benzoxadiazole derivatives, a new class of suicide inhibitors for glutathione S-transferases. Mechanism of action of potential anticancer drugs. J Biol Chem 280:26397–26405

Ritz C, Baty F, Streibig JC, Gerhard D (2015) Dose-response analysis using R. PLOS ONE 10:e0146021

Sabaté S, Tranel P, Hager AG (2016) Genetics of 2,4-D resistance in an Illinois waterhemp population. Proceedings of the 71st Annual Meeting of the North Central Weed Science Society, Des Moines, IA, 71:39

Sabba RP, Ray IM, Lownds N, Sterling TM (2003) Inheritance of resistance to clopyralid and picloram in yellow starthistle (*Centaurea solstitialis* L.) is controlled by a single nuclear recessive gene. J Hered 94:523–527

Salas RA, Burgos NR, Tranel PJ, Singh S, Glasgow L, Scott RC, Nichols RL (2016) Resistance to PPO-inhibiting herbicide in Palmer amaranth from Arkansas. Pest Manag Sci 72:864–869

Salas-Perez RA, Burgos NR, Rangani G, Singh S, Refatti JP, Piveta L, Tranel PJ, Mauromoustakos A, Scott RC (2017) Frequency of Gly-210 deletion mutation among protoporphyrinogen oxidase inhibitor-resistant Palmer amaranth (*Amaranthus palmeri*) populations. Weed Sci 65:718–731

Sarangi D, Jhala AJ (2019) Palmer amaranth (*Amaranthus palmeri*) and velvetleaf (*Abutilon theophrasti*) control in no-tillage conventional (Non–genetically engineered) soybean using overlapping residual herbicide programs. Weed Technol 33:95–105

Sarangi D, Sandell LD, Kruger GR, Knezevic SZ, Irmak S, Jhala AJ (2017) Comparison of herbicide programs for season-long control of glyphosate-resistant common waterhemp (*Amaranthus rudis*) in soybean. Weed Technol 31:53–66

Scheel D, Sandermann H (1981) Metabolism of 2,4-dichlorophenoxyacetic acid in cell suspension cultures of soybean (*Glycine max* L.) and wheat (*Triticum aestivum* L.): I. General results. Planta 152:248–252

Schmitt MR, Edwards GE (1981) Photosynthetic capacity and nitrogen use efficiency of maize, wheat, and rice: A comparison between C3 and C4 photosynthesis. J Exp Bot 32:459–466

Sellers BA, Smeda RJ, Johnson WG, Kendig JA, Ellersieck MR (2003) Comparative growth of six *Amaranthus* species in Missouri. Weed Sci 51:329–333

Shergill LS, Barlow BR, Bish MD, Bradley KW (2018a) Investigations of 2,4-D and multiple herbicide resistance in a missouri waterhemp (*Amaranthus tuberculatus*) population. Weed Sci 66:386–394

Shergill LS, Bish MD, Jugulam M, Bradley KW (2018b) Molecular and physiological characterization of six-way resistance in an *Amaranthus tuberculatus* var. *rudis* biotype from Missouri. Pest Manag Sci 74:2688–2698

Shyam C, Borgato EA, Peterson DE, Dille JA, Jugulam M (2021a) Predominance of metabolic resistance in a six-way-resistant Palmer amaranth (*Amaranthus palmeri*) population. Front Plant Sci 11

Shyam C, Chahal PS, Jhala AJ, Jugulam M (2020) Management of glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) in 2,4-D–, glufosinate-, and glyphosate-resistant soybean. Weed Technol:1–8

Shyam C, Jhala AJ, Kruger G, Jugulam M (2019) Rapid metabolism increases the level of 2,4-D resistance at high temperature in common waterhemp (*Amaranthus tuberculatus*). Sci Rep 9:16695

Shyam C, Nakka S, Putta L, Cuvaca I, Currie RS, Jugulam M (2021b) Genetic basis of chlorsulfuron, atrazine, and mesotrione resistance in a Palmer amaranth (*Amaranthus palmeri*) population. ACS Agric Sci Technol 1:109–114

Singh S, Singh V, Lawton-Rauh A, Bagavathiannan MV, Roma-Burgos N (2018) *EPSPS* gene amplification primarily confers glyphosate resistance among Arkansas Palmer amaranth (*Amaranthus palmeri*) populations. Weed Sci 66:293–300

Singh S, Singh V, Salas-Perez RA, Bagavathiannan MV, Lawton-Rauh A, Roma-Burgos N (2019) Target-site mutation accumulation among ALS inhibitor-resistant Palmer amaranth. Pest Manag Sci 75:1131–1139

Singh VK, Jain M (2015) Genome-wide survey and comprehensive expression profiling of *Aux/IAA* gene family in chickpea and soybean. Front Plant Sci 6:918

Soltani N, Dille JA, Gulden RH, Sprague CL, Zollinger RK, Morishita DW, Lawrence NC, Sbatella GM, Kniss AR, Jha P, Sikkema PH (2018a) Potential yield loss in dry bean crops due to weeds in the United States and Canada. Weed Technol 32:342–346

Soltani N, Dille JA, Robinson DE, Sprague CL, Morishita DW, Lawrence NC, Kniss AR, Jha P, Felix J, Nurse RE, Sikkema PH (2018b) Potential yield loss in sugar beet due to weed interference in the United States and Canada. Weed Technol 32:749–753

Sosnoskie LM, Webster TM, Kichler JM, MacRae AW, Grey TL, Culpepper AS (2012) Pollen-mediated dispersal of glyphosate-resistance in Palmer amaranth under field conditions. Weed Sci 60:366–373

Spiess A (2018) *qpcR*: Modelling and analysis of real-time PCR data. R package version 1.4-1. Available at CRAN.R-project.org/package=qpcR

Steckel GJ, Wax LM, Simmons FW, Phillips WH (1997) Glufosinate efficacy on annual weeds is influenced by rate and growth stage. Weed Technol 11:484–488

Sterling TM, Hall JC (1997) Mechanism of action of natural auxins and the auxinic herbicides. Pages 111-141 *In* Roe RM, Burton JD, Kuhr RJ, ed. Herbicide Activity: Toxicology, Biochemistry and Molecular Biology. Amsterdam: IOS Press

Tanetani Y, Ikeda M, Kaku K, Shimizu T, Matsumoto H (2013) Role of metabolism in the selectivity of a herbicide, pyroxasulfone, between wheat and rigid ryegrass seedlings. J Pestic Sci 38:152–156

Tan X, Calderon-Villalobos LIA, Sharon M, Zheng C, Robinson CV, Estelle M, Zheng N (2007) Mechanism of auxin perception by the *TIR1* ubiquitin ligase. Nature 446:640–645

Tardif FJ, Preston C, Holtum J a. M, Powles SB (1996) Resistance to acetyl-coenzyme a carboxylase-inhibiting herbicides endowed by a single major gene encoding a resistant target site in a biotype of *Lolium rigidum*. Funct Plant Biol 23:15–23

Teale WD, Paponov IA, Palme K (2006) Auxin in action: Signalling, transport and the control of plant growth and development. Nat Rev Mol Cell Biol 7:847–859

Teló GM, Webster EP, Blouin DC, McKnight BM, Rustom SY (2019) Florpyrauxifen-benzyl activity on perennial grass weeds found in Louisiana rice production. Weed Technol 33:246–252

Thimann KV, Koepfli JB (1935) Identity of the growth-promoting and root-forming substances of plants. Nature 135:101–102

Todd OE, Figueiredo MRA, Morran S, Soni N, Preston C, Kubeš MF, Napier R, Gaines TA (2020) Synthetic auxin herbicides: Finding the lock and key to weed resistance. Plant Sci 300:110631

Torra J, Rojano-Delgado AM, Menéndez J, Salas M, de Prado R (2021) Cytochrome P450 metabolism-based herbicide resistance to imazamox and 2,4-D in *Papaver rhoeas*. Plant Physiol Biochem 160:51–61

Torra J, Rojano-Delgado AM, Rey-Caballero J, Royo-Esnal A, Salas ML, De Prado R (2017) Enhanced 2,4-D metabolism in two resistant *Papaver rhoeas* populations from Spain. Front Plant Sci 8

Tranel PJ (2021) Herbicide resistance in *Amaranthus tuberculatus*. Pest Manag Sci 77:43–54

Tranel PJ, Wright TR (2002) Resistance of weeds to ALS-inhibiting herbicides: What have we learned? Weed Sci 50:700–712

Unruh BJ (2013) Influence of nitrogen on weed growth and competition with grain sorghum. MS dissertation. Manhattan, KS: Kansas State University

USDA: U.S. Department of Agriculture (2021) Adoption of Genetically Engineered Crops in the U.S. Available at www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us.aspx.

Van Eerd LL, McLean MD, Stephenson GR, Hall JC (2004) Resistance to quinclorac and ALS-inhibitor herbicides in *Galium spurium* is conferred by two distinct genes. Weed Res 44:355–365

Van Wychen L (2020) Survey of the most common and troublesome weeds in grass crops, pasture, and turf in the United States and Canada. Weed Science Society of America national weed survey dataset. Available at wssa.net/wp-content/uploads/2020-Weed-Survey_grass-crops.xlsx

Varanasi VK, Brabham C, Korres NE, Norsworthy JK (2019) Nontarget site resistance in Palmer amaranth [*Amaranthus palmeri* (S.) Wats.] confers cross-resistance to protoporphyrinogen oxidase-inhibiting herbicides. Weed Technol 33:349–354

Varanasi VK, Brabham C, Norsworthy JK (2018a) Confirmation and characterization of non–target site resistance to fomesafen in Palmer amaranth (*Amaranthus palmeri*). Weed Sci 66:702–709

Varanasi VK, Brabham C, Norsworthy JK, Nie H, Young BG, Houston M, Barber T, Scott RC (2018b) A statewide survey of PPO-Inhibitor resistance and the prevalent target-site mechanisms in Palmer amaranth (*Amaranthus palmeri*) accessions from Arkansas. Weed Sci 66:149–158

Varanasi VK, Godar AS, Currie RS, Dille AJ, Thompson CR, Stahlman PW, Jugulam M (2015) Field-evolved resistance to four modes of action of herbicides in a single kochia (*Kochia scoparia* L. Schrad.) population. Pest Manag Sci 71:1207–1212

Vila-Aiub MM, Neve P, Steadman KJ, Powles SB (2005) Ecological fitness of a multiple herbicide-resistant *Lolium rigidum* population: dynamics of seed germination and seedling emergence of resistant and susceptible phenotypes. J Appl Ecol 42:288–298

Walsh MJ, Owen MJ, Powles SB (2007) Frequency and distribution of herbicide resistance in *Raphanus raphanistrum* populations randomly collected across the Western Australian wheatbelt. Weed Res 47:542–550

Walsh TA, Neal R, Merlo AO, Honma M, Hicks GR, Wolff K, Matsumura W, Davies JP (2006) Mutations in an auxin receptor homolog *AFB5* and in *SGT1b* confer resistance to synthetic picolinate auxins and not to 2,4-dichlorophenoxyacetic acid or indole-3-acetic acid in *Arabidopsis*. Plant Physiol 142:542–552

Wang J, Klessig D, Berry J (1992) Regulation of *C4* gene expression in developing amaranth leaves. Plant Cell 4:173–184

Webster TM, Grey TL (2015) Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) morphology, growth, and seed production in Georgia. Weed Sci 63:264–272

Weinberg T, Stephenson GR, McLean MD, Hall JC (2006) MCPA (4-Chloro-2-ethylphenoxyacetate) resistance in hemp-nettle (*Galeopsis tetrahit* L.). J Agric Food Chem 54:9126–9134

Werck-Reichhart D (2000) Cytochromes P450 for engineering herbicide tolerance. Trends Plant Sci 5:116–23

Whaley CM, Wilson HP, Westwood JH (2007) A new mutation in plant *ALS* confers resistance to five classes of ALS-inhibiting herbicides. Weed Sci 55:83–90

Whitaker JR, York AC, Jordan DL, Culpepper AS (2010) Palmer amaranth (*Amaranthus palmeri*) control in soybean with glyphosate and conventional herbicide systems. Weed Technol 24:403–410

Wickham H (2016) *ggplot2*: Elegant graphics for data analysis. Springer-Verlag New York

Wright RM, Hagen G, Guilfoyle T (1987) An auxin-induced polypeptide in dicotyledonous plants. Plant Mol Biol 9:625–634

Wright TR, Guomin Shan G, Walsh TA, Lira JM, Cui C, Song P, Zhuang M, Arnold NL, Lin G, Yau K, Russell SM, Cicchillo RM, Peterson MA, Simpson DA, Zhou N, Ponsamuel J, Zhang Z (2010) Robust crop resistance to broadleaf and grass herbicides provided by aryloxyalkanoate dioxygenase transgenes. Proc Natl Acad Sci USA 107:20240-20245

Wu C, LeClere S, Liu K, Paciorek M, Perez-Jones A, Westra P, Sammons RD (2021) A dicamba resistance-endowing *IAA16* mutation leads to significant vegetative growth defects and impaired competitiveness in kochia (*Bassia scoparia*). Pest Manag Sci 77:795–804

Wu W, Liu Y, Wang Y, Li H, Liu J, Tan J, He J, Bai J, Ma H (2017) Evolution analysis of the *Aux/IAA* gene family in plants shows dual origins and variable nuclear localization signals. Int J Mol Sci 18

Xiang W, Wang X, Ren T (2006) Expression of a wheat cytochrome P450 monooxygenase cDNA in yeast catalyzes the metabolism of sulfonylurea herbicides. Pestic Biochem Physiol 85:1–6

Yang H, Murphy AS (2009) Functional expression and characterization of *Arabidopsis* ABCB, AUX 1 and PIN auxin transporters in *Schizosaccharomyces pombe*. Plant J 59:179–191

Yuan JS, Tranel PJ, Stewart CN Jr. (2007) Non-target-site herbicide resistance: a family business. Trends Plant Sci 12:6–13.

Yu Q, Powles S (2014) Metabolism-based herbicide resistance and cross-resistance in crop weeds: A threat to herbicide sustainability and global crop production. Plant Physiol 166:1106–1118

Zhao N, Yan Y, Ge L, Zhu B, Liu W, Wang J (2019) Target site mutations and cytochrome P450s confer resistance to fenoxaprop-P-ethyl and mesosulfuron-methyl in *Alopecurus aequalis*. Pest Manag Sci 75:204–214

Zhu J, Geisler M (2015) Keeping it all together: Auxin-actin crosstalk in plant development. J Exp Bot 66:4983–4998

Zimdahl RL (2018) Fundamentals of weed science. 5th ed. Cambridge, MA: Academic Press. Pp 760

Appendix A - Abbreviations

2,4-D	2,4-dichlorophenoxy acetic acid
ABA	Abscisic acid
ABCB	ATP-binding cassette sub-family B
ACC	1-Aminocyclopropane-1-carboxylic acid
ADW	Average dry weight of non-treated control
AICc	Akaike information criteria
ALS	Acetolactate synthase
ARE	Auxin-responsive elements
ARF	Auxin response factors
AUX/IAA	Auxin/indole-3-acetic acid
CHS	Chalcone synthase
DA-EPOST	Days after early post-emergence
DA-LPOST	Days after late post-emergence
DA-PRE	Days after pre-emergence
DAT	Days after treatment
DMO	Dicamba monooxygenase
DW	Dry weight of the sample
EPOST	Early post-emergence
EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase
fb	Followed by
GE	Genetically engineered
GPA	Gallons per acre
GST	Glutathione S-transferases
GR	Glyphosate-resistant
HAT	Hours after treatment
HPLC	High performance liquid chromatography
HPPD	4-hydroxyphenylpyruvate dioxygenase
HR	Herbicide-resistant
HRAC	Herbicide resistance action committee

HT	Herbicide tolerant
KCTR	Kansas conservation tillage resistant
KSS	Kansas susceptible
LPOST	Late post-emergence
LSC	Liquid scintillation counter
MAPK	Mitogen-activated protein kinases
MCPA	2-Methyl-4-chlorophenoxyacetic acid
MFS	Major facilitator superfamily
MOA	Mode of action
MSS	Mississippi susceptible
NBD-Cl	4-chloro-7-nitro-2,1,3-benzoxadiazole
NCED	9-cis-epoxycarotenoid dioxygenase
NT	Non-treated
NTSR	Non-target site resistance
P450	Cytochrome P450 monooxygenases
PBO	Piperonyl butoxide
PCR	Polymerase chain reaction
PIN	PIN-formed
POST	Post-emergence
PPO	Protoporphyrinogen oxidase
PRE	Pre-emergence
PS I	Photosystem I
PS II	Photosystem II
qPCR	Quantitative polymerase chain reaction
RDW	Relative dry weight (% of non-treated control)
RI	Resistance index
RT	Retention time
SAH	Synthetic auxinic herbicide
SCF	Skp1-cullin-F-box
TIR1/AFB	Transport inhibitor response 1/Auxin signaling F-box protein
TSR	Target site resistance

VLCFA	Very-long chain fatty acid
WAT	Weeks after treatment
WSSA	Weed science society of America

Appendix B - Supplementary Material

Figure S2.1 Sequence alignment of acetolactate synthase (*ALS*) gene of representative plants from resistant KCTR and susceptible populations KSS and MSS

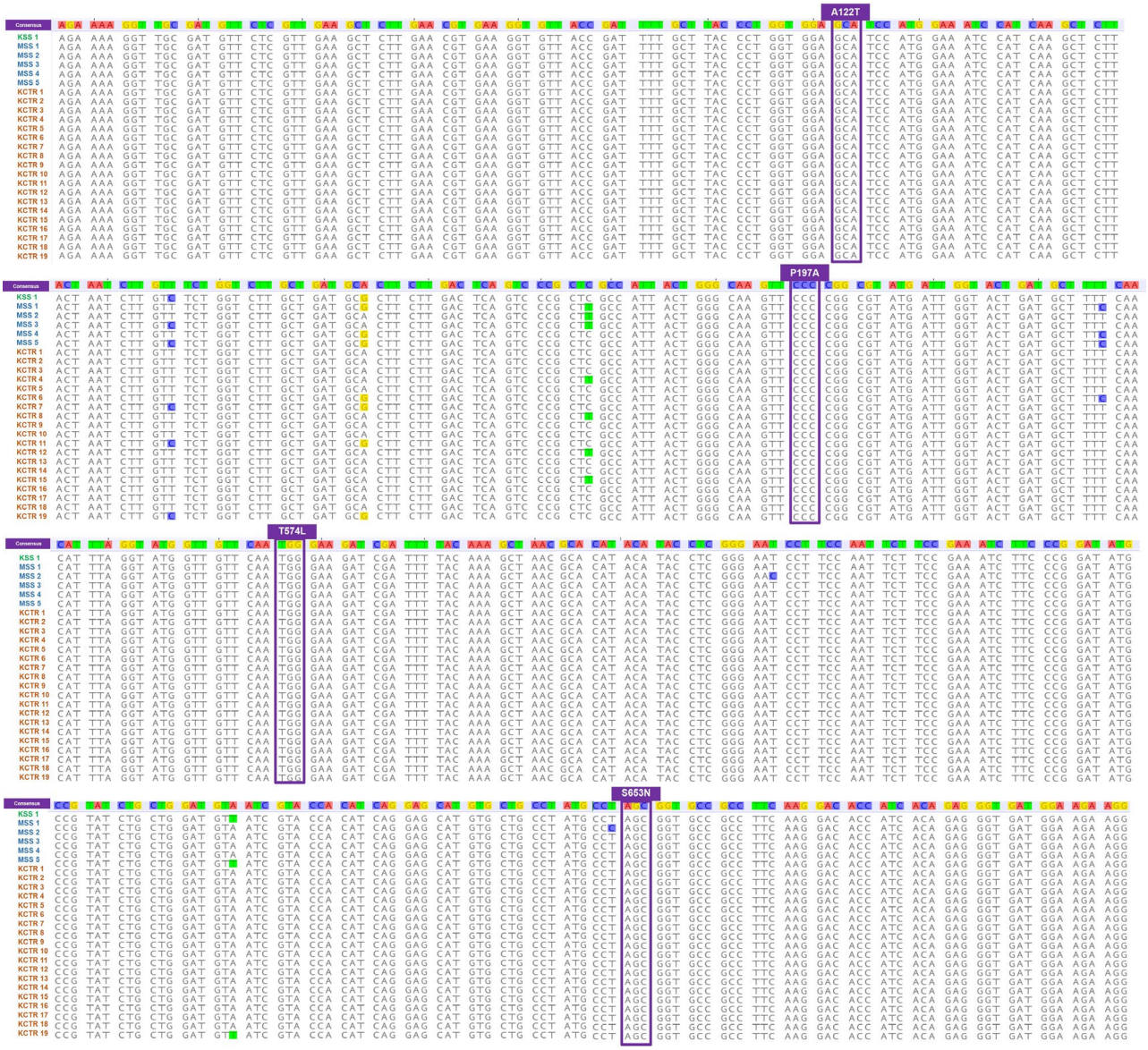


Figure S2.2 Sequence alignment of *psbA* gene of representative plants from resistant KCTR and susceptible populations KSS and MSS

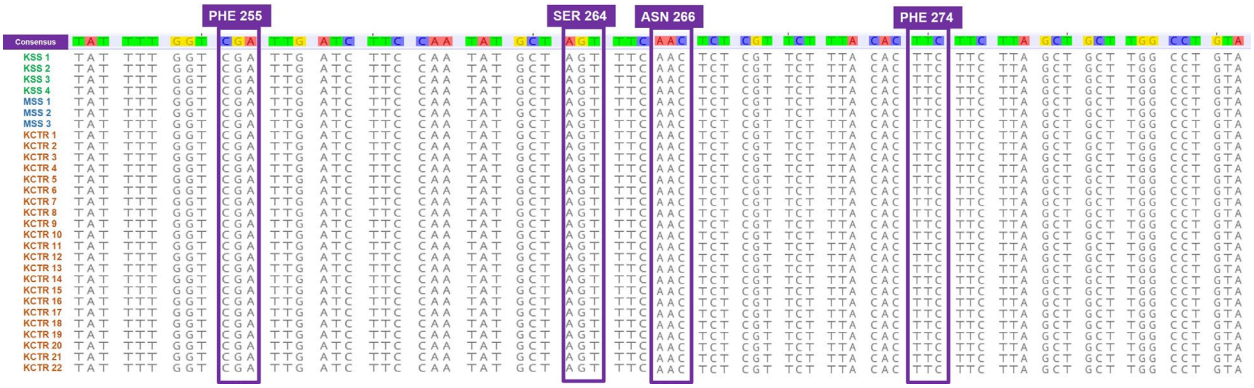
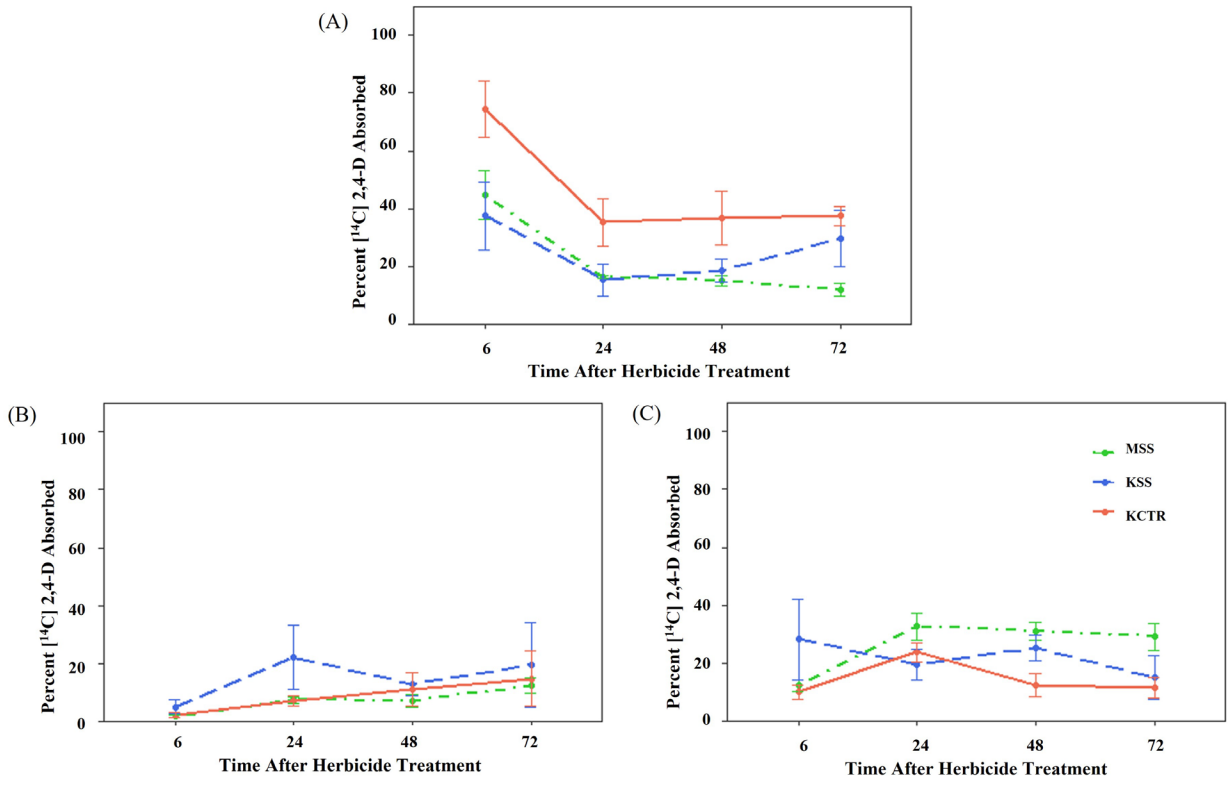


Figure S3.1 Percent [^{14}C] 2,4-D in (A) treated-leaf, (B) above treated-leaf, and (C) below treated-leaf in KSS (susceptible), MSS (susceptible) and KCTR (resistant) Palmer amaranth plants at various time-points after treatment with 2,4-D. Error bars represent standard error of mean.



