Exploring the mechanisms of Palmer amaranth resistance to protoporphyrinogen oxidase-inhibitor herbicides, dynamics of gender, and management strategies

by

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B.S., University of Sao Paulo, 2013
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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 a C4, summer annual, dioecious broadleaf plant and is the most economically damaging and troublesome weed in major crops grown in the USA. Currently, Palmer amaranth is reported to have evolved resistance to nine herbicide sites of action, raising the importance of integrating multiple weed management strategies. The objectives of this dissertation were to 1) quantify the relative level and characterize target- and non-target-site mechanism(s) conferring resistance to protoporphyrinogen oxidase (PPO)-inhibitors (e.g., lactofen and fomesafen) in a multiple-resistant Palmer amaranth population from Kansas (KCTR), 2) compare female and male life cycles of Palmer amaranth to identify differences that could be incorporated into management decisions, and 3) understand the interactions between pre-emergence (PRE) herbicides in the absence or presence of cover crops dead (terminated two weeks prior to planting) or "green" (terminated at the planting day) as well as impacts on soybean yield. Dose-response assay confirmed resistance to PPO-inhibitors in KCTR Palmer amaranth, which was 12.7- to 34.5-times less sensitive to these herbicides compared to a known susceptible Palmer amaranth. Analysis of the *PPO2* gene (the molecular target of PPO-inhibitors) revealed no known mutations nor increased expression of this gene conferring resistance in KCTR. High-pressure liquid chromatography analyses suggested more metabolism of fomesafen at 48, 72 and 96 hours after treatment in KCTR compared to susceptible plants. Additionally, in the presence of malathion, a cytochrome P450-inhibitor, there was increased sensitivity of KCTR to lactofen, suggesting that the KCTR plants metabolized PPO-inhibitors via P450 activity. Interestingly, PRE applications of the PPO-inhibitors fomesafen, flumioxazin, saflufenacil, and sulfentrazone, resulted in complete control of KCTR. A growth chamber assay of comparative emergence of

female and male Palmer amaranth in three populations (KS-1, KS-2, and MS-1) indicated that female seedlings reached 90% emergence with 144 growing degree days (GDD) and males with 150 GDD in KS-1, and in MS-1 females reached 90% emergence with 160 GDD, whereas males needed 190 GDD. However, that pattern was not observed in KS-2, and as the GDD window of difference between female and male emergence was short, anticipated female emergence was not a great opportunity