Appendix C - Publications

Publications from my Ph.D. dissertation not included as a part of this dissertation.

plants

MDPI

Review

Non-Target-Site Resistance to Herbicides: Recent Developments

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Abstract: Non-target-site resistance (NTSR) to herbicides in weeds can be conferred as a result of the alteration of one or more physiological processes, including herbicide absorption, translocation, sequestration, and metabolism. The mechanisms of NTSR are generally more complex to decipher than target-site resistance (TSR) and can impart cross-resistance to herbicides with different modes of action. Metabolism-based NTSR has been reported in many agriculturally important weeds, although reduced translocation and sequestration of herbicides has also been found in some weeds. This review focuses on summarizing the recent advances in our understanding of the physiological, biochemical, and molecular basis of NTSR mechanisms found in weed species. Further, the importance of examining the co-existence of TSR and NTSR for the same herbicide in the same weed species and influence of environmental conditions in the altering and selection of NTSR is also discussed. Knowledge of the prevalence of NTSR mechanisms and co-existing TSR and NTSR in weeds is crucial for designing sustainable weed management strategies to discourage the further evolution and selection of herbicide resistance in weeds.

Keywords: non-target-site resistance; herbicide mode of action; co-existence; environmental conditions

1. Introduction

Herbicide use is indispensable in modern agriculture as it offers exceptional tool for weed management and also facilitates no-till crop production to conserve soil and moisture. However, repeated field applications of herbicides with the same mechanism of action resulted in the selection of herbicide-resistant weeds. The Weed Science Society of America (http//www.wssa.net) defines herbicide resistance as the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. Under continuous selection pressure, i.e., the repeated use of herbicides with the same mode of action, the resistant plants increase in frequency over time, resulting in the domination by individuals resistant to that herbicide. In addition to the selection pressure of herbicides, biological and genetic factors of weed species, properties of herbicides, and agronomic practices also play an important role in the evolution and spread of herbicide resistance [1]. Biological characteristics of highly troublesome weeds, including prolific seed production, high germination percentage, a wide window of emergence, seed dispersal, and longevity, help to maintain a high frequency of resistant individuals in the population. Genetic factors, such as natural mutations conferring herbicide resistance, inheritance of herbicide-resistant genes in the weed population, and fitness cost of resistance genes in the presence or absence of the herbicide, also play an important role in the evolution and spread of herbicide resistance [2,3].

2. Mechanisms of Herbicide Resistance

A key aspect in predicting the evolutionary trajectory of herbicide-resistance traits is understanding the mechanism(s) of herbicide resistance. Mechanisms of herbicide resistance in weeds can be broadly

classified into target-site resistance (TSR) and/or non-target-site resistance (NTSR). The TSR mechanisms largely involve mutation(s) in the target site of action of an herbicide, resulting in an insensitive or less sensitive target protein of the herbicide [1]. In such cases, the TSR is primarily determined by monogenic traits [3]. Additionally, TSR can also evolve as a result of the over-expression or amplification of the target gene [4]. NTSR mechanisms include reduced herbicide uptake/translocation, increased herbicide metabolism, decreased rate of herbicide activation, and/or sequestration [5]. Metabolism-based NTSR involves the increased activity of enzyme complexes such as esterases, cytochrome P450s (CYP450s), glutathione S-transferases (GSTs), and/or Uridine 5′-diphospho (UDP)-glucosyl transferases [1]. NTSR, especially if it involves herbicide detoxification by these enzymes, is usually governed by many genes (polygenic) and may confer resistance to herbicides with completely different modes of action [3,6]. However, monogenic inheritance of NTSR has also been reported in several herbicide-resistant weeds [7–9]. The evolution of NTSR via herbicide detoxification is a serious threat to weed management as it can bestow resistance to multiple herbicides, leaving limited herbicide options for weed control, as well as potential resistances to herbicides not yet commercially available [10]. Comprehensive information on the evolution of TSR-based resistance in weeds are discussed elsewhere in this special issue. In this review recent advances in understanding the mechanisms of NTSR to herbicides with different modes of action are discussed.

3. Known NTSR Mechanisms in Weed Species for Different Herbicide Modes of Actions

3.1. Acetyl CoA Carboxylase (ACCase)-Inhibitors

ACCase is a crucial enzyme that catalyzes the formation of malonyl CoA via the carboxylation of acetyl CoA while using bicarbonate as the source of carbon [11]. Malonyl CoA is needed for de novo fatty acid biosynthesis, and thus, is essential for plant survival. ACCase-inhibitors impede malonyl CoA formation in sensitive grass species, ultimately leading to plant death [11,12]. These herbicides are used as important post-emergence options for managing grass weeds in dicotyledonous crops. To date, 48 weeds have been reported to have evolved resistance to these herbicides [13] via both TSR and NTSR mechanisms. Predominantly, TSR has been reported as the leading mechanism, caused by amino acid substitutions in the carboxyl transferase domain of the ACCase enzyme [14,15].

Metabolic resistance to ACCase-inhibiting herbicides has been documented in Asia minor bluegrass [16], barnyard grass [17], blackgrass [18], Italian ryegrass [19,20], Japanese foxtail [21], rigid ryegrass [22–24], and wild oat [25]. In the majority of these cases, enhanced metabolism mediated by CYP450s was reported. For instance, rapid degradation of diclofop-methyl was observed in rigid ryegrass populations from Australia [22,24]. Interestingly, exposure to low doses of diclofop-methyl acid application rapidly selected for metabolic resistance in rigid ryegrass [26]. Moreover, the metabolites produced in these resistant plants were found to be similar to those in wheat formed via ring hydroxylation and sugar conjugation [26]. This result suggests that in resistant grasses, the metabolism of ACCase-inhibitors occurs through a wheat-like detoxification pathway mediated by CYP450s [25,26]. Studies involving CYP450 or GST inhibitors, such as malathion and piperonyl butoxide (PBO), have been used to indicate the involvement of these detoxification systems. The organophosphate insecticide, malathion can decrease the rate of metabolism and increase the metabolic half-life of herbicides by inhibiting CYP450-based hydroxylation in corn [27,28]. Pre-treatment with CYP450 inhibitors like PBO or malathion has been shown to reduce resistance to ACCase-inhibitors in Asia minor bluegrass [16] and Japanese foxtail [21], indicating the role of CYP450s in enhancing metabolism in these resistant weeds. Conversely, pre-treatment with 2,4-D, a CYP450 inducer, increased the rate of metabolism of diclofop-methyl in susceptible rigid ryegrass populations [29]. Apart from CYP450s, involvement of GSTs and glucosyltransferases (GTs) have also been documented to govern the metabolic resistance to ACCase-inhibitors. Transcriptome analysis of diclofop-resistant rigid ryegrass, led to the identification of four contigs, including two *CYP450s*, one *GT*, and one nitronate monooxygenase (*NMO*) as potential

candidate genes for metabolic resistance to diclofop [24]. Similarly, researchers have reported greater GST activity in resistant plants following ACCase-inhibitor application [16,17,30].

3.2. Acetolactate Synthase (ALS)-Inhibitors

ALS inhibitors were first commercialized in 1982, and by 1998, the number of weed species with resistance to this group of herbicides had surpassed other herbicides [31]. These herbicides, also referred as acetohydroxy acid synthase (AHAS) inhibitors, inhibit ALS or AHAS enzyme, which is vital for the biosynthesis of branched-chain amino acids isoleucine, leucine, and valine [32]. In general, these are broad-spectrum, post-emergence herbicides used for controlling weeds in a variety of crops like wheat and soybean. However, few ALS inhibitors, such as trifloxysulfuron, are also used as pre-emergence options to control weeds. Currently, resistance to ALS inhibitors is reported in 161 weeds globally [13]. TSR caused by single amino acid substitutions has been reported in most of these ALS-inhibitor-resistant weeds. Until recently, detection of these mutations have highlighted more importance on identifying TSR mechanisms compared to NTSR, even though they can co-exist in the same population [33,34]. However, the identification of plants lacking mutations in the ALS domain and surviving herbicide application has led researchers to focus on elucidating the NTSR mechanisms.

Enhanced metabolism conferring resistance to ALS inhibitors has been documented in several grass and broadleaf weeds, such as barnyard grass [35], common waterhemp [36], Palmer amaranth [37], rice barnyard grass [38,39], rigid brome [40], short awn foxtail (*Alopecurus aequalis*) [41,42], and water chickweed (*Myosoton aquaticum*) [33]. Numerous studies have also elucidated the molecular basis of metabolic resistance to ALS inhibitors. Though genes involved in metabolic resistance can be different depending on the weed species and history of herbicide application [43], most of these studies have predominantly identified multiple *CYP450* genes that are either constitutively expressed or upregulated following ALS inhibitor application [38,41,44]. For example, the mechanism of mesosulfuron-methyl resistance in short awn foxtail was studied and two *CYP450* genes, i.e., CYP94A1 and CYP71A4, were identified to be constitutively overexpressed in the resistant plants [41]. In a similar study, two *CYP450* genes, i.e., *CYP81A12* and *CYP81A21*, were identified as candidate genes conferring resistance to bensulfuron-methyl and penoxsulam in rice barnyard grass [38]. Several *CYP450* genes mediating NTSR to ALS inhibitors have been identified in water chickweed [44], ryegrass [45], flixweed [46], and blackgrass [18,47]. In addition to CYP450s, involvement of GSTs, GTs, and ATP-binding cassette (ABC) transporters have also been reported [42,44–46]. For instance, in ALS-inhibitor-resistant water chickweed, four genes—including three *CYP450s* (having homology to *CYP734A1*, *CYP76C1*, and *CYP86B1*) and an *ABC* transporter (having homology to *ABCC10*)—were identified as being highly expressed in all resistant plants [44]. Another commonly used procedure to test the CYP450 mediated metabolic resistance to ALS inhibitors has been the increase in sensitivity upon pre-treatment with CYP450 inhibitors, such as PBO, phorate, and malathion. Such increased sensitivity was observed in rigid ryegrass [48], short awn foxtail [41], Palmer amaranth [37], common waterhemp [36], barnyard grass [35], and rigid brome [40]. Malathion application also reversed 2,4-D-induced protection against chlorsulfuron in susceptible rigid ryegrass from Australia, suggesting the involvement of CYP450s in metabolizing chlorsulfuron [29].

3.3. Synthetic Auxinic Herbicides

Synthetic auxinic herbicides (SAH) are known to mimic the natural plant hormone, indole 3-acetic acid (IAA) [49]. These auxin analogs are mostly used for controlling broadleaf weeds in monocot crops, except quinclorac and quinmerac, which are known to have some grass activity [50]. Despite being introduced as early as 1945, the evolution of resistance to SAH has been slow, and so far, 39 weeds are reported to have developed resistance [13]. In the majority of weeds, NTSR mechanism(s) via (i) reduced uptake, (ii) decreased translocation, and (iii) increased metabolism has been documented. The reduced uptake of SAH is often affected by the properties of the leaf cuticle or other structural barriers that prevent absorption of the herbicide into mesophyll after herbicide application [51]. However, reduced

uptake is a minor mechanism and has been shown to impart resistance in fewer weeds, such as ground ivy [51] and prickly lettuce [52]. However, reduced translocation resulting in the decreased movement of SAH to the site of action is common. Such a reduction in translocation was reported in several weed species, such as wild radish [53], oriental mustard [54], corn poppy [55], and prickly lettuce [52]. For instance, reduced translocation was observed in oriental mustard where approximately 77% of 2,4-D (2,4-dichlorophenoxyacetic acid) was retained in the treated leaves of resistant plants compared to 32% in susceptible plants at 72 h after treatment (HAT) [54]. In another study, the application of auxin efflux inhibitors 1-naphthylphthalamic (NPA) and 2,3,5-triiodobenzoic acid (TIBA) via roots of 2,4-D-susceptible wild radish plants significantly inhibited the translocation of 2,4-D out of the treated leaves, mimicking the 2,4-D-resistant wild radish [53]. Application of the same inhibitors did not affect the translocation of 2,4-D in the resistant biotype, suggesting alteration of the activity of ABC-transporters present in the plasma-membrane that usually facilitate the long-distance transport of 2,4-D [53]. MCPA (2-methyl-4-chlorophenoxyacetic acid) resistance in wild radish from Australia has been attributed to the rapid translocation to the roots [56]. At 48 HAT, a significantly lower amount of MCPA was recovered from resistant plants compared to susceptible plants, suggesting a possible root exudation of MCPA out of the plants [56]. Rapid metabolism of SAH is another major NTSR mechanism reported in several dicot weed species, where similar to the naturally tolerant monocot species, detoxification of herbicides occurs via ring-hydroxylation followed by conjugation, mediated by CYP450s. Such rapid detoxification of SAH has been reported in common waterhemp [36,57] and corn poppy [58]. In 2,4-D-resistant common waterhemp, 2,4-D was found to metabolize at a much faster rate compared to the susceptible plants, resulting in a lower metabolic half-life of 2,4-D [57]. In two 2,4-D-resistant corn poppy populations from Spain, enhanced metabolism was reported [58]. Two hydroxy metabolites were detected in the roots and shoots of the resistant plants, but not in the susceptible plants, suggesting a possible enhanced metabolism of herbicide due to CYP450-based hydroxylation in resistant plants [58]. Increased sensitivity of SAH-resistant biotypes was observed when pre-treated with CYP450-inhibitor malathion followed by herbicide application [36,57,58].

3.4. Photosystem II (PS-II)-Inhibitors

PS-II inhibitors act by competitively binding to the plastoquinone binding site (Q_B) on the D1 protein in the PS-II complex of the chloroplast [59]. This blockage disrupts photosynthesis since plastoquinone is vital for the electron transfer from PS-II to PS-I, and for generating nicotinamide adenine dinucleotide phosphate (NADPH) and ATP. The D1 protein is encoded by chloroplastic *psbA* gene, and hence, TSR to PS-II inhibitors can only be inherited maternally [60]. So far, 74 weed species have been reported to evolve resistance to PS-II inhibitors globally, via both TSR and NTSR mechanisms [13]. TSR to PS-II inhibition as a result of point mutations in the Q_B binding site has been reported in several weeds, such as kochia [61] and wild radish [62].

NTSR to PS-II inhibitors have been documented in annual bluegrass [63], common ragweed [64], common waterhemp [65,66], Palmer amaranth [67,68], and wild radish [62]. In the majority of these cases, the metabolism of PS-II inhibitors was catalyzed by the enhanced activity of GST enzymes [69] and/or CYP450 enzymes [70]. For example, atrazine-resistant Palmer amaranth from Kansas was found to conjugate atrazine 24 times faster than the susceptible plants via enhanced GST-activity [67]. Similarly, the enhanced metabolism of atrazine was found in two common waterhemp populations from Illinois [10] and Nebraska [65]. In the atrazine-resistant common waterhemp from Nebraska, at 6 HAT, approximately 92% of the atrazine was found to be conjugated by GSTs, whereas 92% of atrazine was still retained as a parent compound in susceptible plants [65]. Involvement of a phi-class GST, i.e., AtuGSTF2, was identified in mediating atrazine resistance in common waterhemp from Illinois [66]. Application of GST-inhibitors like 4-chloro-7-nitro-1,2,3-benzoxadiazole (NBD-cl) prior to atrazine application in resistant common waterhemp has resulted in greater sensitivity to atrazine [10]. Similarly, pre-treatment with a CYP450 inhibitor, 1-aminobenzotriazole, has shown increased sensitivity to resistant rigid ryegrass to simazine [70]. Apart from increased metabolism,

reduced absorption and translocation can also impart PS-II-inhibitor resistance. Reduced absorption, translocation and increased metabolism of atrazine were observed in a PS-II-inhibitor (atrazine, diuron, semicarbazone)-resistant annual bluegrass population where known mutations in the *psbA* gene were lacking [63].

3.5. Enolpyruvyl Shikimate-3-Phosphate Synthase (EPSPS)-Inhibitors

Glyphosate, a non-selective, broad-spectrum herbicide, inhibits EPSPS in the shikimate pathway by acting as a transition state analog of phosphoenolpyruvate (PEP), which is a substrate for EPSPS [71]. The shikimate pathway produces the aromatic amino acids tryptophan, tyrosine, and phenylalanine, which are vital for plant growth and development. Additionally, glyphosate can increase carbon flow to the shikimate pathway, resulting in a shortage of carbon for other essential pathways [72]. Currently, there are 44 weeds reported to have evolved resistance to glyphosate [13]. Many of these resistance cases are either by alteration in the target (*EPSPS*) gene [73,74] or amplification and over-expression of the target gene [75–77].

Reduced translocation of glyphosate to meristematic sinks has been reported as the most common NTSR mechanism [78–80]. This mechanism has been reported in Palmer amaranth [81–83], horseweed [84], hairy fleabane [84], Italian ryegrass [85], rigid ryegrass [74,86], common waterhemp [73], Johnsongrass [87,88], sourgrass [89], and giant ragweed [90]. Reduction in translocation has been attributed to the evolution of a transporter that sequesters glyphosate inside the plant vacuole, thus preventing it from reaching the chloroplast [78]. In glyphosate-resistant horseweed, more (>85%) glyphosate was present in the vacuole of the resistant compared to only 15% in the susceptible plants [80]. Such sequestration was irreversible at least up to several days following the glyphosate application [80,91,92]. Similar modified sub-cellular distribution of glyphosate was found in glyphosate-resistant *Conyza bonariensis* [79]. ABC transporter proteins have been proposed to sequester glyphosate via active glyphosate transport [78,93]. Through GS-FLX 454 pyrosequencing, an increased expression of several *ABC* transporter genes was found in glyphosate-resistant horseweed following glyphosate application [94]. However, the role of a specific gene or gene family mediating glyphosate sequestration resulting in NTSR is still unknown. The reduced uptake of glyphosate has also been shown to impart low-level resistance to glyphosate in several weeds, such as Palmer amaranth [81,83], sourgrass [89], and Johnsongrass [87]. An enhanced metabolism of glyphosate is another mechanism responsible for high tolerance to glyphosate and was observed in some biotypes of sourgrass [95], horseweed [96], and *Echinochloa colona* [97]. In sourgrass biotypes with a greater tolerance to glyphosate, more than 56% of glyphosate was metabolized into aminomethylphosphonic acid (AMPA), glyoxylate, and sarcosine at 168 HAT compared to 10% in susceptible biotypes [95]. Similar, rapid metabolism of glyphosate was observed in resistant horseweed populations where almost 100% of glyphosate metabolized into glyoxylate, sarcosine, and AMPA within 96 HAT [96]. Through RNA-seq analysis, an aldo-keto reductase (AKR) contig with greater expression and activity, exhibiting metabolic resistance to glyphosate was identified in an *Echinochloa colona* population from Australia [97]. Further, glyphosate metabolites, such as AMPA and glyoxalate, were also found in *Escherichia coli* expressing the *AKR* gene (*EcAKR4-1*), which was similar to the resistant *Echinochloa colona* plants [97].

3.6. 4-Hydroxyphenylpyruvate Dioxygenase (HPPD) and Carotenoid-Inhibitors

HPPD enzyme is required for catalyzing the conversion of 4-hydroxyphenylpyruvate (HPP) to 2,5-dihydroxyphenylacetate (homogentisate) to produce plastoquinone and tocopherols in the carotenoid biosynthesis pathway [98]. Plastoquinone is essential for the electron transfer from PS-II to PS-I and also as a co-factor of phytoene desaturase (PDS), required for carotenoid formation [99]. Hence, most of these herbicides inhibit carotenoid formation, ultimately resulting in photo-oxidation of chlorophyll molecules and lipid peroxidation of the cell membranes by forming singlet oxygen [100]. So far, NTSR to HPPD-inhibitors has been reported most often. However, TSR caused by higher *HPPD* gene and protein expression has been reported in mesotrione-resistant Palmer amaranth [99].

Enhanced metabolism was the primary NTSR mechanism reported in HPPD-inhibitors-resistant Palmer amaranth [99,101,102], common waterhemp [10,36,103–105], and rice barnyardgrass [106]. In mesotrione-resistant Palmer amaranth, more than 90% of mesotrione was metabolized at 24 HAT [99]. Rapid 4-hydroxylation, followed by glycosylation and a higher expression of certain CYP450 enzymes, were identified in tembotrione-resistant Palmer amaranth compared to the susceptible biotype [102]. Similarly, increased mesotrione metabolism via 4-hydroxylation of the dione ring was reported in mesotrione-resistant common waterhemp from Nebraska [105]. Pre-treatment with CYP450-inhibitors has been shown to increase the sensitivity of resistant common waterhemp populations to mesotrione [10,36,103]. CYP450s belonging to sub-family CYP81A were found to impart metabolic-resistance to clomazone in rice barnyard grass [106]. *Arabidopsis* lines transformed with CYP81A12, CYP81A21, CYP81A15, and CYP81A24 showed increased resistance to clomazone, indicating their involvement in metabolizing clomazone [106].

3.7. Protoporphyrinogen Oxidase (PPO)-Inhibitors

PPO inhibitors are important broad-spectrum herbicides that growers can use to control weeds resistant to ALS inhibitors and glyphosate [107]. PPO inhibitors impede the PPO enzyme, which is required for catalyzing the conversion of protoporphyrinogen IX to protoporphyrin IX in the last step of plant heme and chlorophyll biosynthesis [108,109]. The inhibition of the PPO enzyme leads to the accumulation of intermediates in the cytosol, which are photoactively oxidized, ultimately leading to the production of highly reactive oxygen species (ROS). These ROS attack lipids and proteins in cell membranes and cause lipid peroxidation, leading to plant death [110]. So far, 13 weeds have evolved resistance to PPO inhibitors [13]. The most common mechanism of resistance reported was a single amino acid deletion (Gly210) or substitution in the *PPX2* (e.g., Arg98Leu) gene [107].

NTSR-based PPO inhibitor resistance has been reported in two pigweed species: Palmer amaranth [111] and common waterhemp [112]. A common waterhemp population resistant to carfentrazone-ethyl lacked the presence of known mutations previously reported to confer TSR to PPO inhibitors but exhibited increased sensitivity to carfentrazone-methyl when pre-treated with malathion [112]. This suggests the possible involvement of CYP450 in conferring resistance to carfentrazone-ethyl in common waterhemp [112]. Similarly, the absence of known mutations was reported in fomesafen-resistant Palmer amaranth [111]. The same population was further found to be cross-resistant to flumioxazin, acifluorfen, and saflufenacil [113]. Involvement of both CYP450s and GSTs was reported to mediate fomesafen resistance in Palmer amaranth due to increased sensitivity when pre-treated with malathion or NBD-cl [111,113].

3.8. Photosystem I (PS-I)-Inhibitors

Paraquat is a non-selective, fast-acting herbicide that diverts electrons from PS-I, leading to the inhibition of photosynthesis. Paraquat accepts a single electron in order to generate a reduced cation radical, that on further reaction with oxygen, generates a superoxide ion [114]. In the presence of light, paraquat catalyzes the production of superoxide ions, which eventually form hydroxy radicals and result in lipid peroxidation [114,115]. Only a single NTSR mechanism, i.e., reduced translocation via vacuolar sequestration, has been reported in horseweed [84], hairy fleabane [84], rigid ryegrass [116], and Italian ryegrass [117]. The amount of paraquat present in the leaf protoplast of resistant and susceptible rigid ryegrass was estimated and a 2–3-fold higher retention of herbicide was found in leaves of resistant plants, indicating possible sequestration of the herbicide in vacuoles [116]. A similar mechanism of paraquat resistance was reported in Italian ryegrass [117]. However, the molecular basis of such sequestration is still unknown.

3.9. Very Long Chain Fatty Acid (VLCFA) Synthesis-Inhibitors

VLCFA inhibiting herbicides are known to affect several steps in the elongation of the carbon chain of very long chain fatty acids [118]. VLCFAs are required for the formation of triacylglycerols, waxes, phospholipids, and complex sphingolipids, which are essential for various plant functions [119]. For instance, phospholipids and sphingolipids are required during cell division, as well as for maintaining membrane trafficking pathways [119–121]. The ever-increasing occurrence of ACCase and ALS-inhibitor resistance has led growers to rely more on VLCFA inhibitors, which are important pre-emergence herbicides for controlling grasses.

NTSR mechanisms to very long chain fatty acid (VLCFA) inhibitors have been studied in ryegrass populations from Australia [122], U.K., and France [123], as well as Palmer amaranth from Arkansas [124]. Metabolic resistance to pyroxasulfone was reported in a rigid ryegrass population from Australia, where approximately 88% of parental applied herbicides was metabolized within 24 HAT [122]. Pyroxasulfone metabolites were formed via glutathione conjugation and two putative *GST* genes were 2–6-fold constitutively overexpressed in resistant ryegrass populations [122]. Interestingly, continuous sub-optimal application of pyroxasulfone can rapidly select for resistant biotypes of rigid ryegrass [125]. Application of herbicides at sub-optimal doses can favor the selection of several minor resistance alleles and facilitate their accumulation in cross-pollinating weeds like rigid ryegrass, leading to rapid evolution of polygenic NTSR [126,127]. Moreover, such selection can promote the evolution of cross-resistance to other VLCFA inhibitors like prosulfocarb and triallate [128]. In flufenacet-resistant ryegrass populations from the U.K. and France, enhanced metabolism due to conjugation by GSTs was reported [123]. Similarly, enhanced metabolism of flufenacet was observed in resistant blackgrass [129]. Recently, resistance to *s*-metolachlor was documented in Palmer amaranth from Arkansas. A reduction in root growth was observed when the resistant accessions were kept for germination in agar solution containing a GST-inhibitor, NBD-cl, indicating the role of GSTs in mediating the resistance [124].

4. Influence of Environmental Factors on NTSR Mechanisms

NTSR mechanisms can be affected by changes in environmental conditions [130,131]. Factors like the mode of action of herbicides and the physiology of weed species can contribute significantly in the alteration of NTSR under different environmental conditions. Both herbicide-resistant and susceptible biotypes have shown increased and decreased tolerance to herbicides under different environmental conditions. NTSR mechanisms are suspected to develop gradually in response to biotic and abiotic stresses, which enable them to adapt to the growing conditions [132]. Changing environmental conditions can seriously affect herbicide efficacy and favor the selection of more tolerant biotypes. Hence, information on how NTSR mechanisms behave in different weed species in varying environmental conditions can be very crucial to mitigate such selection. The effect of environmental conditions, such as temperature, CO_2 concentration, and relative humidity, on NTSR mechanisms has been studied in several weed species. Altered temperature regimes were shown to impact herbicide absorption [133–136], translocation and sequestration [133,134,136–141], and metabolism [30,139,142]. For example, exposure to high temperature was found to reduce pinoxaden sensitivity of grass species, such as *Brachypodium hybridum* [142]. A higher level of inactive glucose-conjugated pinoxaden metabolites in these grasses was observed under high- versus low-temperature conditions [142]. Such increased detoxification of pinoxaden in *Brachypodium hybridum* was associated with a possible increased enzymatic activity of reactive oxygen species scavengers [30]. In another study, the suppression of vacuolar sequestration of glyphosate at low temperature was found to result in the increased sensitivity of glyphosate-resistant horseweed [137]. Recently, poor control of kochia due to the reduced absorption of glyphosate and translocation of dicamba at high temperature was reported [133]. Decreased efficacy of mesotrione in controlling Palmer amaranth due to rapid metabolism at high temperature was documented [139]. Conversely, improved 2,4-D efficacy at high temperature due to increased translocation was found in both glyphosate-resistant and -susceptible common and giant ragweed [134]. Additionally, increased absorption of glyphosate in common ragweed, and

increased absorption and translocation of glyphosate in giant ragweed, improved glyphosate efficacy at high temperature [134]. Apart from temperature, changes in CO_2 concentrations can affect herbicide translocation and sequestration in weeds [140,143]. In horseweed and lambsquarter, an increase in glyphosate translocation was found at elevated CO_2 levels and increased temperature, leading to a reduced glyphosate sensitivity [140]. Similarly, in *Echinochloa colona*, high CO_2 and increased temperature reduced the efficacy of cyhalofop-butyl by decreasing translocation [143]. Altering relative humidity (RH) can also affect herbicide translocation in pigweeds [144]. For instance, in Palmer amaranth, redroot pigweed, and common waterhemp, a higher translocation of glufosinate was found at high, compared to low, RH [144]. These findings indicate the need to further elucidate and evaluate the impact of environmental conditions on the sensitivity of weeds to herbicides to slow evolution of herbicide resistance.

5. Coexistence of TSR and NTSR Mechanisms

Numerous cases of TSR have been reported as a result of single nucleotide polymorphisms resulting in amino acid substitutions in the target sites of several herbicides, such as PS-I, ALS, and ACCase inhibitors, and glyphosate. Novel mechanisms of TSR in weeds, such as the deletion of codons, leading to PPO-inhibitor resistance in common waterhemp and Palmer amaranth [107,145], as well as gene amplification-based resistance to glyphosate [75,77] and ACCase inhibitors [146], were reported. These findings will help in identifying the precise genetic elements involved in the evolution of TSR in resistant weeds. Similarly, recent advances have also helped to understand the physiological and molecular basis of NTSR in weed species. Interestingly, several cases of coexistence of these mechanisms have been reported. For instance, ALS-inhibitor-resistant water chickweed [33] and barnyard grass [147], ACCase-inhibitor-resistant *Vulpia bromoides* [148], Italian ryegrass [19], short awn foxtail [41], PS-II inhibitor-resistant wild radish [62], EPSPS-inhibitor-resistant rigid ryegrass [74], common waterhemp [73], HPPD-inhibitor-resistant Palmer amaranth [99], and microtubule inhibitor-resistant rigid ryegrass [34] have been identified with TSR and NTSR mechanisms in the same populations. Therefore, if TSR mechanisms are found to contribute to herbicide resistance, it is also necessary to investigate the NTSR mechanisms and vice versa. Although deciphering both types of resistances in the same weed species can be challenging, understanding the coexistence of TSR and NTSR mechanisms for the same herbicide is valuable for predicting possible cross-resistance to other herbicides, thereby assisting in management of resistance.

6. Conclusions and Prospects

Weed management practices can impact the mechanisms by which weeds evolve resistance to herbicides. Additionally, a key aspect in predicting the evolutionary trajectory of herbicide-resistant traits is understanding the mechanism(s) of herbicide resistance. More importantly, understanding the relationship between the weed management tactics and their influence on evolutionary mechanisms (TSR or NTSR) that determine herbicide resistance in weed species will help to formulate effective future strategies to manage these increasingly problematic weeds. It has been proposed that a lower rate of herbicides result in the evolution of polygenic traits, whereas high herbicide doses may favor monogenic target-site-based resistances [127]. Likewise, Gressel [149] proposed that suboptimal herbicide use rates can result in the evolution of polygenic herbicide resistance. Understanding the type of selection pressure leading to the evolution of NTSR mechanisms, especially metabolic resistance, is extremely valuable and needed to sustain the limited herbicide portfolio and develop integrated weed management strategies.

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References

1. Powles, S.B.; Yu, Q. Evolution in action: Plants resistant to herbicides. *Annu. Rev. Plant Biol.* **2010**, *61*, 317–347. [CrossRef] [PubMed]
2. Jasieniuk, M.; Brûlé-Babel, A.L.; Morrison, I.N. The evolution and genetics of herbicide resistance in weeds. *Weed Sci.* **1996**, *44*, 176–193. [CrossRef]
3. Délye, C.; Jaseniuk, M.; Le Corre, V. Deciphering the evolution of herbicide resistance in weeds. *Trends Genet.* **2013**, *29*, 649–658. [CrossRef] [PubMed]
4. Sammons, R.D.; Gaines, T.A. Glyphosate resistance: State of knowledge. *Pest Manag. Sci.* **2014**, *70*, 1367–1377. [CrossRef] [PubMed]
5. Devine, M.D.; Eberlein, C.V. Physiological, biochemical and molecular aspects of herbicide resistance based on altered target sites. In *Herbicide Activity: Toxicology, Biochemistry and Molecular Biology*; Roe, R.M., Burton, J.D., Kuhr, R.J., Eds.; IOS: Amsterdam, The Netherlands, 1997; pp. 159–185.
6. Preston, C. Inheritance and linkage of metabolism-based herbicide cross-resistance in rigid ryegrass (*Lolium rigidum* Gaud.). *Weed Sci.* **2003**, *51*, 4–12. [CrossRef]
7. Yamada, T.; Kambara, Y.; Imaishi, H.; Ohkawa, H. Molecular cloning of novel Cytochrome P450 species induced by chemical treatments in cultured tobacco cells. *Pestic. Biochem. Physiol.* **2000**, *68*, 11–25. [CrossRef]
8. Gion, K.; Inui, H.; Takakuma, K.; Yamada, T.; Kambara, Y.; Nakai, S.; Fijuwara, H.; Miyamura, T.; Imaishi, H.; Ohkawa, H. Molecular mechanisms of herbicide-inducible gene expression of tobacco CYP71AH11 metabolizing the herbicide chlorotoluron. *Pestic. Biochem. Physiol.* **2014**, *108*, 49–57. [CrossRef]
9. Huffman, J.; Hausman, N.; Hager, A.; Riechers, D.; Tranel, P. Genetics and inheritance of nontarget-site resistances to atrazine and mesotrione in a waterhemp (*Amaranthus tuberculatus*) population from Illinois. *Weed Sci.* **2015**, *63*, 799–809. [CrossRef]
10. Ma, R.; Kaundun, S.S.; Tranel, P.J.; Riggins, C.W.; McGinness, D.L.; Hager, A.G.; Hawkes, T.; McIndie, E.; Riechers, D.E. Distinct detoxification mechanisms confer resistance to mesotrione and atrazine in a population of waterhemp. *Plant Physiol.* **2013**, *163*, 363–377. [CrossRef]
11. Kaundun, S.S. Resistance to acetyl-CoA carboxylase-inhibiting herbicides. *Pest. Manag. Sci.* **2014**, *70*, 1405–1417. [CrossRef]
12. Devine, M.D. Acetyl-CoA carboxy lase inhibitors. In *Herbicide Classes in Development*; Boger, P., Wakabayashi, K., Hirai, K., Eds.; Springer: Berlin, Germany, 2002; pp. 103–137.
13. Heap, I. The International Survey of Herbicide Resistant Weeds. Available online: www.weedscience.org (accessed on 7 August 2019).
14. Yu, L.P.; Kim, Y.S.; Tong, T. Mechanism for the inhibition of the carboxyltransferase domain of acetyl-coenzyme A carboxylase by pinoxaden. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 22072–22077. [CrossRef] [PubMed]
15. Zhang, Z.Y.H.; Shen, Y.; Tong, L. Crystal structure of the carboxyltransferase domain of acetyl-coenzyme A carboxylase. *Science* **2003**, *299*, 2064–2067. [CrossRef] [PubMed]
16. Zhao, N.; Ge, L.; Yan, Y.; Bai, S.; Wang, D.; Liu, W.; Wang, J. Trp-1999-Ser mutation of acetyl CoA carboxylase and cytochrome P450s-involved metabolism confer resistance to fenoxaprop-*P*-ethyl in *Polypogon fugax*. *Pest Manag. Sci.* **2019**. [CrossRef] [PubMed]
17. Huan, Z.; Zhang, H.; Hou, Z.; Zhang, S.; Zhang, Y.; Liu, W.T.; Bi, Y.; WANG, J. Resistance level and metabolism of barnyard-grass (*Echinochloa crusgalli* (L.) Beauv.) populations to quizalofop-p-ethyl in Heilongjiang province. *China Agric. Sci. China* **2011**, *10*, 1914–1922. [CrossRef]
18. Délye, C.; Gardin, J.A.C.; Bucansaud, K.; Chauvel, B.; Pett, C. Non-target-site-based resistance should be the centre of attention for herbicide resistance research: *Alopecurus myosuroides* as an illustration. *Weed Res.* **2011**, *51*, 433–437. [CrossRef]
19. Kaundan, S.S. An aspartate to glycine change in the carboxyl transferase domain of acetyl CoA carboxylase and non-target-site mechanism(s) confer resistance to ACCase inhibitor herbicides in a *Lolium multiflorum* population. *Pest Manag. Sci.* **2010**, *66*, 1249–1256. [CrossRef] [PubMed]
20. Kaundan, S.S.; Bailly, G.C.; Dale, R.P.; Hutchings, S.J.; McIndoe, E. A Novel W1999S Mutation and Non-Target Site Resistance Impact on Acetyl-CoA Carboxylase Inhibiting Herbicides to Varying Degrees in a UK *Lolium multiflorum* Population. *PLoS ONE* **2013**, *8*, e58012. [CrossRef]
21. Feng, Y.; Gao, Y.; Zhang, Y.; Dong, L.; Li, J. Mechanisms of resistance to pyroxsulam and ACCase inhibitors in Japanese foxtail (*Alopecurus japonicus*). *Weed Sci.* **2016**, *64*, 695–704. [CrossRef]

22. Busi, R.; Vila-Aiub, M.M.; Powles, S.B. Genetic control of a cytochrome P450 metabolism-based herbicide resistance mechanism in *Lolium rigidum*. *Heredity* **2011**, *106*, 817–824. [CrossRef]
23. Han, H.; Yu, Q.; Owen, M.J.; Cawthray, G.R.; Powles, S.B. Widespread occurrence of both metabolic and target-site herbicide resistance mechanisms in *Lolium rigidum* populations. *Pest Manag. Sci.* **2016**, *72*, 255–263. [CrossRef]
24. Gaines, T.A.; Lorentz, L.; Figge, A.; Herrmann, J.; Maiwald, F.; Ott, M.C.; Han, H.; Busi, R.; Yu, Q.; Powles, S.B. RNA-Seq transcriptome analysis to identify genes involved in metabolism-based diclofop resistance in *Lolium rigidum*. *Plant J.* **2014**, *78*, 865–876. [CrossRef] [PubMed]
25. Ahmad-Hamdani, M.S.; Yu, Q.; Han, H.; Cawthray, G.R.; Wang, S.F.; Powles, S.B. Herbicide resistance endowed by enhanced rates of herbicide metabolism in wild oat (*Avena* spp.). *Weed Sci.* **2013**, *61*, 55–62. [CrossRef]
26. Yu, Q.; Han, H.; Cawthray, G.R.; Wang, S.F.; Powles, S.B. Enhanced rates of herbicide metabolism in low herbicide-dose selected resistant *Lolium rigidum*. *Plant Cell Environ.* **2013**, *36*, 818–827. [CrossRef] [PubMed]
27. Kreuz, K.; Fonné-Pfister, R. Herbicide-insecticide interaction in maize: Malathion inhibits cytochrome P450-dependent primisulfuron metabolism. *Pestic. Biochem. Phys.* **1992**, *43*, 232–240. [CrossRef]
28. Baerg, R.J.; Barrett, M.; Polge, N.D. Insecticide and Insecticide Metabolite Interactions with Cytochrome P450 Mediated Activities in Maize. *Pestic. Biol. Phys.* **1996**, *55*, 10–20. [CrossRef]
29. Han, H.; Yu, Q.; Cawthray, G.R.; Powles, S.B. Enhanced herbicide metabolism induced by 2,4-D in herbicide susceptible *Lolium rigidum* provides protection against diclofop-methyl. *Pest Manag. Sci.* **2013**, *69*, 996–1000. [CrossRef]
30. Matzrafi, M.; Shaar-Moshe, L.; Rubin, B.; Peleg, Z. unraveling the transcriptional basis of temperature-dependent pinoxaden resistance in *Brachypodium hybridum*. *Front. Plant Sci.* **2017**, *8*, 1064. [CrossRef]
31. Tranel, P.; Wright, T.R. Resistance of Weeds to ALS-Inhibiting Herbicides: What Have We Learned? *Weed Sci.* **2002**, *50*, 700–712. [CrossRef]
32. Umbarger, H.E. Amino acid biosynthesis and its regulation. *Annu. Rev. Biochem.* **1978**, *47*, 533–606. [CrossRef]
33. Bai, S.; Zhang, F.; Li, Z.; Wang, H.; Wang, Q.; Wang, J.; Liu, W.; Bai, L. Target-site and non-target-site-based resistance to tribenuron-methyl in multiply-resistant *Myosoton aquaticum* L. *Pestic. Biochem. Phys.* **2019**, *155*, 8–14. [CrossRef]
34. Chen, J.; Chu, Z.; Han, H.; Goggin, D.E.; Yu, Q.; Sayer, C.; Powles, S.B. A Val-202-Phe α-tubulin mutation and enhanced metabolism confer dinitroaniline resistance in a single *Lolium rigidum* population. *Pest. Manag. Sci.* **2019**. [CrossRef]
35. Riar, D.S.; Norsworthy, J.K.; Bond, J.A.; Bararpour, M.T.; Wilson, M.J.; Scott, R.C. Resistance of *Echinochloa crus-galli* populations to acetolactate synthase-inhibiting herbicides. *Int. J. Agron.* **2012**, *2012*, 893953. [CrossRef]
36. Shergill, L.S.; Bish, M.D.; Jugulam, M.; Bradley, K.W. Molecular and physiological characterization of six-way resistance in an *Amaranthus tuberculatus* var. rudis biotype from Missouri. *Pest. Manag. Sci.* **2018**, *74*, 2688–2698. [CrossRef]
37. Nakka, S.; Thompson, C.R.; Peterson, D.E.; Jugulam, M. Target site–based and non-target site based resistance to ALS inhibitors in Palmer amaranth (*Amaranthus palmeri*). *Weed Sci.* **2017**, *65*, 681–689. [CrossRef]
38. Iwakami, S.; Endo, M.; Saika, H.; Okuno, J.; Nakamura, N.; Yokoyama, M.; Watanabe, H.; Toki, S.; Uchino, A.; Inamura, T. Cytochrome P450 CYP81A12 and CYP81A21 are associated with resistance to two acetolactate synthase inhibitors in *Echinochloa phyllopogon*. *Plant Physiol.* **2014**, *165*, 618–629. [CrossRef]
39. Iwakami, S.; Kamidate, Y.; Yamaguchi, T.; Ishizaka, M.; Endo, M.; Suda, H.; Nagai, K.; Sunohara, Y.; Toki, S.; Uchino, A.; et al. CYP81A P450s are involved in concomitant cross-resistance to acetolactate synthase and acetyl-CoA carboxylase herbicides in *Echinochloa phyllopogon*. *New Phytol.* **2019**, *221*, 2112–2122. [CrossRef]
40. Owen, M.J.; Goggin, D.E.; Powles, S.B. Non-target-site-based resistance to ALS-inhibiting herbicides in six *Bromus rigidus* populations from Western Australian cropping fields. *Pest Manag. Sci.* **2012**, *68*, 1077–1082. [CrossRef]
41. Zhao, N.; Yan, Y.; Ge, L.; Zhu, B.; Liu, W.; Wang, J. Target site mutations and cytochrome P450s confer resistance to fenoxaprop-*P*-ethyl and mesosulfuron-methyl in *Alopecurus aequalis*. *Pest Manag. Sci.* **2019**, *75*, 204–214. [CrossRef]

42. Zhao, N.; Li, W.; Bai, S.; Guo, W.; Yuan, G.; Wang, F.; Liu, W.; Wang, J. Transcriptome profiling to identify genes involved in mesosulfuron-methyl resistance in *Alopecurus aequalis*. *Front. Plant Sci.* **2017**, *8*, 1391. [CrossRef]
43. Yu, Q.; Powles, S. Metabolism-Based Herbicide Resistance and Cross-Resistance in Crop Weeds: A Threat to Herbicide Sustainability and Global Crop Production. *Plant Physiol.* **2014**, *166*, 1106–1118. [CrossRef]
44. Liu, W.; Bai, S.; Zhao, S.; Li, W.; Zhang, L.; Wang, J. Non-target site-based resistance to tribenuron-methyl and essential involved genes in *Myosoton aquaticum* (L.). *BMC Plant Biol.* **2018**, *18*, 225. [CrossRef]
45. Duhoux, A.; Carrere, S.; Gouzy, J.; Bonin, L.; Delye, C. RNA-Seq analysis of rye-grass transcriptomic response to an herbicide inhibiting acetolactate-synthase identifies transcripts linked to non-target-site-based resistance. *Plant Mol. Biol.* **2015**, *87*, 473–487. [CrossRef]
46. Yang, Q.; Deng, W.; Li, X.; Yu, Q.; Bai, L.; Zheng, M. Target-site and non-target-site based resistance to the herbicide tribenuron-methyl in flixweed (*Descurainia sophia* L.). *BMC Genom.* **2016**, *17*, 551. [CrossRef]
47. Gardin, J.A.C.; Gouzy, J.; Carrere, S.; Delye, C. ALOMY base, a resource to investigate non-target-site-based resistance to herbicides inhibiting acetolactate-synthase (ALS) in the major grass weed *Alopecurus myosuroides* (black-grass). *BMC Genom.* **2015**, *16*, 590. [CrossRef]
48. Busi, R.; Gaines, T.A.; Powles, S.B. Phorate can reverse P450 metabolism-based herbicide resistance in *Lolium rigidum*. *Pest Manag. Sci.* **2017**, *73*, 410–417. [CrossRef]
49. Busi, R.; Goggin, D.E.; Heap, I.M.; Horak, M.J.; Jugulam, M.; Masters, R.A.; Napier, R.M.; Riar, D.S.; Satchivi, N.M.; Torra, J.; et al. Weed resistance to synthetic auxin herbicides. *Pest Manag. Sci.* **2018**, *74*, 2265–2276. [CrossRef]
50. Grossmann, K. Auxin herbicides: Current status of mechanism and mode of action. *Pest. Manag. Sci.* **2010**, *66*, 113–120. [CrossRef]
51. Kohler, E.A.; Throssell, C.S.; Reicher, Z.J. 2,4-D rate response, absorption, and translocation of two ground ivy (*Glechoma hederacea*) populations. *Weed Technol.* **2004**, *18*, 917–923. [CrossRef]
52. Riar, D.S.; Burke, I.C.; Yenish, J.P.; Bell, J.; Gill, K. Inheritance and physiological basis for 2,4-D resistance in prickly lettuce (*Lactuca serriola* L.). *J Agric. Food Chem.* **2011**, *59*, 9417–9423. [CrossRef]
53. Goggin, D.E.; Cawthray, C.B. 2,4-D resistance in wild radish: Reduced herbicide translocation via inhibition of cellular transport. *J. Exp. Bot.* **2016**, *67*, 3223–3323. [CrossRef]
54. Dang, H.T.; Malone, J.M.; Boutsalis, P.; Krishnan, M.; Gill, G.; Preston, C. Reduced translocation in 2,4-D-resistant oriental mustard populations (*Sisymbrium orientale* L.) from Australia. *Pest Manag. Sci.* **2018**, *74*, 1524–1532. [CrossRef]
55. Rey-Caballero, J.; Menendez, J.; Gine-Bordonaba, J.; Salas, M.; Alcantara, R.; Torra, J. Unravelling the resistance mechanisms to 2,4-D (2,4-dichlorophenoxyacetic acid) in corn poppy (*Papaver rhoeas*). *Pestic Biochem. Phys.* **2016**, *133*, 67–72. [CrossRef]
56. Jugulam, M.; DiMeo, N.; Veldhuis, L.J.; Walsh, M.; Hall, J.C. Investigation of MCPA (4-Chloro-2-ethylphenoxyacetate) resistance in wild radish (*Raphanus raphanistrum* L.). *J. Agric. Food Chem.* **2013**, *61*, 12516–12521. [CrossRef]
57. Figueiredo, M.R.; Leibhart, L.J.; Reicher, Z.J.; Tranel, P.J.; Nissen, S.J.; Westra, P.; Bernards, M.L.; Kruger, G.R.; Gaines, T.A.; Jugulam, M. Metabolism of 2,4-dichlorophenoxyacetic acid contributes to resistance in a common waterhemp (*Amaranthus tuberculatus*) population. *Pest Manag. Sci.* **2018**, *74*, 2356–2362. [CrossRef]
58. Torra, J.; Rojano-Delgado, A.M.; Rey-Caballero, J.; Royo-Esnal, A.; Salas, M.L.; De Prado, R. Enhanced 2,4-D metabolism in two resistant *Papaver rhoeas* populations from Spain. *Front. Plant Sci.* **2017**, *8*, 1584. [CrossRef]
59. Gronwald, J. Resistance to photosystem II inhibiting herbicides. In *Herbicide Resistance in Plants: Biology and Biochemistry*; Lewis Publishers: Boca Raton, FL, USA, 1994; pp. 27–60.
60. Goloubinoff, P.; Edelman, M.; Hallick, R.B. Chloroplast-coded atrazine resistance in Solanum nigrum: *psbA* loci from susceptible and resistant biotypes are isogenic except for a single codon change. *Nucleic Acids Res.* **1984**, *12*, 9489–9496. [CrossRef]
61. Varanasi, V.K.; Godar, A.S.; Currie, R.S.; Dille, A.J.; Thompson, C.R.; Stahlman, P.W.; Jugulam, M. Field-evolved resistance to four modes of action of herbicides in a single kochia (*Kochia scoparia* L. Schrad.) population. *Pest. Manag. Sci.* **2015**, *71*, 1207–1212. [CrossRef]
62. Lu, H.; Yu, Q.; Han, H.; Owen, M.J.; Powles, S.B. Metribuzin resistance in a wild radish (Raphanus raphanistrum) population via both psbA Gene mutation and enhanced metabolism. *J. Agric. Food Chem.* **2019**, *67*, 1353–1359. [CrossRef]

63. Svyantek, A.; Aldahir, P.; Chen, S.; Flessner, M.; McCullough, P.; Sidhu, S.; McElroy, J. Target and nontarget resistance mechanisms induce annual bluegrass (*Poa annua*) resistance to atrazine, amicarbazone, and diuron. *Weed Tech.* **2016**, *30*, 773–782. [CrossRef]
64. Simard, M.J.; Laforest, M.; Soufiane, B.; Benoit, D.K.; Tardif, F.J. Linuron-resistant common ragweed (*Ambrosia artemisiifolia*) populations in Québec carrot fields: Presence and distribution of target site and non-target site resistant biotypes. *Can. J. Plant Sci.* **2018**, *98*, 345–352. [CrossRef]
65. Vennapusa, A.R.; Faleco, F.; Vieira, B.; Samuelson, S.; Kruger, G.R.; Werle, R.; Jugulam, M. Prevalence and mechanism of atrazine resistance in waterhemp (*Amaranthus tuberculatus*) from Nebraska. *Weed Sci.* **2018**, *66*, 595–602. [CrossRef]
66. Evans, A.F.; O'Brien, S.R.; Ma, R.; Hager, A.G.; Riggins, C.W.; Lambert, K.N.; Riechers, D.E. Biochemical characterization of metabolism-based atrazine resistance in *Amaranthus tuberculatus* and identification of an expressed GST associated with resistance. *Plant Biotechnol. J.* **2017**, *15*, 1238–1249. [CrossRef]
67. Nakka, S.; Godar, A.; Thompson, C.R.; Peteron, D.E.; Juglam, M. Rapid detoxification via Glutathione S-transferase (GST)-conjugation confers high level of atrazine resistance in Palmer amaranth (*Amaranthus palmeri*). *Pest Manag. Sci.* **2017**, *73*, 2236–2243. [CrossRef]
68. Chahal, P.S.; Jugulam, M.; Jhala, A.J. Mechanism of atrazine resistance in atrazine- and HPPD inhibitor-resistant Palmer amaranth (*Amaranthus palmeri* S. Watson) from Nebraska. *Can. J. Plant Sci.* **2019**. [CrossRef]
69. Anderson, M.P.; Gronwald, J.W. Atrazine resistance in a velvetleaf (*Abutilon theophrasti*) biotype due to enhanced glutathione S-transferase activity. *Plant Physiol.* **1991**, *96*, 104–109. [CrossRef]
70. Burnet, M.W.M.; Loveys, B.R.; Holtum, J.A.M.; Powles, S.B. Increased detoxification is a mechanism of simazine resistance in *Lolium rigidum*. *Pestic. Biochem. Physiol.* **1993**, *46*, 207–218. [CrossRef]
71. Duke, S.O.; Powles, S.B. Glyphosate: A once-in-a-century herbicide. *Pest. Manag. Sci.* **2008**, *64*, 319–325. [CrossRef]
72. Siehl, D.L. Inhibitors of EPSP synthase, glutamine synthase and histidine synthesis. In *Herbicide Activity: Toxicology, Biochemistry and Molecular Biology*; Roe, R.M., Burton, J.D., Kuhr, R.J., Eds.; IOS Press: Amsterdam, The Netherlands, 1997; pp. 37–67.
73. Nandula, V.K.; Ray, J.D.; Ribeiro, D.N.; Pan, Z.; Reddy, K.N. Glyphosate resistance in tall waterhemp (*Amaranthus tuberculatus*) from Mississippi is due to both altered target-site and nontarget-site mechanisms. *Weed Sci.* **2013**, *61*, 374–383. [CrossRef]
74. Bostamam, Y.; Malone, J.M.; Dolman, F.C.; Boutsalis, P.; Preston, C. Rigid ryegrass (*Lolium rigidum*) populations containing a target site mutation in EPSPS and reduced glyphosate translocation are more resistant to glyphosate. *Weed Sci.* **2012**, *60*, 474–479. [CrossRef]
75. Jugulam, M.; Niehues, K.; Godar, A.S.; Koo, D.H.; Danilova, T.; Friebe, B.; Sehgal, S.; Varanasi, V.K.; Wiersma, A.; Westra, P.; et al. Tandem amplification of a chromosomal segment harboring 5-Enolpyruvylshikimate-3-Phosphate synthase locus confers glyphosate resistance in *Kochia scoparia*. *Plant Physiol.* **2014**, *166*, 1200–1207. [CrossRef]
76. Koo, D.H.; Molin, W.T.; Saski, C.A.; Jiang, J.; Putta, K.; Jugulam, M.; Friebe, B.; Gill, B.S. Extra-chromosomal circular DNA (eccDNA) based amplification and transmission of herbicide resistance in crop weed *Amaranthus palmeri*. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 3332–3337. [CrossRef] [PubMed]
77. Gaines, T.A.; Zhang, W.; Wang, D.; Bukun, B.; Chisholm, S.T.; Shaner, D.L.; Nissen, S.J.; Patzoldt, W.L.; Tranel, P.J.; Culpepper, A.S.; et al. Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1029–1034. [CrossRef] [PubMed]
78. Shaner, D.L. The role of translocation as a mechanism of resistance to glyphosate. *Weed Sci.* **2009**, *57*, 118–123. [CrossRef]
79. Kleinman, Z.; Rubin, B. Non-target-site glyphosate resistance in *Conyza bonariensis* is based on modified subcellular distribution of the herbicide. *Pest Manag. Sci.* **2017**, *73*, 246–253. [CrossRef]
80. Ge, X.; d'Avignon, D.A.; Acerman, J.J.H.; Sammons, R.D. Rapid vacuolar sequestration: The horseweed glyphosate resistance mechanism. *Pest Manag. Sci.* **2010**, *66*, 345–348. [CrossRef]
81. Palma-Bautista, C.; Torra, J.; Garcia, M.J.; Bracamonte, E.; Rojano-Delgado, A.M.; la Cruz, R.A.; Prado, R.D. Reduced absorption and impaired translocation endows glyphosate resistance in *Amaranthus palmeri* harvested in glyphosate-resistant soybean from Argentina. *J. Agric. Food Chem.* **2019**, *67*, 1052–1060. [CrossRef]

82. Dominguez-Valenzuela, J.A.; Gherekhloo, J.; Fernandez-Moreno, P.T.; Cruz-Hipolito, H.E.; Cruz, R.A.L.; Sanchez-Gonalez, E.; Prado, R.D. First confirmation and characterization of target and non-target site re sistance to glyphosate in Palmer amaranth (*Amaranthus palmeri*) from Mexico. *Plant Physiol. Biochem.* **2017**, *115*, 212–218. [CrossRef]
83. Nandula, V.K.; Reddy, K.N.; Koger, C.H.; Poston, D.H.; Rimando, A.M.; Duke, S.O.; Bond, J.A.; Ribeiro, D.N. Multiple resistance to glyphosate and pyrithiobac in Palmer Amaranth (*Amaranthus palmeri*) from Mississippi and response to flumiclorac. *Weed Sci.* **2012**, *60*, 179–188. [CrossRef]
84. Moretti, M.L.; Hanson, B.D. Reduced translocation is involved in resistance to glyphosate and paraquat in *Conyza bonariensis* and *Conyza canadensis* from California. *Weed Res.* **2016**, *57*, 25–34. [CrossRef]
85. Gonzalez-Torralva, F.; Gil-Humanes, J.; Barro, F.; Brants, I.; Prado, R.D. Target site mutation and reduced translocation are present in a glyphosate-resistant *Lolium multiflorum* Lam. biotype from Spain. *Plant Physiol. Biochem.* **2012**, *58*, 16–22. [CrossRef]
86. Adu-Yeboah, P.; Malone, J.M.; Preston, C. Reduced glyphosate translocation in two glyphosate-resistant populations of rigid ryegrass (*Lolium rigidum*) from fence lines in South Australia. *Weed Sci.* **2014**, *62*, 4–10. [CrossRef]
87. Vila-Aiub, M.; Balbi, M.C.; Distefano, A.J.; Fernandez, L.; Hopp, E.; Yu, Q.; Powles, S.B. Glyphosate resistance in perennial *Sorghum halepense* (Johnsongrass), endowed by reduced glyphosate translocation and leaf uptake. *Pest Manag. Sci.* **2012**, *68*, 430–436. [CrossRef] [PubMed]
88. Riar, D.S.; Norsworthy, J.K.; Johnson, D.B.; Scott, R.C.; Bagavathiannan, M. Glyphosate resistance in a johnsongrass (*Sorghum halepense*) biotype from Arkansas. *Weed Sci.* **2011**, *59*, 299–304. [CrossRef]
89. Carvalho, L.B.D.; Alves, P.L.D.C.A.; Gonzalez-Torralva, F.; Cruz-Hipolito, H.E.; Rojano-Delgado, A.M.; Prado, R.D.; Gil-Humanes, J.; Barro, F.; Castro, M.D.L.D. Pool of resistance mechanisms to glyphosate in *Digitaria insularis*. *J. Agric. Food Chem.* **2012**, *602*, 615–622. [CrossRef]
90. Nandula, V.K.; Wright, A.A.; Horn, C.R.V.; Westra, P.; Reddy, K.N. Glyphosate resistance in giant ragweed (*Ambrosia trifida* L.) from Mississippi is partly due to reduced translocation. *Am. J. Plant Sci.* **2015**, *6*, 2104–2113. [CrossRef]
91. Ge, X.; d'Avignon, D.A.; Acerman, J.J.H.; Collavo, A.; Sattin, M.; Ostrander, E.L.; Hall, E.L.; Sammons, R.D.; Preston, C. Vacuolar glyphosate-sequestration correlates with glyphosate resistance in ryegrass (*Lolium spp.*) from Australia, South America, and Europe: A ^{31}P NMR investigation. *J. Agric. Food Chem.* **2012**, *605*, 1243–1250. [CrossRef]
92. Ge, X.; d'Avignon, D.A.; Acerman, J.J.H.; Sammons, R.D. In vivo ^{31}P-nuclear magnetic resonance studies of glyphosate uptake, vacuolar sequestration, and tonoplast pump activity in glyphosate-resistant horseweed. *Plant Physiol.* **2014**, *168*, 377. [CrossRef]
93. Yuan, J.S.; Abercrombie, L.G.; Cao, Y.; Halfhill, M.D.; Zhou, X.; Peng, Y. Functional genomics analysis of glyphosate resistance in *Conyza canadensis* (horseweed). *Weed Sci.* **2010**, *58*, 109–117. [CrossRef]
94. Peng, Y.; Abercrombie, L.L.G.; Yuan, J.S.; Riggins, C.W.; Sammons, R.D.; Tranel, P.J.; Stewart, C.N.S., Jr. Characterization of the horseweed (*Conyza canadensis*) transcriptome using GS-FLX 454 pyrosequencing and its application for expression analysis of candidate non-target herbicide resistance genes. *Pest Manag. Sci.* **2010**, *66*, 1053–1062. [CrossRef]
95. Carvalho, L.B.D.; Rojano-Delgado, A.M.; Alves, P.L.D.C.; Prado, R.D. Differential content of glyphosate and its metabolites in *Digitaria insularis* biotypes. *Commun. Plant Sci.* **2013**, *3*, 17–20. [CrossRef]
96. González-Torralva, F.; Rojano-Delgado, A.M.; de Castro, M.D.L.; Mülleder, N.; Prado, R.D. Two non-target mechanisms are involved in glyphosate-resistant horseweed (*Conyza canadensis* L. Cronq.) biotypes. *J. Plant Physiol.* **2012**, *169*, 1673–1679. [CrossRef] [PubMed]
97. Pan, L.; Yu, Q.; Han, H.; Mao, L.; Nyporko, A.; Fan, L.; Bai, L.; Stephen, B.; Powles, S.B. AKR metabolizes glyphosate and confers resistance in *Echinochloa colona*. *Plant Physiol.* **2019**. [CrossRef] [PubMed]
98. Beaudegnies, R.; Edmunds, A.J.F.; Fraser, T.E.M.; Hall, R.G.; Hawkes, T.R.; Mitchell, G.; Schaetzer, J.; Wendeborn, S.; Wibley, J. Herbicidal 4-hydroxyphenylpyruvate dioxygenase inhibitors—a review of the triketone chemistry story from a Syngenta perspective. *Bioorg. Med. Chem.* **2009**, *17*, 4134–4152. [CrossRef] [PubMed]
99. Nakka, S.; Godar, A.; Wani, P.S.; Thompson, C.R.; Peterson, D.E.; Roelofs, J.; Jugulam, M. Physiological and molecular characterization of hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor resistance in Palmer amaranth (*Amaranthus palmeri* S.Wats.). *Front. Plant Sci.* **2017**, *8*, 555. [CrossRef] [PubMed]

100. Siefermann, H.D. The light harvesting and protective functions of carotenoids in photosynthetic membranes. *Physiol. Plant.* **1987**, *69*, 561–568. [CrossRef]
101. Thompson, C.R.; Peterson, D.E.; Lally, N.G. Characterization of HPPD-resistant Palmer amaranth. In Proceedings of the Weed Science Society of America Annual Meetings, Waikoloa, Hawaii, 6–9 February 2012.
102. Küpper, A.; Peter, F.; Zöllner, P.; Lorentz, L.; Tranel, P.J.; Beffa, R.; Gaines, T.A. Tembotrione detoxification in 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitor-resistant Palmer amaranth (*Amaranthus palmeri* S. Wats.). *Pest. Manag. Sci.* **2018**, *74*, 2325–2334. [CrossRef]
103. Oliveira, M.C.; Gaines, T.A.; Dayan, F.E.; Patterson, E.L.; Jhala, A.J.; Knezevic, S.Z. Reversing resistance to tembotrione in an *Amaranthus tuberculatus* (var. rudis) population from Nebraska, USA with cytochrome P450 inhibitors. *Pest Manag. Sci.* **2017**, *74*, 2296–2305. [CrossRef]
104. Hausman, N.E.; Singh, S.; Tranel, P.J.; Riechers, D.E.; Kaundun, S.S.; Polge, N.D.; Thomas, D.A.; Hager, A.G. Resistance to HPPD-inhibiting herbicides in a population of waterhemp (*Amaranthus tuberculatus*) from Illinois, United States. *Pest Manag. Sci.* **2011**, *67*, 258–261. [CrossRef]
105. Kaundun, S.S.; Hutchings, S.J.; Dale, R.P.; Howell, A.; Morris, J.A.; Kramer, V.C.; Shivrain, V.K.; Mcindoe, E. Mechanism of resistance to mesotrione in an *Amaranthus tuberculatus* population from Nebraska, USA. *PLoS ONE* **2017**, *12*, e0180095. [CrossRef]
106. Guo, F.; Iwakami, S.; Yamaguchi, T.; Uchino, A.; Sunohara, Y.; Matsumoto, H. Role of CYP81A cytochrome P450s in clomazone metabolism in *Echinochloa phyllopogon*. *Plant Sci.* **2019**, *283*, 31–328. [CrossRef]
107. Salas, R.A.; Burgos, N.R.; Tranel, P.J.; Singh, S.; Glasgow, L.; Scott, R.C.; Nichols, R.L. Resistance to PPO-inhibiting herbicide in Palmer amaranth from Arkansas. *Pest Manag. Sci.* **2016**, *72*, 864–869. [CrossRef] [PubMed]
108. Deybach, J.C.; Dasilva, V.; Grandchamp, B.; Nordmann, Y. The mitochondrial location of protoporphyrinogen oxidase. *Eur. J. Biochem.* **1985**, *149*, 431–435. [CrossRef] [PubMed]
109. Beale, S.I.; Weinstein, J.D. Tetrapyrrole metabolism in photosynthetic organisms. In *Biosynthesis of Heme and Chlorophylls*; Dailey, H.A., Ed.; McGraw-Hill: New York, NY, USA, 1990; pp. 287–391.
110. Sherman, T.D.; Becerril, J.M.; Matsumoto, H.; Duke, M.V.; Jacobs, J.M.; Jacobs, N.J.; Duke, S.O. Physiological basis for differential sensitivities of plant species to protoporphyrinogen oxidase-inhibiting herbicides. *Plant Physiol.* **1991**, *97*, 280–287. [CrossRef] [PubMed]
111. Varanasi, V.K.; Brabham, C.; Norsworthy, J.K. Confirmation and characterization of non–target site resistance to fomesafen in Palmer amaranth (*Amaranthus palmeri*). *Weed Sci.* **2018**, *66*, 702–709. [CrossRef]
112. Obenland, A.A.; Ma, R.; O'Brien, S.R.; Lygin, A.V.; Riechers, D.E. Carfentrazone-ethyl resistance in an *Amaranthus tuberculatus* population is not mediated by amino acid alterations in the PPO2 protein. *PLoS ONE* **2019**, *14*, e0215431. [CrossRef]
113. Varanasi, V.; Brabham, C.; Korres, N.; Norsworthy, J. Nontarget site resistance in Palmer amaranth [*Amaranthus palmeri* (S.) Wats.] confers cross-resistance to protoporphyrinogen oxidase-inhibiting herbicides. *Weed Technol.* **2019**, *33*, 349–354. [CrossRef]
114. Hawkes, T.R. Mechanisms of resistance to paraquat in plants. *Pest. Manag. Sci.* **2014**, *70*, 1316–1323. [CrossRef]
115. Gutteridge, J.M.C. Lipid peroxidation initiated by superoxide-dependent hydroxyl radicals using complexed iron and hydrogen peroxide. *FEBS Lett.* **1984**, *172*, 245–249. [CrossRef]
116. Yu, Q.; Huang, S.; Powles, S. Direct measurement of paraquat in leaf protoplasts indicates vacuolar paraquat sequestration as a resistance mechanism in *Lolium rigidum*. *Pestic. Biochem. Physiol.* **2010**, *98*, 104–109. [CrossRef]
117. Brunharo, C.A.C.G.; Hanson, B.D. Vacuolar sequestration of paraquat is involved in the resistance mechanism in *Lolium perenne* L. spp. *multiflorum*. *Front. Plant Sci.* **2017**, *8*, 1485. [CrossRef]
118. Tanetani, Y.; Kaku, K.; Kawai, K.; Fujioka, T.; Shimizu, T. Action mechanism of a novel herbicide, pyroxasulfone. *Pestic. Biochem. Phys.* **2009**, *95*, 47–55. [CrossRef]
119. Busi, R. Resistance to herbicides inhibiting the biosynthesis of very-long-chain fatty acids. *Pest. Manag. Sci.* **2014**, *70*, 1378–1384. [CrossRef] [PubMed]
120. Lechelt-Kunze, C.; Meissner, R.C.; Drewes, M.; Tietjen, K. Flufenacet herbicide treatment phenocopies the fiddlehead mutant in Arabidopsis thaliana. *Pest Manag. Sci.* **2003**, *59*, 847–856. [CrossRef] [PubMed]

121. Markham, J.E.; Molino, D.; Gissot, L.; Bellec, Y.; Hématy, K.; Marion, J.; Belcram, K.; Palauqui, J.C.; Satiat-JeuneMaitre, B.; Faure, J.D. Sphingolipids containing very-long-chain fatty acids define a secretory pathway for specific polar plasma membrane protein targeting in Arabidopsis. *Plant Cell* **2011**, *23*, 2362–2378. [CrossRef] [PubMed]
122. Busi, R.; Porri, A.; Gaines, T.A.; Powles, S.B. Pyroxasulfone resistance in *Lolium rigidum* is metabolism-based. *Pestic. Biochem. Physiol.* **2018**, *148*, 74–78. [CrossRef]
123. Dücker, R.; Zölner, P.; Lümmen, P.; Ries, S.; Collavo, A.; Beffa, R. Glutathione transferase plays a major role in flufenacet resistance of ryegrass (*Lolium* spp.) field populations. *Pest Manag. Sci.* **2019**. [CrossRef]
124. Brabham, C.; Norsworthy, J.; Houston, M.; Varanasi, V.; Barber, T. Confirmation of S-metolachlor resistance in Palmer amaranth (*Amaranthus palmeri*). *Weed Technol.* **2019**, 1–7. [CrossRef]
125. Busi, R.; Gaines, T.A.; Walsh, M.J.; Powles, S.B. Understanding the potential for resistance evolution to the new herbicide pyroxasulfone: Field selection at high doses versus recurrent selection at low doses. *Weed Res.* **2012**, *52*, 489–499. [CrossRef]
126. Manalil, S.; Busi, R.; Renton, M.; Powles, S. Rapid Evolution of herbicide resistance by low herbicide dosages. *Weed Sci.* **2011**, *59*, 210–217. [CrossRef]
127. Neve, P.; Powles, S. High survival frequencies at low herbicide use rates in populations of *Lolium rigidum* result in rapid evolution of herbicide. *Herdity* **2005**, *95*, 485–492. [CrossRef]
128. Busi, R.; Powles, S.B. Cross-resistance to prosulfocarb and triallate in pyroxasulfone-resistant *Lolium rigidum*. *Pest. Manag. Sci.* **2013**, *69*, 1379–1384. [CrossRef]
129. Dücker, R.; Zöllner, P.; Parcharidou, E.; Ries, S.; Lorentz, L.; Beffa, R. Enhanced metabolism causes reduced flufenacet sensitivity in black-grass (*Alopecurus myosuroides* Huds.) field populations. *Pest. Manag. Sci.* **2019**. [CrossRef]
130. Ramesh, K.; Matloob, A.; Aslam, F.; Florentine, S.K.; Chauhan, B.S. Weeds in a changing climate: Vulnerabilities, consequences, and implications for future weed management. *Front. Plant Sci.* **2017**, *8*, 95. [CrossRef] [PubMed]
131. Matzrafi, M. Climate change exacerbates pest damage through reduced pesticide efficacy. *Pest. Manag. Sci.* **2019**, *75*, 9–13. [CrossRef] [PubMed]
132. Cramer, G.R.; Urano, K.; Delrot, S.; Pezzotti, M.; Shinozaki, K. Effects of abiotic stress on plants: A systems biology perspective. *BMC Plant Biol.* **2011**, *11*, 163. [CrossRef]
133. Ou, J.; Stahlman, P.W.; Jugulam, M. Reduced absorption of glyphosate and decreased translocation of dicamba contribute to poor control of kochia (*Kochia scoparia*) at high temperature. *Pest Manag. Sci.* **2018**, *74*, 1134–1142. [CrossRef]
134. Ganie, Z.A.; Jugulam, M.; Jhala, A.J. Temperature influences efficacy, absorption, and translocation of 2,4-D or glyphosate in glyphosate-resistant and glyphosate-susceptible common ragweed (*Ambrosia artemisiifolia*) and giant ragweed (*Ambrosia trifida*). *Weed Sci.* **2017**, *65*, 588–602. [CrossRef]
135. Hatterman-Valenti, H.; Pitty, A.; Owen, M. Environmental effects on velvetleaf (*Abutilon theophrasti*) epicuticular wax deposition and herbicide absorption. *Weed Sci.* **2011**, *59*, 14–21. [CrossRef]
136. Palma-Bautista, C.; la Cruz, R.A.; Rojano-Delgado, A.M.; Dellaferrera, I.; Domínguez-Martínez, P.A.; Prado, R.D. Low temperatures enhance the absorption and translocation of ^{14}C-glyphosate in glyphosate-resistant *Conyza sumatrensis*. *J. Plant Physiol.* **2019**, *240*, 153009. [CrossRef]
137. Ge, X.; d'Avignon, D.A.; Acerman, J.J.H.; Duncan, B.; Spaur, M.B.; Sammons, R.D. Glyphosate-resistant horseweed made sensitive to glyphosate: Low-temperature suppression of glyphosate vacuolar sequestration revealed by ^{31}P NMR. *Pest Manag. Sci.* **2011**, *67*, 1215–1221. [CrossRef]
138. Purba, E.; Preston, C.; Powles, S.B. The mechanism of resistance to paraquat is strongly temperature dependent in resistant *Hordeum leporinum* Link and *H. glaucum* Steud. *Planta* **1995**, *196*, 464. [CrossRef]
139. Godar, A.S.; Varanasi, V.K.; Nakka, S.; Prasad, P.V.; Thompson, C.R.; Mithila, J. Physiological and molecular mechanisms of differential sensitivity of palmer amaranth (*Amaranthus palmeri*) to mesotrione at varying growth temperatures. *PLoS ONE* **2015**, *10*, e0126731. [CrossRef] [PubMed]
140. Matzrafi, M.; Brunharo, C.; Tehranchian, P.; Hanson, B.D.; Jasieniuk, M. Increased temperatures and elevated CO_2 levels reduce the sensitivity of *Conyza canadensis* and *Chenopodium album* to glyphosate. *Sci. Rep.* **2019**, *9*, 2228. [CrossRef]
141. Ghanizadeh, H.; Harrington, K.C.; James, T.K. Glyphosate-resistant population of *Lolium perenne* loses resistance at winter temperatures. *N. Z. J. Agric. Res.* **2015**, *58*, 423–431. [CrossRef]

142. Matzrafi, M.; Seiwert, B.; Reemtsma, T.; Rbin, B.; Peleg, Z. Climate change increases the risk of herbicide-resistant weeds due to enhanced detoxification. *Planta* **2016**, *244*, 1217–1227. [CrossRef] [PubMed]
143. Refatti, J.P.; de Avila, L.A.; Camargo, E.R.; Ziska, L.H.; Oliviera, C.; Salas-Perez, R.; Rouse, C.E.; Roma-Burgos, N. High [CO_2] and temperature increase resistance to cyhalofop-butyl in multiple-resistant *Echinochloa colona*. *Front. Plant Sci.* **2019**. [CrossRef]
144. Coetzer, E.; Al-Khatib, K.; Loughin, T. Glufosinate efficacy, absorption, and translocation in amaranth as affected by relative humidity and temperature. *Weed Sci.* **2001**, *49*, 8–13. [CrossRef]
145. Patzoldt, W.L.; Hager, A.G.; McCormick, J.S.; Tranel, P.J. A codon deletion confers resistance to herbicides inhibiting protoporphyrinogen oxidase. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 12329–12334. [CrossRef]
146. Laforest, M.; Soufiane, B.; Simard, M.; Obeid, K.; Page, E.; Nurse, R.E. Acetyl-CoA carboxylase overexpression in herbicide-resistant large crabgrass (*Digitaria sanguinalis*). *Pest. Manag. Sci.* **2017**, *73*, 2227–2235. [CrossRef]
147. Fang, J.; Zhang, Y.; Liu, T.; Yan, B.; Li, J.; Dong, L. Target-Site and metabolic resistance mechanisms to penoxsulam in barnyardgrass (*Echinochloa crus-galli* (L.) P. Beauv). *J. Agric. Food Chem.* **2019**, *67*, 8085–8095. [CrossRef]
148. Yu, Q.; Friesen, L.J.S.; Zhang, X.Q.; Powles, S.B. Tolerance to acetolactate synthase and acetyl-coenzyme A carboxylase inhibiting herbicides in *Vulpia bromoides* is conferred by two co-existing resistance mechanisms. *Pestic. Biochem. Phys.* **2004**, *78*, 21–30. [CrossRef]
149. Gressel, J. Low pesticide rates may hasten the evolution of resistance by increasing mutation frequencies. *Pest. Manag. Sci.* **2011**, *67*, 253–257. [CrossRef] [PubMed]

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Rapid metabolism increases the level of 2,4-D resistance at high temperature in common waterhemp (*Amaranthus tuberculatus*)

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Common waterhemp emerges throughout the crop growing season in the Midwestern United States, and as a result, the seedlings are exposed to a wide range of temperature regimes. Typically, 2,4-D is used in the Midwest to control winter annual broad-leaf weeds before planting soybean and in an early post-emergence application in corn and sorghum; however, the evolution of 2,4-D-resistant common waterhemp in several Midwestern states may limit the use of 2.4-D for controlling this problem weed. Moreover, temperature is one of the crucial factors affecting weed control efficacy of 2,4-D. This research investigated the effect of temperature on efficacy of 2,4-D to control 2,4-D susceptible (WHS) and -resistant (WHR) common waterhemp. Do se-response of WHS and WHR to 2,4-D was assessed at two temperature regimes, high (HT; 34/20 °C, d/n) and low (LT; 24/10 °C, d/n). Whole plant dose response study indicated an increased level of 2,4-D resistance in WHR at HT compared to LT. Additional investigation of the physiological mechanism of this response indicated that both WHS and WHR common waterhemp plants rapidly metabolized ^{14}C 2,4-D at HT compared to LT. In conclusion, a rapid metabolism of 2,4-D conferred increased level of resistance to 2,4-D in WHR at HT. Therefore, application of 2,4-D when temperatures are cooler can improve control of 2,4-D resistant common waterhemp.

Common waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] is one of the most troublesome weeds that can cause extensive yield loss in major agronomic crops in the Midwestern United States. Season-long interference of common waterhemp can result in up to 56% and 74% yield loss in soybean[1] and corn[2], respectively. Biological characteristics of common waterhemp, such as continuous emergence pattern, high fecundity, and adaptability to diverse environment conditions make this species difficult to control. Moreover, the evolution of multiple herbicide resistance has reduced herbicide options for the management of common waterhemp. A synthetic auxinic herbicide (SAH), 2,4-dichloro-phenoxy acetic acid (2,4-D), has been a valuable post-emergence (POST) option to control many broadleaf weeds including common wateremp; however, the evolution of common waterhemp resistant to 2,4-D can affect the utility of 2,4-D-resistant corn and soybean. Common waterhemp resistant to 2,4-D was first documented in 2009 in Nebraska[3], followed by Illinois[4], and more recently in Missouri[5]. The WHR (2,4-D resistant common waterhemp) population from Nebraska is 8-10-fold resistant to 2,4-D compared to a known susceptible (WHS) population[3]. Further, a rapid metabolism of 2,4-D, possibly mediated by cytochrome P-450 monooxygenases, has been reported to confer resistance in this population[6]. Similarly, 2,4-D resistance in common waterhemp population from Missouri was also attributed to a rapid metabolism mediated by cytochrome P-450 monooxygenases[5].

Reproductive success of common waterhemp is often attributed to its broader window of emergence[7,8]. Such emergence pattern demands a PRE (pre-emergence) followed by a POST herbicide program for effective control and to reduce crop yield loss[9,10]. Moreover, studies show increased ecological advantage to common waterhemp

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Population	Temperature (°C)	Effective herbicide dose	Resistance Index (RI)	Regression parameters	
		GR_{50} (g ae ha^{-1})		b	d
WHS	24/10	107 (±26)	—	0.88 (±0.14)	99.88 (±6.00)
	34/20	178 (±43)	—	0.76 (±0.11)	101.27 (±5.80)
WHR	24/10	1001 (±237)	9.35	0.81 (±0.14)	100.39 (±5.95)
	34/20	3696 (±1138)	20.76	0.65 (±0.16)	100.57 (±5.58)

Table 1. Regression parameters estimated from the whole-plant 2,4-D dose- response study based on dry shoot biomass of 2,4-D–susceptible (WHS) and –resistant (WHR) common waterhemp grown under low (24/10°C, d/n) and high (34/20 °C, d/n) temperature regimes at 4 weeks after treatment (WAT). [a]Data combined from two runs. [b]GR_{50} is the effective 2,4-D doses (g ae ha^{-1}) required for 50% reduction in shoot dry biomass. [c]RI is calculated as a ratio of GR_{50} ofthe WHR population to GR_{50} of the WHS population. [c]Values in parenthesis are standard error of mean.

cohorts emerging early in the season than later[11]. Temperature is one of the critical environmental factors that can fluctuate throughout the growing season. In Kansas, the early emerging waterhemp is exposed to a lower day/night temperature ranging from 18.4–29.0/3.1–20.6 °C (d/n; average 24.7/11.6 °C), while late in the season diurnal temperatures ranges from 28.2–40.5/15.1–27.1 °C (d/n; average 34/21.2 °C)[12]. Temperature can affect the growth and development of common waterhemp[13], which in turn can influence the efficacy of POST herbicide application[14]. Below optimal efficacy of POST-herbicide not only results in reduced weed control but can also select resistant biotypes due to increasing chances of survival and seed production.

2,4-D, is widely used for managing dicotyledonous weeds in several crops and non-crop areas. Additionally, 2,4-D choline/glyphosate/glufosinate-resistant corn (Enlist™ corn) is commercially available from 2018 growing season in the United States and 2,4-D- choline/glyphosate/glufosinate-resistant soybean (Enlist™ soybean) is likely to be commercially available in the near future. In sensitive dicotyledonous weeds, 2,4-D is absorbed through root, stem, and leaves and gradually translocates systemically to meristems[15]. Plant species tolerant to 2,4-D naturally degrade this herbicide into inactive metabolites, thus preventing the active ingredient to translocate further[16]. For instance, in corn, 2,4-D is metabolized via ring hydroxylation mediated by cytochrome P-450 monooxygenases[17,18]. Similar to monocotyledonous weeds, in many 2,4-D-resistant dicotyledonous weeds such as corn poppy (*Papavar rhoeas*)[19], common waterhemp[5,6], degradation was possibly mediated by cytochrome P-450 monooxygenases. Apart from metabolism, reduced absorption and/or translocation of 2,4-D have also been found to bestow 2,4-D resistance in several dicotyledonous weeds such as corn poppy[20], prickly lettuce (*Lactuca serriola*)[21] and wild radish (*Raphanus raphanistrum*)[22].

The effect of temperature on herbicide efficacy often vary depending on weed species and herbicide site of action. For example, Ganie *et al.*[23] found that efficacy of 2,4-D was improved to control giant ragweed (*Ambrosia trifida*) and common ragweed (*Ambrosia artemisiifolia*) at temperature 29/17 °C, d/n due to increased 2,4-D translocation compared to 20/11 °C, d/n temperature. In contrast, Ou *et al.*[24] reported reduced control of kochia (*Kochia scoparia*) at a higher temperature (32.5/22.5 °C, d/n) compared to a lower temperature (17.5/7.5 °C, d/n) due to reduced absorption of glyphosate and reduced translocation of dicamba. Scientific literature is not existing on effect of temperature on efficacy of 2,4-D for control of 2,4-D-resistant and susceptible common waterhemp. Understanding the effect of temperature on efficacy of 2,4-D as a post-emergence option will help to better facilitate control of common waterhemp. The objectives of this research were (1) to evaluate the efficacy of 2,4-D on WHS and WHR control at a high (HT; 34/20 °C, d/n) and low (LT; 24/10 °C, d/n) temperature regimes, and (2) to investigate the uptake, translocation, and metabolism of ^{14}C 2,4-D in WHS and WHR common waterhemp at aforementioned temperature regimes.

Results

2,4-D dose-response experiment. WHS and WHR exhibited varying response to 2,4-D at HT or LT regime (Fig. 1). At 4WAT, the amount of 2,4-D required to reduce 50% (GR_{50}) growth of WHS and WHR plants grown at HT regime were 178 and 3,696 g ae ha^{-1} and while at LT regime were 107 and 1,001 g ae ha^{-1}, respectively (Table 1). Thus, the resistance indices of WHR relative to WHS grown at HT and LT regimes were ~20 and ~10, respectively, suggesting that WHR common waterhemp showed increased level of resistance to 2,4-D at HT compared to LT (Fig. 1, Table 1). "CompParm" function in R indicated that there is significant diference between GR_{50} of WHR at HT and LT ($p < 0.05$), WHR and WHS at HT ($p < 0.01$), WHR and WHS at LT ($p < 0.001$). However, there was no significant difference between GR_{50} of WHS at HT and LT. This suggstes reduction in efficacy of 2,4-D at HT to control 2,4-D-resistant common waterhemp (Fig. 1).

The test for 'lack of fit' in 'drc' was non-significant ($p = 0.88$), suggesting that the data fitted the regression model reasonably. Root means square error (RMSE) values of the 2,4-D dose-response experiments conducted at HT and LT ranged from 1.82 to 2.48 for WHS and 2.65 to 2.04 for WHS respectively, indicating a good fit.

^{14}C 2,4-D absorption and translocation experiment. Regression analysis of ^{14}C 2,4-D absorption indicated that temperature did not affect the absorption or translocation of ^{14}C 2,4-D in both WHS and WHR and there was no significant difference between A_{max} (upper limit of absorption) and A_{90} (the time required to achieve 90% of maximum absorption) of WHR and WHS at HT and LT conditions. A_{max} for WHS and WHR at HT and LT regimes were 96.31 (±3.70), 92.73 (±3.61), and 93.43 (±2.54), and 95.35 (±3.16) %, respectively (Table 2).

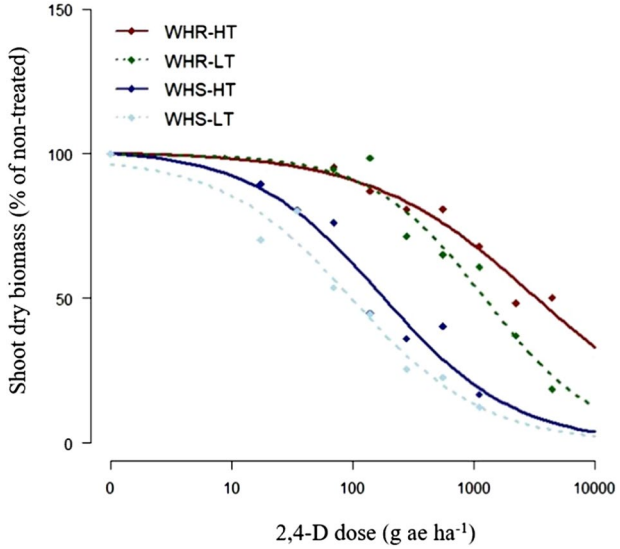

Figure 1. Whole-plant 2,4-D dose-response of 2,4-D susceptible (WHS) and -resistant common waterhemp (WHR) at low (LT; 24/10 °C, d/n) and high (HT; 34/20 °C, d/n) temperature regimes based on dry shoot biomass at 4 weeks after treatment (WAT).

Population	Temperature (°C)	Absorption		Translocation	
		A_{max}	A_{90}	T_{max}	T_{90}
WHS	24/10	92.73 (±3.61)	13 (±7.38)	79.18 (±14.03)	119.73 (±70.20)
	34/20	96.31 (±3.70)	18 (±6.19)	75.69 (±14.39)	111.63 (±55.07)
WHR	24/10	95.35 (±3.16)	22.12 (±7.61)	73.78 (±18.92)	120.59 (±94.74)
	34/20	93.43 (±2.54)	16.43 (±5.17)	70.83 (±14.39)	113.12 (±77.17)

Table 2. Regression parameter estimates of ^{14}C 2,4-D absorption and translocation of 2,4-D- susceptible (WHS) and -resistant (WHR) common waterhemp at low (24/10 °C, d/n) and high (34/20 °C, d/n) temperature regimes using rectangular hyperbola model. [a]Data combined from two runs. [b]A_{max} and T_{max} is the maximum absorption or translocation (%), A_{90} or T_{90} is the time (h) required to achieve 90% of the maximum absorption or translocation. [c]Values in parenthesis are standard error of mean.

Moreover, A_{90} was also similar in WHS and WHR plants at HT or LT regimes i.e., 18 (±6.19), 13 (±7.38), 16.43 (±5.17), and 22.12 (±7.61) hours, respectively (Table 2). Similarly, there was no significant difference between T_{max} (upper limit of translocation) and T_{90} (the time required to achieve 90% of maximum translocation) between WHS and WHR at two temperature regimes, which indicated that temperature regimes did not affect ^{14}C 2,4-D translocation. The predicted T_{max} for WHS and WHR at HT and LT regimes were 75.69 (±14.39), 79.18 (±14.03) and 70.83 (±14.39), and 73.78 (±18.92) %, respectively (Table 2). The time required to achieve 90% of the maximum translocation of 2,4-D in WHS and WHR plants were 111.63 (±55.07), 119.73 (±70.20) and 113.12 (±77.17), 120.59 (±94.74) hours, respectively, at HT and LT regimes (Table 2).

^{14}C 2,4-D metabolism experiment. The HPLC chromatographs indicated that the retention time of the parent ^{14}C 2,4-D (used as standard) was 11.96 min (Fig. 2). Peaks of parent 2,4-D were much taller in WHR at LT compared to HT at 24 and 72 HAT. However, such difference was not observed at 6 HAT in WHR plants (Fig. 3b) At 6 HAT, the mean 2,4-D retention by WHR and WHS common waterhemp at HT and LT temperature regimes was 69.3, 69.3%, and 85.1, 95.3%, respectively (Fig. 3a,b). Twenty-four HAT, WHR plants retained 20.2 and 47.7% of parent 2,4-D at HT (Figs 2d and 3b) and LT (Figs 2c, 3b), respectively. Whereas, WHS retained 82.3 (Figs 2b and 3a) at HT and 86.1 (Figs 2a and 3a) % at LT, respectively. This validates that, metabolism of 2,4-D plays a key role in bestowing 2,4-D resistance in WHR (Fig. 2). More importantly, this indicates that at 24 HAT, WHR plants grown at LT retained approximately 27% more parent 2,4-D than at HT (Figs 2c,d and 3b). This indicates rapid metabolism of 2,4-D in WHR plants grown at HT compared to LT. Also, at 72 HAT, the WHR plants grown at HT conditions metabolized close to 100% of the parent 2,4-D while those at LT still retained 9.4% (Fig. 3b). At 72 HAT the WHS plants retained 33.7, 54.5% of parent 2,4-D at HT and LT conditions, respectively (Fig. 3a). Overall, the rate of 2,4-D metabolism increased both in WHR and WHS at HT (Fig. 3a,b).

The two-way analysis of parent 2,4-D retained in WHR followed by mean comparison using LSD ($p = 0.05$) suggested that there is a significant difference in % parent 2,4-D present in WHR at 24 HAT (Fig. 3b) with more 2,4-D being retained in plants grown at LT. In case of WHS plants, such difference was observed at 72 HAT (Fig. 3a) with more 2,4-D retained at LT compared to HT.

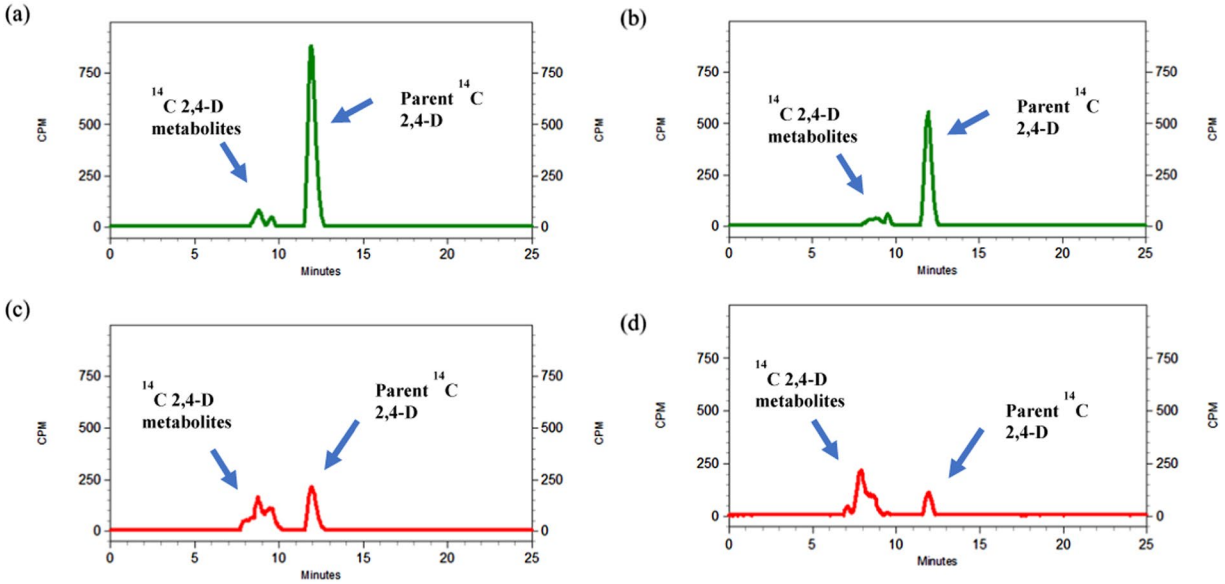

Figure 2. ^{14}C 2,4-D parent compound and its metabolites in (**a**,**b**) 2,4-D–susceptible (WHS) and (**c**,**d**) 2,4-D resistant (WHR) common waterhemp populations at 24 hours after treatment (HAT) at (**a**,**c**) low temperature regime (24/10 °C, d/n) and (**b**,**d**) high temperature regime (34/20 °C, d/n).

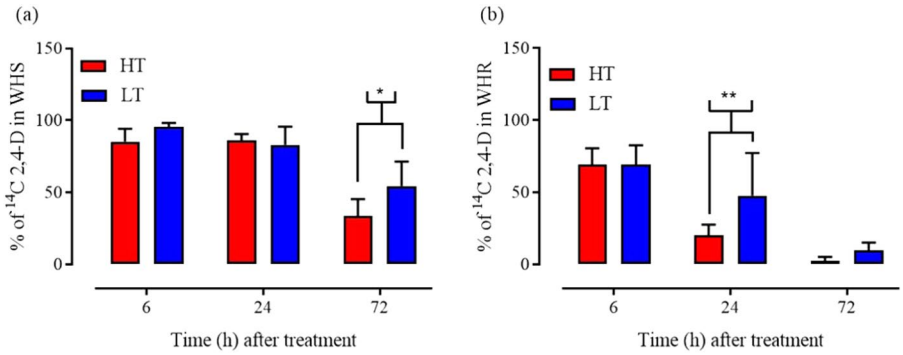

Figure 3. Percentage of ^{14}C 2,4-D parent compound in (**a**) 2,4-D susceptible (WHS) and (**b**) resistant (WHR) common waterhemp populations at 6, 24, and 72 hours after treatment (HAT) at low (LT; 24/10 °C, d/n) and high (HT; 34/20 °C, d/n) temperature regimes. Data combined over two runs. *P-value < 0.05, **P-value < 0.001, ***P-value < 0.0001, indicates the level of significance of difference in means, and error bars represent standard error of mean).

Discussions

The time of emergence of common waterhemp under field conditions depends on various factors including, soil temperature, moisture, and seed dormancy. Especially, in the Midwestern United States, common waterhemp emergence occurs over a wider time frame compared to other summer annual weed species[25]. The average diurnal temperatures in May and July, the two-major seasons for waterhemp cohort emergence, are around 24/10 °C and 34/20 °C in Kansas (Fig. 4)[12]. The dose-response study results demonstrated reduced efficacy of 2,4-D at HT (34/20 °C) compared to LT (24/10 °C) for controlling both WHS and WHR common waterhemp. In contrast, Ganie *et al.*[23] reported improved efficacy of 2,4-D or glyphosate at HT (29/17 °C) compared with LT (20/11 °C) for common and giant ragweed control regardless of susceptibility or resistance to glyphosate. Godar *et al.*[26] reported reduced efficacy of mesotrione for Palmer amaranth (*Amaranthus palmeri*) control at high (40/30 °C) compared to low (25/15 °C) temperature due to reduced translocation coupled with rapid metabolism of mesotrione and increased 4-hydroxyphenylpyruvate dioxygenase (HPPD)-gene expression. However, as previously reported by Figueiredo *et al.*[6] the data from this study also showed no difference in 2,4-D absorption or translocation between WHR and WHS (Table 2). The maximum limit of ^{14}C 2,4-D absorption in this study was found to be 95% in WHR and WHS common waterhemp (Table 2). Previous studies have shown that 2,4-D absorption can range from 10–99% depending on several factors such as environment, weed species and other application factors[27]. Similar to our findings, Coetzer *et al.*[28] reported no effect of temperature on glufosinate absorption in Palmer amaranth.

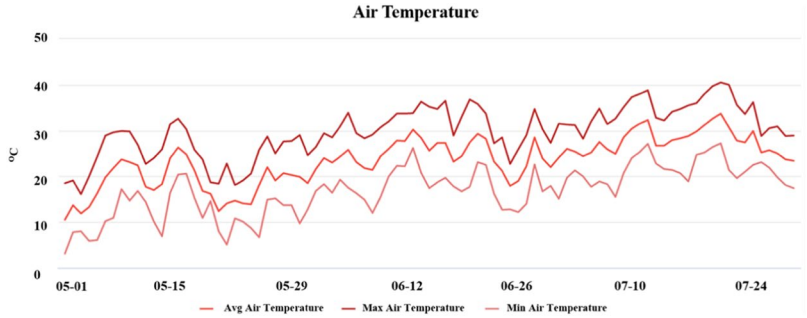

Figure 4. Average, maximum, and minimum air temperature in Kansas during May to July, a typical common waterhemp emergence time in the state (KSU, Mesonet 2018).

High temperature increased the rate of metabolism of 2,4-D both in WHR and WHS common waterhemp. Similar to these findings, Johnson and Young[29], reported a 6–7-fold higher susceptibility of common waterhemp to mesotrione at 18 °C compared to 32 °C. Likewise, Olsen *et al.*[30] reported decreased metabolism of MON 37500 in several grass weeds (*Aegilops cylindrica*, *Avena fatua*, *Bromus tectorum*) grown at cool air temperature. Gallaher *et al.*[31] observed rapid metabolism of primisulfuron and nicosulfuron in broadleaf signalgrass (*Brachiaria platyphylla*) at high (30/20 °C) compared to low (20/10 °C) temperature.

The auxinic herbicide-tolerant monocotyledonous weeds are known to metabolize 2,4-D via ring hydroxylation mediated by cytochrome P-450 monooxygenases, an enzyme family predominantly involved in metabolizing xenobiotics in plants[16,32]. A possible involvement of these enzymes in 2,4-D degradation has been documented in many dicotyledonous weeds, resistant to this herbicide. For example, cytochrome P-450 mediated 2,4-D degradation has been reported in 2,4-D-resistant corn poppy[19]. Figueiredo *et al.*[6] reported a 7-fold reduction in GR_{50} of WHR (the same common waterhemp) with pre-treatment of malathion (a cytochrome P-450-inhibitor) followed by 2,4-D compared to plants treated with 2,4-D alone, indicating a possible involvement of cytochrome P-450s in 2,4-D metabolism in common waterhemp. Thus, it is likely that a rapid metabolism of 2,4-D in WHR plants grown at HT is facilitated by increased activity of cytochrome P-450 enzymes. Previously, Viger *et al.*[33] reported rapid metabolism of metolachlor at a high temperature (30 °C) compared to a low temperature (21 °C), which was associated with a five-fold increase in glutathione-S-transferase (GST) activity in corn. Therefore, the possible increased cytochrome P-450 enzyme activity may be an example of common waterhemp adaptation to high temperature stress. Studies have shown that plant response to stress, including abiotic stress can lead to further selection of resistant weed biotypes[34]. Hence, application of 2,4-D at the most effective temperature regime is important to control common waterhemp and reduce further selection of 2,4-D resistance.

In conclusion, the results of this research demonstrate that 2,4-D efficacy can be improved at low temperature regime (24/10 °C, d/n) to manage common waterhemp. Thus, applying 2,4-D when day temperature is lower than 30 °C is desirable for common waterhemp control; however, apart from air temperature other abiotic factors such as light intensity, relative humidity, and plant factors such as leaf orientation also play key role in affecting herbicide efficacy. Our studies were conducted in growth chambers where apart from temperature all other factors were kept constant. This is particularly important to reduce common waterhemp competition and crop yield loss and reduce selection for resistance. In general, the efficacy of auxinic herbicides for controlling dicotyledonous weeds depends on several factors including time of application[34–37]. Additionally, efficacy of 2,4-D is species dependent as improved efficacy at HT has been noticed for control of common and giant ragweed. Therefore, further studies are needed to assess the interaction of other abiotic and plant factors that can influence 2,4-D efficacy for controlling common waterhemp.

Materials and Methods

Plant materials and growth conditions. WHS and WHR common waterhemp from Nebraska, USA were used in this study[3,6]. Common waterhemp resistant to 2,4-D (WHR) has been confirmed in a native grass little bluestem (*Schizachyrium scoparium*) production field in southeastern Nebraska where 2,4-D was applied for over 10 years[6]. The susceptible population (WHS) was collected from a soybean field near Auburn, Nebraska[3,6].

WHS and WHR common waterhemp seeds were germinated in plastic trays (25 × 15 × 2.5 cm) filled with potting mix (Fafard® ultra container potting mix, Sungro Horticulture, Agawam, MA). After emergence, individual seedlings at 2–3 leaf stage were transplanted into plastic pots (6 × 6 × 6 cm) and kept in the greenhouse maintained at 25/20° C day/night (d/n), 15 hours of photoperiod supplemented with 120 μmol m^{-2} s^{-1} illumination provided with sodium vapor lamps along with 60 ± 10% relative humidity. At 7 days after transplanting, half of the small and uniform seedlings (4-leaf stage) were transferred in growth chambers set at HT (34/20°C, d/n) and the rest were transferred in a separate growth chamber set at LT (24/10°C, d/n). Temperature regimes were selected based on the average diurnal temperatures during mid-May to mid-June in Kansas, USA[12]. Incandescent and fluorescent bulbs were used in growth chambers to maintain light level of 750 μmol m^{-2} s^{-1} (15/9 hrs, d/n condition) and relative humidity was maintained at 60 ± 10% throughout the study. Plants were watered daily and fertilized once a week after transplanting.

2,4-D dose-response experiment. Ten to 12 cm tall WHS and WHR common waterhemp plants grown at HT or LT were treated with several rates of 2,4-D (2,4-D Amine 4, Winfield Solutions, LLC, St. Paul MN,

USA). Specifically, the WHS plants were treated at 0, 17.5, 35, 70, 140, 560, 1,120 g ae ha^{-1} 2,4-D whereas, the WHR plants were treated with 0, 70, 140, 280, 560, 1,120, 2,240, 4,480 g ae ha^{-1} 2,4-D, using a bench-type sprayer (Research Track Sprayer, Generation III, De Vries Manufacturing, Hollandale, MN, USA) equipped with a single flat-fan nozzle (80015LP TeeJet tip, Spraying Systems Co., Wheaton, IL, USA) delivering 187 L ha^{-1} at 220 kPa in a single pass at 3.2 km h^{-1}. The treated plants were transferred back in respective growth chambers 30 min after 2,4-D application. At 4 weeks after treatment (WAT), above-ground biomass from each plant was harvested and placed in paper bags and dried in an oven at 60 °C for 72 hours (h) to measure dry shoot biomass. Percent dry shoot biomass was calculated relative to the non-treated control for each common waterhemp population as follows:

$$Shoot\ biomass\ (\%) = \frac{biomass\ of\ each\ sample \times 100}{biomass\ of\ the\ \text{non-treated}\ sample}$$

^{14}C 2,4-D absorption and translocation experiment. WHS and WHR seedlings, raised and grown in the greenhouse (as described above) were transferred to growth chambers maintained at high (HT: 34/20°C, d/n) and low (LT: 24/10 °C, d/n) temperatures. ^{14}C 2,4-D working solution was prepared by mixing ^{14}C 2,4-D [3.3 kBq μl^{-1} with a specific activity of 5.5 MBq $mmol^{-1}$ (Dow AgroSciences, Indianapolis, IN, USA)] with commercially available 2,4-D (2,4-D Amine 4, Winfield Solutions, LLC, St. Paul MN, USA) to obtain 560 g ae ha^{-1} 2,4-D in a carrier volume of 187 L. Ten to 12 cm tall (8 to10 leaf stage) plants were treated with ten 1-µl droplets of ^{14}C 2,4-D working solution on the adaxial surface of the fourth youngest fully expanded leaf using Wiretrol® (10 µL; Drummond Scientific Co., Broomall, PA, USA). After 30 minutes, the treated plants were returned to respective growth chambers maintained at HT or LT. The plants were harvested at 6, 24, and 72 hours after treatment (HAT), and separated into treated-leaf (TL), tissue above treated-leaf (ATL), and below treated-leaf (BTL). TL were washed with 5 ml of wash solution containing 10% (v/v) aqueous solution of ethanol and 0.5% Tween-20 in 20-ml scintillation vials for 1 minute to remove excess unabsorbed 2,4-D from the leaf surface. The leaf rinsate was mixed with 15 ml of scintillation cocktail [Ecolite-(R), MP Biomedicals, LLC. Santa Ana, CA, USA] to measure the radioactivity using liquid scintillation counter (LSC; Beckman Coulter LS6500 Liquid Scintillation Counter, Beckman Coulter Inc., Fullerton, CT, USA). Plant sections were oven dried at 60 °C for 72 h, and then combusted for 3 min using a biological oxidizer (OX-501, RJ Harvey Instrument, Tappan, NY, USA). The ^{14}C 2,4-D was recovered in a scintillation cocktail [Carbon-14 (C14) Cocktail, RJ Harvey Instrument, Tappan, NY, USA] and the radioactivity was measured using a LSC. The data was converted into percentages using the following equations[26],

$$Percentage\ absorption\ (percentR_{absorbed}) = \frac{(R_{applied} - R_{rinsate}) \times 100}{R_{applied}}$$

$$Percentage\ translocation = (100 - percent\ R_{TL})$$

$$Percentage\ radioactivity\ recovered\ in\ treated\ leaf = \frac{R_{TL} \times 100}{R_{absorbed}}$$

In the above equations, $R_{absorbed}$ is the radioactivity absorbed; $R_{applied}$ is total amount of radioactivity applied on the plant; $R_{rinsate}$ is the radioactivity recovered in leaf rinsate; and R_{TL} is the radioactivity recovered in the treated leaf (TL).

^{14}C 2,4-D Metabolism experiment. The WHS and WHR common waterhemp plants (10–12 cm tall) grown under high and low temperature regimes (as described above) were used. The adaxial surface of the fourth youngest fully expanded leaf was treated with 10-µl droplets of ^{14}C 2,4-D working solution containing ^{14}C 2,4-D (5 kBq μl^{-1} with a specific activity of 5.5 MBq $mmol^{-1}$) and commercial 2.4-D and plants were returned to growth chambers. Treated plants were harvested at 6, 24, and 72 HAT. At each harvest time, the TL was washed as described in absorption and translocation experiment to remove excess unabsorbed 2,4-D from the leaf surface. Above-ground plant tissue including the TL was wrapped in aluminum foil and flash frozen in liquid nitrogen to store at −80 °C. The frozen plant tissue was later grinded using a mortar and pestle. The ^{14}C 2,4-D, and its metabolites were extracted with 15 ml of 90% aqueous acetone in a centrifuge tube and preserved at 4 °C for at least 16 hours. After 16 hours, the tubes were centrifuged at 5,000 × g for 10 minutes. The supernatant was transferred to a new centrifuge tube and concentrated at 45°C for 1.5–2 h with a rotary evaporimeter (Centrivap, Labconco, Kansas City, MO). The final volume of the supernatant was maintained around 600 µL and transferred to a 1.5 ml microcentrifuge tube and centrifuged at 10,000 × g for 10 minutes. The radioactivity of the supernatant solution was measured with the liquid scintillation counter and normalized by diluting the samples with 50% acetonitrile (1:1 v/v acetonitrile:water). The final solutions were analyzed using reversed-phase high-performance liquid chromatography (HPLC) (BeckmanCoulter system Gold 126 solvent module, Beckman Coulter Inc., Fullerton, CA, USA) to resolve the solution contents into parent ^{14}C 2,4-D and its metabolites.

Experimental design and statistical analysis. The experiments were arranged in a split-plot design with four replications and repeated in time. Growth chambers were switched between two experimental runs to avoid effect of growth chamber on plant response. The dose-response experiments were arranged in a two-way

factorial combination of temperature regimes (HT and LT) as main factor and herbicide doses for each common waterhemp population as sub-plot factor.

Relative shoot biomass data obtained from the whole plant dose-response study were analyzed using the 'drc' package (drc 1.2, Christian Ritz and Jens Strebig, R2.5, Kurt Hornik, online) in R (R statistical software, R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org) as per Knezevic *et al.*[38] A dose-response regression model was constructed using the three-parameter log-logistic equation.

$$Y = \left\{\frac{d}{1} + \exp[b(logX - loge)]\right\}$$

In equation above, Y is response variable (% reduction in biomass compared to control), b denotes relative slope around e, e is GR_{50} (effective dose to reduce biomass of the population by 50%) and d is the upper limit of the model. The ratio of GR_{50} values of WHS and WHR common waterhemp in HT and LT conditions were calculated to determine the level of resistance or the resistance index. Estimated GR_{50} values were then compared with each other using the "compParm" function in 'drc' package in R.

Fitness of the log-logistic regression model used above was assessed through the "Lack-of-fit" test in 'drc' using "modelFit" function. Further, root mean square error (RMSE) was calculated to test the goodness of fit of the data. The formula used for RMSE[25] was:

$$RMSE = \left[\frac{1}{n}\sum_{i=1}^{n}(P_i - O_i)^2\right]$$

where, n is the number of observations and O_i and P_i are the observed and predicted value of the observations respectively.

Absorption, translocation, and metabolism experiments, treatments were arranged in a two-way factorial combination with temperature regime (HT and LT) as the main-factor and harvesting time (6, 24, and 72 HAT) as sub-factor for each common waterhemp population. The percentage of herbicide absorbed and translocated were used to fit asymptotic regression, rectangular hyperbola (RHB), and linear model according to Kniss *et al.*[39] using 'drc' and '*qPCR*' packages in R. After fitting the data to these three models, the bias-corrected Akaike information criteria (AICc) of each model was obtained and compared. For analyzing both 2,4-D absorption and translocation, the RHB model was selected due to the lowest AICc values. The RHB model used is:

$$Absorption = \frac{(A_{max} \times t)}{[(10/90) \times A_{90} + t]}$$

$$Translocation = \frac{(T_{max} \times t)}{[(10/90) \times T_{90} + t]}$$

In the above equations, absorption is percent herbicide absorbed expressed in terms of percentage herbicide applied to the plant, A_{max} is the maximum herbicide absorption in time t, and A_{90} is the time required for 90% of the absorption to occur. Similarly, translocation is the percent herbicide translocated expressed in terms of percentage herbicide absorbed in the plant, T_{max} is the maximum herbicide translocation in time t, and T_{90} is the time required for 90% of the translocation to occur. A_{max}, A_{90}, T_{max}, and T_{90} parameters of WHR and WHS at each temperature regime were compared using the "compParm" function in the 'drc' package.

In metabolism experiments, chromatographs obtained from HPLC profiling were used for visual assessment of ^{14}C 2,4-D degradation. Percent parent ^{14}C 2,4-D present in each sample was determined and analyzed using GraphPad Prism 7.04® (GraphPad Software, San Diego, CA) at $p = 0.05$ and comparisons were made between HT and LT conditions in each biotype.

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References

1. Bensch, C. N., Hoprak, M. J. & Peterson, D. Interference of redroot pigweed (*Amaranthus retroflexus*), Palmer amaranth (*A. palmeri*), and common waterhemp (*A. rudis*) in soybean. *Weed Sci.* **51**, 37–43 (2003).
2. Steckel, L. E. & Sprague, C. L. Common waterhemp (*Amaranthus rudis*) interference in corn. *Weed Sci.* **52**, 359–364 (2004).
3. Bernards, M. L., Crespo, R. J., Kruger, G. R., Gaussoin, R. & Tranel, P. J. A waterhemp (*Amaranthus tuberculatus*) population resistant to 2,4-D. *Weed Sci.* **60**, 379–384 (2012).
4. Heap, I. The International survey of herbicide resistant weeds. Online. Internet. Monday, December 24. Available, www.weedscience.org (2018).
5. Shergill, L. S., Barlow, B. R., Bish, M. D. & Bradley, K. W. Investigations of 2,4-D and multiple herbicide resistance in a Missouri waterhemp (*Amaranthus tuberculatus*) population. *Weed Sci.* **66**, 386–394 (2018).
6. Figueiredo, M. R. A. *et al*. Metabolism of 2,4-dichlorophenoxyacetic acid contributes to resistance in a common waterhemp population (*Amaranthus tuberculatus*) population. *Pest Manag. Sci.* **74**, 2356–2362, https://doi.org/10.1002/ps.4811 (2018).
7. Wu, C. & Owen, M. D. K. When is the best time to emerge: Reproductive phenology and success of natural common waterhemp (*Amaranthus rudis*) cohorts in the Midwest United States? *Weed Sci.* **62**, 107–117 (2014).
8. Hartzler, R. G., Battles, B. A. & Nordby, D. Effect of common waterhemp (*Amaranthus rudis*) emergence date on growth and fecundity in soybean. *Weed Sci.* **52**, 242–245 (2004).
9. Jhala, A. J., Sandell, L. D., Sarangi, D., Kruger, G. R. & Knezevic, S. Z. Control of glyphosate-resistant common waterhemp (*Amaranthus rudis*) in glufosinate-tolerant soybean. *Weed Technol.* **31**, 32–45 (2017).

10. Sarangi, D. *et al*. Pollen-mediated gene flow from glyphosate-resistant common waterhemp (*Amaranthus rudis* Sauer): consequences for dispersal of resistance genes. *Nature Sci. Rep.* **7**, 44913 (2017).
11. Uscanga-Mortera, E., Clay, S. A., Forcella, E. & Gunsolus, J. Common waterhemp growth and fecundity as influenced by emergence date and competing crop. *Agron. J.* **99**, 1265–1270 (2007).
12. KSU Mesonet. Average air temperature, max air temperature and minimum air temperature in Kansas during the common waterhemp emergence time, http://mesonet.ksu.edu (2018).
13. Guo, P. & Al-Khatib, K. Temperature effects on germination and growth of redroot pigweed (*Amaranthus retroflexus*), Palmer amaranth (*A. palmeri*), and common waterhemp (*A. rudis*). *Weed Sci.* **51**, 869–875 (2003).
14. Coetzer, E., Al-Khatib, K. & Peterson, D. E. Glufosinate efficacy on *Amaranthus* species in glufosinate-resistant soybeans. *Weed Technol.* **16**, 326–331 (2002).
15. Munro, I. C. *et al*. A comprehensive, integrated review and evaluation of the scientific evidence relating to the safety of the herbicide 2,4-D. *J. Am. Coll. Toxicol.* **11**, 560–664 (1992).
16. Peterson, M. A., McMaster, S. A., Riechers, D. E., Skelton, J. & Stahlman, P. W. 2,4-D past, present, and future: A review. *Weed Technol.* **30**, 303–345 (2016).
17. Schroder, P. & Collins, C. Conjugating enzymes involved in xenobiotic metabolism of organic xenobiotics in plants. *Intl. J. Phytoremediation* **4**, 247–265 (2002).
18. Montgomery, M. L., Chang, Y. L. & Freed, V. H. Comparative metabolism of 2,4-D by bean and corn plants. *J. Agric. Food Chem.* **19**, 1219–1221 (1971).
19. Torra, J. *et al*. Enhanced 2,4-D metabolism in two resistant *Papaver rhoeas* populations from Spain. *Front. Plant Sci.* **8**, 1584 (2017).
20. Rey-Caballero, J. *et al*. Unravelling the resistance mechanisms to 2,4-D (2,4-dichlorophenoxyacetic acid) in corn poppy (*Papaver rhoeas*). *Pest Biochem. Physiol.* **133**, 67–72 (2016).
21. Riar, D. S., Burke, I. C., Yenish, J. P., Bell, J. & Gill, K. Inheritance and physiological basis of 2,4-D resistance in prickly lettuce (*Lactuca serriola* L.). *J. Agric. Food Chem.* **59**, 9417–9423 (2011).
22. Goggin, D. E., Cawthray, G. R. & Powles, S. B. 2,4-D resistance in wild radish: reduced herbicide translocation via inhibition of cellular transport. *J. Exp. Bot.* **11**, 3223–3235 (2016).
23. Ganie, Z., Jugulam, M. & Jhala, A. J. Temperature influences efficacy, absorption, and translocation of 2,4-D or glyphosate in glyphosate-resistant and glyphosate-susceptible common ragweed (*Ambrosia artemisiifolia*) and giant ragweed (*Ambrosia trifida*). *Weed Sci.* **65**, 588–602 (2017).
24. Ou, J., Stahlman, P. W. & Jugulam, M. Reduced absorption of glyphosate and decreased translocation of dicamba contribute to poor control of kochia (*Kochia scoparia*) at high temperature. *Pest Manag. Sci*., https://doi.org/10.1002/ps.4463 (2016).
25. Roman, E. S., Murphy, S. D. & Swanton, C. J. Simulation of *Chenopodium album* seedling emergence. *Weed Sci.* **48**, 217–224 (2000).
26. Godar, A. S. *et al*. Physiological and molecular mechanisms of differential sensitivity of Palmer amaranth (*Amaranthus palmeri*) to mesotrione at varying growth temperatures. *PLoS ONE* **10**, 2012673 (2015).
27. Shergill, L. S., Bish, M. D., Jugulam, M. & Bradley, K. W. Molecular and physiological characterization of six-way resistance in *Amaranthus tuberculatus* var. *rudis* biotype from Missouri. *Pest Manag. Sci.* **74**, 2688–2698 (2018).
28. Coetzer, E., Al-Khatib, K. & Loughin, T. M. Glufosinate efficacy, absorption, and translocation in amaranth as affected by relative humidity and temperature. *Weed Sci.* **49**, 8–13 (2001).
29. Johnson, B. C. & Young, B. G. Influence of temperature and relative humidity on the foliar activity of mesotrione. *Weed Sci.* **50**, 157–161 (2002).
30. Olsen, B. L. S., Al-Khatib, K., Stahlman, P. & Isakson, P. J. Efficacy and metabolism of MON 37500 in *Triticum aestivum* and weedy grass species as affected by temperature and soil moisture. *Weed Sci.* **48**, 541–548 (2000).
31. Gallaher, K., Mueller, T. C., Hayes, R. M., Schwartz, O. & Barrett, M. Absorption, translocation, and metabolism of primisulfuron and nicosulfuron in broadleaf signalgrass (*Brachiaria platyphylla*) and corn. *Weed Sci.* **47**, 8–12 (1999).
32. Hatzios, K., Hock, B. & Elstner, E. Metabolism and elimination of toxicants. Pages 469 – 518 in Plant Toxicology. 4th edn. Boca Raton, FL: CRC Press (2005).
33. Viger, P. R., Eberline, C. V., Fuerst, E. P. & Gronwald, J. W. Effects of CGA-154281 and temperature on metolachlor absorption and metabolism, glutathione content, and glutathione-*S*-transferase activity in corn (*Zea mays*). *Weed Sci.* **39**, 324–328 (1991).
34. Dyer, W. E. Stress-induced evolution of herbicide resistance and related pleiotropic effects. *Pest Manag. Sci.* **74**, 1759–1768 (2018).
35. Johnston, C. R., Eure, P. M., Grey, T. L. & Culpepper, A. S. Time of application influences translocation of auxinic herbicides in Palmer amaranth. *Weed Sci.* **66**, 4–14 (2018).
36. Stewart, C. L., Nurse, R. E. & Sikkema, P. H. Time of day impacts postemergence weed control in corn. *Weed Technol.* **23**, 346–355 (2009).
37. Skuterud, R., Bjugstad, N., Tyldum, A. & Tørresen, K. S. Effect of herbicides applied at different times of the day. *Crop Prot.* **17**, 41–46 (1998).
38. Knezevic, S. Z., Streibig, J. C. & Ritz, C. Utilizing R software package for dose-response studies: the concept and data analysis. *Weed Technol.* **21**, 840–848 (2007).
39. Kniss, A. R., Vassios, J. D., Nissen, S. J. & Ritz, C. Nonlinear regression analysis of herbicide absorption studies. *Weed Sci.* **59**, 601–610 (2011).

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Author contributions

C.S., A.J.J., G.K. and M.J. conceived the research plan, C.S. conducted the experiments and statistical analysis, C.S. and, M.J. wrote the article with contributions from A.J.J. and G.K.

Competing interests

The authors declare no competing interests.

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Genetic Basis of Chlorsulfuron, Atrazine, and Mesotrione Resistance in a Palmer Amaranth (*Amaranthus palmeri*) Population

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ABSTRACT: A population of Palmer amaranth (*Amaranthus palmeri* S. Watson) in Kansas (KSR) was found to have evolved resistance to three commonly used herbicides, i.e., chlorsulfuron, atrazine, and mesotrione. Our previous research confirmed the predominance of metabolic resistance to these herbicides in KSR, although a small percentage of plants also showed target-site alteration conferring resistance. In this research, we investigated the inheritance of resistance to these three herbicides in KSR Palmer amaranth. F_1 and F_2 progeny were generated by a conventional breeding approach. On the basis of the response of F_1 and F_2 progeny to three herbicide applications and χ^2 analyses, we found that the resistance of KSR Palmer amaranth to chlorsulfuron and atrazine is controlled by single nuclear genes. However, resistance to mesotrione was found to be imparted by multiple nuclear alleles in this population. Single-gene resistance can spread rapidly, while multiple-gene traits are slower to evolve and spread. Regardless, adoption of integrated weed management strategies can minimize the spread of resistance.

KEYWORDS: *inheritance, multiple herbicide resistance, screening, segregation*

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INTRODUCTION

Palmer amaranth (*Amaranthus palmeri* S. Watson) is the most troublesome weed in the United States.[1] Yield losses due to Palmer amaranth interference have been reported in crops like corn (*Zea mays* L.) and soybean (*Glycine max* L.).[2,3] Extensive herbicide selection and obligate outcrossing coupled with high fecundity have enabled Palmer amaranth to evolve resistance to eight herbicide modes of action (MOAs), viz., acetolactate synthase (ALS), photosystem II (PS II), 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), 4-hydroxyphenyl-pyruvate dioxygenase (HPPD), microtubule, protoporphyrinogen oxidase (PPO), and long-chain fatty acid (LCFA) inhibitors and synthetic auxinic herbicides.[4] Populations of Palmer amaranth with resistance to more than one MOA are prevalent throughout the United States, leading to fewer herbicide options for management.[5−7]

Target-site (TSR)- and/or non-target-site (NTSR)-based herbicide resistance mechanisms have been reported in a single population of Palmer amaranth.[5,7−10] In general, TSR involves alterations in the gene encoding the herbicide target protein, resulting in a direct reduction in the efficacy of the herbicide.[11] Such modifications include amino acid substitutions, deletions, increased target gene copy number, increased levels of gene and/or protein expression, and increased enzyme activity.[5,7−10,12,13] TSR is generally conferred by a single gene. Contrary to TSR, NTSR mechanisms involve alterations in physiological processes within plants before the herbicide reaches the target site, including reduced absorption, translocation, or increased detoxification of herbicides.[8−10,14] This type of resistance is typically polygenic,[15] although instances of NTSR inherited by a single gene have been reported.[16] Herbicide resistance inherited by a single major gene results in the faster spread of resistance within a population in comparison to polygenic traits that require several sexual recombinations to accumulate multiple minor alleles.[17] Inheritance of herbicide resistance alleles also varies depending on the genetic background of weed species and the rates of application of herbicides along with selection pressure.[18]

Previous research suggests that in common waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer], metabolism-based resistance to HPPD inhibitors (e.g., mesotrione) was conferred by multiple genes,[16,19] whereas metabolic resistance to PS-II inhibitors (e.g., atrazine) was conferred by a single gene.[16] TSR to ALS inhibitor (e.g., chlorsulfuron) in common sowthistle (*Sonchus oleraceus* L.) was found to be governed by a single gene.[20] Despite an increase in the incidence of herbicide resistance, limited information about the inheritance of chlorsulfuron, atrazine, and mesotrione resistance in Palmer amaranth is available. Understanding the inheritance of herbicide resistance can provide information about the rate of resistance evolution and its spread, which in turn might help in formulating effective weed management strategies.[21] Such knowledge can also enrich the perspective of the genetic structure of weed populations under selection pressure.[22]

A population of Palmer amaranth from Kansas (KSR) was documented with >275-, 178−237-, and 10−18-fold resistance to ALS, PS-II, and HPPD inhibitors, respectively (based on

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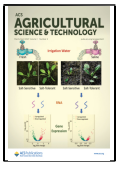

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Table 1. List of Herbicides Used in This Study and Recommended Field Application Rates

mode of action (MOA)	herbicide	formulation	manufacturer	feld recommended rate (1×; g ha^{-1})	adjuvants
ALS inhibitor	chlorsulfuron	Dupont Glean XP	Dupont, Wilmington, DE	18	0.25% (v/v) NIS
PS II inhibitor	atrazine	Aatrex 4L	Syngenta Crop Protection, Research Triangle Park, NC	2240	1% (v/v) COC
HPPD inhibitor	mesotrione	Callisto	Syngenta Crop Protection	105	1% (v/v) COC and 2.5% (v/v) AMS

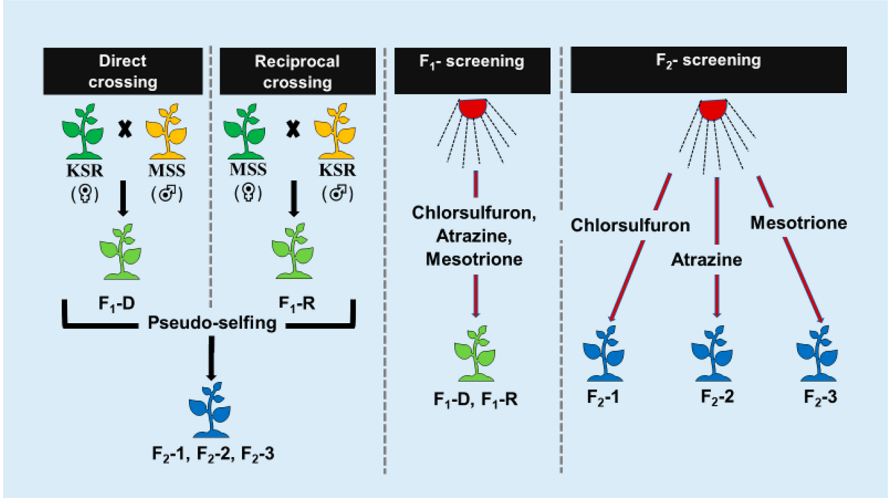

Figure 1.

greenhouse studies), compared to known susceptible populations of MSS and KSS.[8−10] Predominantly, metabolic resistance to these three herbicides was found in KSR.[8−10] More than 70% of chlorsulfuron-resistant KSR plants lacked the commonly found mutation (e.g., Pro197Ser) in the *ALS* gene, which bestows resistance to sulfonylurea herbicides. Cytochrome P450-monooxygenases (P450s) are involved in detoxifying several xenobiotics in plants, including herbicides.[23] Application of P450 inhibitor malathion was found to reverse chlorsulfuron resistance, indicating P450-mediated metabolism in KSR.[8] KSR plants that survived application of atrazine did not possess any mutation (e.g., Ser264Gly) in the *PsbA* gene and exhibited rapid detoxification of atrazine via glutathione *S*-transferase (GST) conjugation.[9] Additionally, mesotrione (HPPD inhibitor) resistance in KSR was predominantly conferred by rapid detoxification, although an increased level of expression of the *HPPD* transcript was found in some KSR plants.[10] Resistance to both atrazine and mesotrione was reported to be governed by nuclear genes in KSR.[6,9]

This study was conducted to investigate the inheritance of chlorsulfuron, atrazine, and mesotrione resistance in KSR Palmer amaranth. Specific objectives were (1) to generate F_1 and F_2 families by crossing KSR with known multiple-herbicide-susceptible (MSS) plants and (2) to assess the segregation (resistance or susceptibility) of progenies from F_1 and F_2 families to determine the nature and number of genes controlling the resistance to the three herbicides.

MATERIALS AND METHODS

Plant Material and Growth Condition. Two Palmer amaranth populations from Kansas (KSR and KSS) and one from Mississippi (MSS) were used in this study. KSR is resistant to and both KSS and MSS are susceptible to ALS , PS II, and HPPD inhibitors. KSR, KSS, and MSS were the same populations used in our previous research.[8−10] All of the experiments were conducted in a greenhouse facility maintained at 25 °C/20 °C day/night (d/n), 15 h/9 h (d/n) photoperiod with a photosynthetic photon flux density of 250 μmol m^{-2} s^{-1} and a relative humidity of 60 ± 10%. Seeds of Palmer amaranth were sown in small plastic trays (25 cm × 15 cm × 2.5 cm) filled with a potting mixture (Fafard ultra container potting mix, Sungro Horticulture, Agawam, MA). Upon emergence, individual seedlings at the two- to three-leaf stage were transplanted into pots (6 cm × 6 cm × 6 cm). The plants were watered adequately and fertilized (Miracle GRO All-purpose plant food 24-8-16) once a week until they were harvested.

Generation of Direct and Reciprocal F_1 Families. KSR plants (10−12 cm tall) were treated with a recommended field rate of mesotrione (Table 1; n = 20). The survivors were identified 3 weeks after treatment (WAT) and transplanted into new pots (15 cm × 10 cm × 15 cm). Once the plants begin to form inflorescences (before flower opening), direct and reciprocal crosses were performed by individually pairing survivor female and male plants of KSR with male and female plants of MSS, respectively, and enclosing them with pollination bags to prevent pollen contamination. At maturity, seeds were collected from each female parent plant, cleaned, and stored at 4 °C. In total, two F_1 families were obtained; one each from direct and reciprocal crosses [F_1-D and F_1-R (Figure 1)].

Screening of F_1 Plants for Chlorsulfuron, Atrazine, and Mesotrione Resistance. Seeds of F_1 generation were sown, and the emerged seedlings were grown in the greenhouse as described above. Screening experiments with three herbicides were conducted separately using 10−12 cm tall F_1 plants. For chlorsulfuron, atrazine, and mesotrione screening, 21−36 F_1 plants were treated with Glean, Aatrex, and Callisto, respectively (Table 1). At the time of application, adjuvants such as a non-ionic surfactant (NIS; Southern Ag, Rubonia, FL), a crop oil concentrate (COC; Agridex, Helena Chemical Co., Collierville, TN), and ammonium sulfate (AMS; Liquid N-Pak, Winfield Solutions, St. Paul, MN) were applied as per commercial product guidelines (Table 1). All herbicide applications were performed using a bench-type sprayer (Research Track Sprayer,

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Generation III, De Vries Manufacturing, Hollandale, MN) equipped with a single flat-fan nozzle (80015LP TeeJet tip, Spraying Systems Co., Wheaton, IL) delivering 187 L ha^{-1} at 222 kPa in a single pass at 4.8 km h^{-1}. After treatment, the plants were transferred back to the greenhouse and arranged in a completely randomized design. At 3 WAT, the percent survival of F_1 progenies was tabulated.

Generation of F_2 Families. Palmer amaranth is a dioecious plant; therefore, to generate F_2 families, pseudoselfing was performed, as shown in Figure 1. In brief, individual female and male plants of the F_1 generation that survived recommended field rates of each of the herbicide applications described above (Table 1) were randomly selected, paired, and crossed to generate F_2-1, F_2-2, and F_2-3 accordingly (Figure 1).

Screening of F_2 Families for Chlorsulfuron, Atrazine, and Mesotrione Resistance. Because of a shortage of MSS seeds, another population of Palmer amaranth (KSS; susceptible to all three herbicides) was used as a susceptible control in this study. Seeds of F_2 families along with KSR and KSS were grown in the greenhouse as described above. On the basis of segregation observed between KSR and KSS in greenhouse experiments, the discriminatory doses selected for testing for F_2 segregation were the recommended field rate of the three herbicides (Table 1). Therefore, 70−80 progenies representing each F_2 family along with 12 plants each of KSR and KSS were treated at 10−12 cm tall with recommended field rates of chlorsulfuron, atrazine, and mesotrione (Table 1) to identify live or dead (resistance or susceptible phenotype) plants at 3 WAT (Figure 1). In addition to the recommended field rate, seedlings from individual F_2 families were treated with smaller doses of these three herbicides, to check for segregation at lower rates. The null hypothesis (H_0) of this experiment for each screening scenario was set as "resistance to each herbicide is controlled by a single gene". The following single-gene locus model was used (eq 1):

$$\text{Exp } F_2 = 0.25 \times \text{Obs KSR} + 0.5 \times \text{Obs } F_1 + 0.25 \times \text{Obs KSS} \quad (1)$$

where Exp F_2 is the expected segregation frequency of the F_2 families and Obs is the observed segregation frequency of the KSR, F_1, and KSS phenotype. On the basis of this hypothesis, we expect a segregation ratio of 3:1 (resistant:susceptible plants). Thus, the χ^2 goodness of fit test was used to analyze the data by comparing the segregation frequency of the observed with the expected. The p value obtained from the analysis of the data from each F_2 family was compared at the $\alpha = 0.05$ significance level. The model was rejected if the obtained p value was <0.05.

RESULTS AND DISCUSSION

Inheritance of Chlorsulfuron Resistance. Although the F_1 progeny were generated by crossing mesotrione-resistant KSR and MSS, the seedlings from both F_1-D and F_1-R were found to be resistant to chlorsulfuron. This suggests that the KSR parents used in the direct or reciprocal crosses were resistant to chlorsulfuron. In response to chlorsulfuron treatment, 47% of F_1-D plants and 78% of F_1-R plants survived (Table 2), indicating that chlorsulfuron resistance in KSR is governed by nuclear allele(s). However, segregation of F_1-D and F_1-R progenies for chlorsulfuron resistance specifies that both male and female KSR plants used to generate F_1 progenies were heterozygous for the trait. Segregation of F_2-1 progenies into chlorsulfuron-resistant or -susceptible phenotypes at the recommended field rate (1×) as well as half of that rate (0.5×) corroborated our expected 3:1 (resistant:susceptible) ratio and was supported by the goodness of fit test with a p value of 0.554 ($\alpha = 0.05$) (Table 3). Interestingly, at the lower rates of chlorsulfuron (0.125× and 0.25×) used in this study, segregation of F_2-1 failed to fit the 3:1 ratio with a p value of 0.0455 ($\alpha = 0.05$) (Table 4). Overall, this study indicates the involvement of a single dominant nuclear allele imparting chlorsulfuron resistance in KSR.

ALS is a nuclear gene, and in several ALS inhibitor-resistant weeds, the resistance is governed by nuclear allele(s).[24] For example, TSR to ALS inhibitors in common sowthistle (*S. oleraceus*) and kochia [*Bassia scoparia* (L.)] was reported to be controlled by nuclear alleles.[20,25] Similarly, NTSR to ALS inhibitor chlorsulfuron was reported to be conferred by nuclear

Table 2. Response of F_1 Progenies of Palmer Amaranth (KSR) in Response to Treatment with Chlorsulfuron, Atrazine, and Mesotrione 21 Days after Treatment (DAT)[a]

F_1 family	herbicide	no. of plants treated	no. of living plants	survival rate[b] (%)
F_1-D	chlorsulfuron	32	15	47
	atrazine	32	6	50
	mesotrione	32	5	16
F_1-R	chlorsulfuron	36	28	78
	atrazine	30	12	40
	mesotrione	21	7	33

[a]F_1-D and F_1-R represent F_1 progeny derived from direct and reciprocal crosses performed using KSR and MSS plants, respectively. [b]The survival rate was calculated on the basis of the response to the recommended field rate of the herbicides listed in Table 1.

Table 3. Segregation of Chlorsulfuron Resistance or Susceptibility of Palmer Amaranth (KSR) F_2-1 Progenies

		observed segregation of plants				
chlorsulfuron dose (g ha^{-1})	total no. of plants	resistant	susceptible	R:S[a]	χ^2[b]	p value[c]
2.25	12	12	0	3:1	4	0.0455
4.5	12	12	0	3:1	4	0.0455
9	12	10	2	3:1	0.444	0.505
18	77	60	17	3:1	0.351	0.554

[a]R:S represents the expected segregation ratio of a trait for a single gene qualitative trait model into resistant and susceptible phenotypes. [b]$\chi^2 = \sum[(O_i - E_i)^2/E_i]$, where O_i is the observed frequency count for the ith level of the categorical variable and E_i is the expected frequency count for the ith level of the categorical variable. [c]The p value is obtained from the test and compared with respect to the $p \leq 0.05$ significance level.

Table 4. Segregation of Atrazine Resistance or Susceptibility of Palmer Amaranth (KSR) F_2-2 Progenies

		observed segregation of plants				
atrazine dose (g ha^{-1})	total no. of plants	resistant	susceptible	R:S[a]	χ^2[b]	p value[c]
280	12	12	0	3:1	4	0.0455
560	12	10	2	3:1	0.444	0.505
1120	12	11	1	3:1	1.778	0.182
2240	72	55	17	3:1	0.074	0.785

[a]R:S represents the expected segregation ratio of a trait for a single gene qualitative trait model into resistant and susceptible phenotypes. [b]$\chi^2 = \sum[(O_i - E_i)^2/E_i]$, where O_i is the observed frequency count for the ith level of the categorical variable and E_i is the expected frequency count for the ith level of the categorical variable. [c]The p value is obtained from the test and compared with respect to the $p \leq 0.05$ significance level.

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allele(s) in rigid ryegrass (*Lolium rigidum* Gaudin).[26] In KSR Palmer amaranth, a predominance (~70%) of NTSR to ALS inhibitors was found, although ~30% of resistant plants exhibited the mutation (Pro197Ser) in the *ALS* gene.[8] However, the Pro197Ser mutation in the *ALS* gene confers resistance to only sulfonylurea herbicides such as chlorsulfuron and thifensulfuron.[27] Interestingly with only the Pro197Ser mutation, KSR is cross-resistant to two other classes of ALS inhibitors, i.e., sulfonyl aminocarbonyl-triazolinones (propoxycarbazone) and pyrimidinyl-benzoates (pyrithiobac), but is susceptible to imidazolines (imazamox).[8] Increased sensitivity to chlorsulfuron was also found in KSR Palmer amaranth after pretreatment of malathion.[8] Malathion pretreatment has been shown to increase the sensitivity of weeds with metabolic resistance to sulfonylurea herbicides,[28] pyrimidinyl-benzoates,[29] and sulfonylaminocarbonyl-triazolinones.[30] Overall, the absence of mutations in the *ALS* gene that confer cross-resistance to propoxycarbazone and pyrithiobac and reversal of resistance with malathion application indicate the existence of a more complex mechanism and inheritance pattern of ALS inhibitor resistance in KSR Palmer amaranth.

Inheritance of Atrazine Resistance. Both F_1-D and F_1-R progenies generated by crossing mesotrione-resistant KSR and MSS survived atrazine application (Table 2), suggesting that the KSR parent used in the direct or reciprocal crosses was also resistant to atrazine. Similar to the response to chlorsulfuron, both F_1-D (50%) and F_1-R (40%) progenies survived atrazine application, indicating that a nuclear allele(s) governs atrazine resistance in KSR (Table 2). Segregation of F_1-D and F_1-R progenies for atrazine resistance also indicated that both male and female KSR plants used for generating F_1s were heterozygous for the trait. Segregation of atrazine resistance in the F_2-2 progenies at rates lower than 0.25× atrazine did not follow the 3:1 ratio. However, at doses higher than 0.25×, F_2-2 progenies segregated into a 3:1 ratio (resistant:susceptible). Overall, the segregation analysis at the recommended field rate of atrazine suggests a single nuclear gene inheritance of atrazine resistance in KSR Palmer amaranth (Table 5). A χ^2 goodness of fit test at the recommended field rate of atrazine supported our hypothesis of a single gene inheritance of atrazine resistance, with a p value of 0.7 ($\alpha = 0.05$).

Previously, we reported metabolic resistance (NTSR) to atrazine in KSR Palmer amaranth.[9] Similar to the findings of this study, NTSR to atrazine in common waterhemp populations from Illinois and Iowa was also found to be governed by single nuclear alleles.[16,31] The target site of atrazine, *psbA*, is a chloroplast-encoded gene. Thus, TSR (e.g., Ser264Gly in the *psbA* gene) to atrazine is a maternally inherited trait. For example, rapeseed (*Brassica campestris* L.), atrazine resistance was inherited cytoplasmically through the female parent.[32] However, NTSR to triazine was mediated by nuclear alleles, e.g., as reported in atrazine-resistant common waterhemp.[31] In agreement with that, on the basis of the F_1 response as well as previous research,[9] NTSR to atrazine in KSR is inherited by nuclear allele(s). Furthermore, on the basis of the F_2-2 segregation pattern, it is reasonable to assume that an incompletely dominant gene confers atrazine resistance in KSR Palmer amaranth. An increased level of expression of *AtuGSTF2*, a ϕ class GST, was found to be strongly correlated with atrazine resistance in common waterhemp.[33] Information about specific GSTs involved in atrazine resistance in Palmer amaranth needs to be gathered.

Inheritance of Mesotrione Resistance. The response of F_1 plants to mesotrione application suggested that 16% of F_1-D plants and 33% of F_1-R plants were found to be resistant to this herbicide, indicating that the KSR parental plants selected for generating F_1 families were heterozygous for this trait. This also indicated that mesotrione resistance is a nuclear trait. The segregation of F_2-3 progenies at the recommended field or lower rates did not follow the expected 3:1 (resistance:susceptible) ratio, which strongly suggests that more than one gene governs mesotrione resistance (Table 5). The estimated p value for screening at the recommended field rate of mesotrione from the χ^2 goodness of fit test was 0.015 (which is less than $\alpha = 0.05$).

The response of F_1 progenies to mesotrione in this study as well as previous work[6] indicated that the mesotrione resistance in KSR is inherited via nuclear allele(s). Nuclear inheritance of mesotrione resistance was also reported in mesotrione-resistant common waterhemp populations from Illinois and Nebraska.[16,19] Mesotrione resistance in KSR was predominantly metabolic, although an increased level of *HPPD* gene expression was also found in some plants.[10] Therefore, further investigation is necessary to prove that the two mechanisms of mesotrione resistance are linked and inherited together. Metabolic resistance to mesotrione in KSR is suspected to be mediated by P450 enzyme activity.[10] P450s are known to be involved in the detoxification of mesotrione in corn.[34] P450-based detoxification of mesotrione and tembotrione was reported in common waterhemp population from Illinois and Palmer amaranth from Nebraska.[35,36] Interestingly, more recently, in an atrazine- and mesotrione-resistant common waterhemp population from Illinois, Jacobs et al.[37] suggested that the gene conferring atrazine resistance was also involved in mesotrione resistance, although additional genes are also required to confer mesotrione resistance. The results of this study suggest that the mesotrione resistance in KSR Palmer amaranth is polygenic. Therefore, the possibility of involvement of multiple P450s or numerous loci modulating P450 activity along with the gene governing atrazine resistance needs to be investigated.

This study confirms that a single allele(s) controls resistance to chlorsulfuron and atrazine in KSR Palmer amaranth, while mesotrione resistance is polygenic. Nuclear inheritance of chlorsulfuron, atrazine, and mesotrione resistance in KSR reported previously implies that the resistance can be spread via both pollen and seed. This is particularly important

Table 5. Segregation of Mesotrione Resistance or Susceptibility of Palmer Amaranth (KSR) F_2-3 Progenies

		observed segregation of plants				
mesotrione dose (g ha^{-1})	total no. of plants	resistant	susceptible	R:S[a]	χ^2[b]	p value[c]
13.125	12	12	0	3:1	4	0.0455
26.25	12	12	0	3:1	4	0.0455
52.5	12	12	0	3:1	4	0.0455
105	82	71	11	3:1	5.87	0.015

[a]R:S represents the expected segregation ratio of a trait for a single gene qualitative trait model into resistant and susceptible phenotypes. [b]$\chi^2 = \sum[(O_i - E_i)^2/E_i]$, where O_i is the observed frequency count for the ith level of the categorical variable and E_i is the expected frequency count for the ith level of the categorical variable. [c]The p value is obtained from the test and compared with respect to the $p \leq 0.05$ significance level.

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considering that both intraspecific and interspecific transfer of resistant alleles via pollen has been reported in Palmer amaranth.[38] Similarly, single-gene-controlled chlorsulfuron and atrazine resistance can spread faster than polygenic mesotrione resistance (unless TSR and NTSR coexist). Overall, such transfer of resistance alleles via pollen indicates a possible spread of multiple resistance from KSR to different Palmer amaranth populations as well as to other cross-compatible *Amaranthus* species.

The result of this study sheds light on the complexity of the genetic architecture of chlorsulfuron, atrazine, and mesotrione resistance in KSR Palmer amaranth. Selection for ALS inhibitors is thought to have happened first in KSR, followed by the evolution of PS II and HPPD inhibitor resistance.[4] In particular, a predominance of NTSR for ALS inhibitors in KSR may have laid the foundation for the selection of metabolic resistance to PS-II and HPPD inhibitors, even though there was no selection pressure for HPPD inhibitors in this population.[39] Hence, the role of P450s as well as GSTs potentially involved in herbicide detoxification needs to be explored in KSR. The male and female mesotrione-resistant KSR plants used to generate F_1 families were also resistant to chlorsulfuron and atrazine, indicating that these plants were resistant to all three MOAs. Interestingly, metabolic resistance via P450s and GSTs can impart cross-resistance to herbicides belonging to different MOAs.[40] For instance, P450s from subfamily CYP81A have been reported to endow cross-resistance to ALS and ACCase inhibitors to late watergrass (*Echinochloa phyllopogon* Stapf.).[41] Therefore, the possibility of the same P450s or GSTs being involved in detoxification of chlorsulfuron, atrazine, and mesotrione cannot be ruled out, and further research on this is warranted. Metabolism-based NTSR to herbicides in weeds is one of the prime challenges for effective weed management with herbicides. In-depth knowledge of mechanisms and the genetic basis of the evolution of herbicide resistance in weeds will help to draft the best field programs/herbicide combinations for resistance management. Incorporation of multiple herbicide MOAs should be followed to reduce selection pressure and slow the spread of resistant allele dispersion. Moreover, growers should be encouraged to adopt integrated weed management techniques involving cultural, mechanical, and chemical methods to reduce selection pressure and prevent further evolution of herbicide resistance.

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ABBREVIATIONS USED

ALS, acetolactate synthase; EPSPS, 5-enolpyruvyl-shikimate-3-phosphate synthase; F_1-D, F_1 family from direct crossing; F_1-R, F_1 family from reciprocal crossing; F_2-1, F_2-2, and F_2-3, F_2 families; GR_{50}, effective dose to reduce above-ground shoot biomass by 50%; GST, glutathione *S*-transferase; HPPD, 4-hydroxyphenylpyruvate dioxygenase; KSR, Kansas resistant Palmer amaranth population; KSS, Kansas susceptible Palmer amaranth population; LCFA, long-chain fatty acid; MOA, mode of action; MSS, Mississippi susceptible Palmer amaranth population; NTSR, non-target-site resistance; P450, cytochrome P450 monooxygenases; PPO, protoporphyrinogen oxidase; PS II, photosystem II; TSR, target-site resistance; WAT, weeks after treatment

REFERENCES

(1) Van Wychen, L. Survey of the Most Common and Troublesome Weeds in Broadleaf Crops, Fruits & Vegetables in the United States and Canada. Weed Science Society of America National Weed Survey Dataset. 2019. http://wssa.net/wp-content/uploads/2019-Weed-Survey_broadleaf-crops.xlsx (accessed 2020-04-20).

(2) Bensch, C. N.; Horak, M. J.; Peterson, D. Interference of Redroot Pigweed (*Amaranthus retroflexus*), Palmer Amaranth (*A. palmeri*), and Common Waterhemp (*A. rudis*) in Soybean. *Weed Sci.* **2003**, *51*, 37−43.

(3) Massinga, R. A.; Currie, R. S.; Horak, M. J.; Boyer, J. Interference of Palmer Amaranth in Corn. *Weed Sci.* **2001**, *49*, 202−208.

(4) Heap I. The International Survey of Herbicide Resistant Weeds. http://www.weedscience.org (accessed 2020-04-20).

(5) Shyam, C.; Borgato, E. A.; Peterson, D. E.; Dille, J. A.; Jugulam, M. Predominance of Metabolic Resistance in a Six-Way-Resistant Palmer Amaranth (*Amaranthus palmeri*) Population. *Front. Plant Sci.* **2021**, DOI: 10.3389/fpls.2020.614618.

(6) Thompson, C. R.; Peterson, D.; Lally, N. G. Characterization of HPPD Resistant Palmer Amaranth. Proceedings of the 52nd Annual Conference of Weed Science Society of America, Waikoloa, HI, 2012 (http://wssaabstracts.com/public/9/proceedings.html).

(7) Chaudhari, S.; Varanasi, V. K.; Nakka, S.; Bhowmik, P. C.; Thompson, C. R.; Peterson, D. E.; Currie, R. S.; Jugulam, M. Evolution of Target and Non-Target Based Multiple Herbicide Resistance in a Single Palmer Amaranth (*Amaranthus palmeri*) Population from Kansas. *Weed Technol.* **2020**, *34*, 447−453.

(8) Nakka, S.; Thompson, C. R.; Peterson, D. E.; Jugulam, M. Target Site-Based and Non-Target Site Based Resistance to ALS Inhibitors in Palmer Amaranth (*Amaranthus Palmeri*). *Weed Sci.* **2017**, *65*, 681−689.

(9) Nakka, S.; Godar, A. S.; Thompson, C. R.; Peterson, D. E.; Jugulam, M. Rapid Detoxification via Glutathione *S* -Transferase (GST) Conjugation Confers a High Level of Atrazine Resistance in

Palmer Amaranth (*Amaranthus palmeri*). *Pest Manage. Sci.* **2017**, *73*, 2236−2243.
(10) Nakka, S.; Godar, A. S.; Wani, P. S.; Thompson, C. R.; Peterson, D. E.; Roelofs, J.; Jugulam, M. Physiological and Molecular Characterization of Hydroxyphenylpyruvate Dioxygenase (HPPD)-Inhibitor Resistance in Palmer Amaranth (*Amaranthus palmeri* S. Wats.). *Front. Plant Sci.* **2017**, DOI: 10.3389/fpls.2017.00555.
(11) Délye, C. Unravelling the Genetic Basis of Non-Target-Site-Based Resistance (NTSR) to Herbicides: A Major Challenge for Weed Science in the Forthcoming Decade. *Pest Manage. Sci.* **2013**, *69*, 176−187.
(12) Gaines, T. A.; Zhang, W.; Wang, D.; Bukun, B.; Chisholm, S. T.; Shaner, D. L.; Nissen, S. J.; Patzoldt, W. L.; Tranel, P. J.; Culpepper, A. S.; Grey, T. L.; Webster, T. M.; Vencill, W. K.; Sammons, R. D.; Jiang, J.; Preston, C.; Leach, J. E.; Westra, P. Gene Amplification Confers Glyphosate Resistance in *Amaranthus palmeri*. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 1029−1034.
(13) Salas, R. A.; Burgos, N. R.; Tranel, P. J.; Singh, S.; Glasgow, L.; Scott, R. C.; Nichols, R. L. Resistance to PPO-Inhibiting Herbicide in Palmer Amaranth from Arkansas. *Pest Manage. Sci.* **2016**, *72*, 864−869.
(14) Palma-Bautista, C.; Torra, J.; Garcia, M. J.; Bracamonte, E.; Rojano-Delgado, A. M.; Alcántara-De la Cruz, R.; De Prado, R. Reduced Absorption and Impaired Translocation Endows Glyphosate Resistance in *Amaranthus palmeri* Harvested in Glyphosate-Resistant Soybean from Argentina. *J. Agric. Food Chem.* **2019**, *67*, 1052−1060.
(15) Preston, C. Herbicide Resistance in Weeds Endowed by Enhanced Detoxification: Complications for Management. *Weed Sci.* **2004**, *52*, 448−453.
(16) Huffman, J.; Hausman, N. E.; Hager, A. G.; Riechers, D. E.; Tranel, P. J. Genetics and Inheritance of Nontarget-Site Resistances to Atrazine and Mesotrione in a Waterhemp (*Amaranthus tuberculatus*) Population from Illinois. *Weed Sci.* **2015**, *63*, 799−809.
(17) Délye, C.; Jasieniuk, M.; Le Corre, V. Deciphering the Evolution of Herbicide Resistance in Weeds. *Trends Genet.* **2013**, *29*, 649−658.
(18) Ffrench-Constant, R. H.; Daborn, P. J.; Le Goff, G. The Genetics and Genomics of Insecticide Resistance. *Trends Genet.* **2004**, *20*, 163−170.
(19) Oliveira, M. C.; Gaines, T. A.; Jhala, A. J.; Knezevic, S. Z. Inheritance of Mesotrione Resistance in an *Amaranthus tuberculatus* (Var. *rudis*) Population from Nebraska, USA. *Front. Plant Sci.* **2018**, DOI: 10.3389/fpls.2018.00060.
(20) Boutsalis, P.; Powles, S. B. Inheritance and mechanism of resistance to herbicides inhibiting acetolactate synthase in *Sonchus oleraceus* L. *Theor. Appl. Genet.* **1995**, *91*, 242−247.
(21) Maxwell, B. D.; Roush, M. L.; Radosevich, S. R. Predicting the Evolution and Dynamics of Herbicide Resistance in Weed Populations. *Weed Technol.* **1990**, *4*, 2−13.
(22) Renton, M.; Busi, R.; Neve, P.; Thornby, D.; Vila-Aiub, M. Herbicide Resistance Modelling: Past, Present and Future. *Pest Manage. Sci.* **2014**, *70*, 1394−1404.
(23) Pandian, B. A.; Sathishraj, R.; Djanaguiraman, M.; Prasad, P. V. V.; Jugulam, M. Role of Cytochrome P450 Enzymes in Plant Stress Response. *Antioxidants* **2020**, *9*, 454.
(24) Tranel, P. J.; Wright, T. R. Resistance of Weeds to ALS-Inhibiting Herbicides: What Have We Learned? *Weed Sci.* **2002**, *50*, 700−712.
(25) Thompson, C. R.; Thill, D. C.; Mallory-Smith, C. A.; Shafii, B. Characterization of Chlorsulfuron Resistant and Susceptible Kochia (*Kochia scoparia*). *Weed Technol.* **1994**, *8*, 470−476.
(26) Preston, C. Inheritance and Linkage of Metabolism-Based Herbicide Cross-Resistance in Rigid Ryegrass (*Lolium rigidum*). *Weed Sci.* **2003**, *51*, 4−12.
(27) Yu, Q.; Han, H.; Powles, S. B. Mutations of the *ALS* Gene Endowing Resistance to ALS-Inhibiting Herbicides in *Lolium rigidum* Populations. *Pest Manage. Sci.* **2008**, *64*, 1229−1236.
(28) Liu, W.; Wu, C.; Guo, W.; Du, L.; Yuan, G.; Wang, J. Resistance Mechanisms to an Acetolactate Synthase (ALS) Inhibitor in Water Starwort (*Myosoton aquaticum*) Populations from China. *Weed Sci.* **2015**, *63*, 770−780.
(29) Fischer, A. J.; Bayer, D. E.; Carriere, M. D.; Ateh, C. M.; Yim, K. O. Mechanisms of Resistance to Bispyribac-Sodium in an *Echinochloa phyllopogon* Accession. *Pestic. Biochem. Physiol.* **2000**, *68*, 156−165.
(30) Park, K. W.; Fandrich, L.; Mallory-Smith, C. A. Absorption, Translocation, and Metabolism of Propoxycarbazone-Sodium in ALS-Inhibitor Resistant *Bromus tectorum* Biotypes. *Pestic. Biochem. Physiol.* **2004**, *79*, 18−24.
(31) Patzoldt, W. L.; Dixon, B. S.; Tranel, P. J. Triazine Resistance in *Amaranthus tuberculatus* (Moq) Sauer that is Not Site-of-Action Mediated. *Pest Manage. Sci.* **2003**, *59*, 1134−1142.
(32) Machado, V. S.; Bandeen, J. D. Genetic Analysis of Chloroplast Atrazine Resistance in *Brassica campestris*: Cytoplasmic Inheritance. *Weed Sci.* **1982**, *30*, 281−285.
(33) Evans, A. F.; O'Brien, S. R.; Ma, R.; Hager, A. G.; Riggins, C. W.; Lambert, K. N.; Riechers, D. E. Biochemical Characterization of Metabolism-Based Atrazine Resistance in *Amaranthus tuberculatus* and Identification of an Expressed GST Associated with Resistance. *Plant Biotechnol. J.* **2017**, *15*, 1238−1249.
(34) Mitchell, G.; Bartlett, D. W.; Fraser, T. E.; Hawkes, T. R.; Holt, D. C.; Townson, J. K.; Wichert, R. A. Mesotrione: A New Selective Herbicide for Use in Maize. *Pest Manage. Sci.* **2001**, *57*, 120−128.
(35) Küpper, A.; Peter, F.; Zöllner, P.; Lorentz, L.; Tranel, P. J.; Beffa, R.; Gaines, T. A. Tembotrione Detoxification in 4-Hydroxyphenylpyruvate Dioxygenase (HPPD) Inhibitor-Resistant Palmer Amaranth (*Amaranthus palmeri* S. Wats.). *Pest Manage. Sci.* **2018**, *74*, 2325−2334.
(36) Ma, R.; Kaundun, S. S.; Tranel, P. J.; Riggins, C. W.; McGinness, D. L.; Hager, A. G.; Hawkes, T.; McIndoe, E.; Riechers, D. E. Distinct Detoxification Mechanisms Confer Resistance to Mesotrione and Atrazine in a Population of Waterhemp. *Plant Physiol.* **2013**, *163*, 363−377.
(37) Jacobs, K. E.; Butts-Wilmsmeyer, C. J.; Ma, R.; O'Brien, S. R.; Riechers, D. E. Association Between Metabolic Resistances to Atrazine and Mesotrione in a Multiple-Resistant Waterhemp (*Amaranthus tuberculatus*) Population. *Weed Sci.* **2020**, *68*, 358−366.
(38) Oliveira, M. C.; Gaines, T. A.; Patterson, E. L.; Jhala, A. J.; Irmak, S.; Amundsen, K.; Knezevic, S. Z. Interspecific and Intraspecific Transference of Metabolism-Based Mesotrione Resistance in Dioecious Weedy *Amaranthus*. *Plant J.* **2018**, *96*, 1051−1063.
(39) Nandula, V. K.; Riechers, D. E.; Ferhatoglu, Y.; Barrett, M.; Duke, S. O.; Dayan, F. E.; Goldberg-Cavalleri, A.; Tétard-Jones, C.; Wortley, D. J.; Onkokesung, N.; Brazier-Hicks, M.; Edwards, R.; Gaines, T.; Iwakami, S.; Jugulam, M.; Ma, R. Herbicide Metabolism: Crop Selectivity, Bioactivation, Weed Resistance, and Regulation. *Weed Sci.* **2019**, *67*, 149−175.
(40) Jugulam, M.; Shyam, C. Non-Target-Site Resistance to Herbicides: Recent Developments. *Plants* **2019**, 8.
(41) Iwakami, S.; Kamidate, Y.; Yamaguchi, T.; Ishizaka, M.; Endo, M.; Suda, H.; Nagai, K.; Sunohara, Y.; Toki, S.; Uchino, A.; Tominaga, T.; Matsumoto, H. CYP81A P450s are Involved in Concomitant Cross-Resistance to Acetolactate Synthase and Acetyl-COA Carboxylase Herbicides in *Echinochloa phyllopogon*. *New Phytol.* **2019**, *221*, 2112−2122.

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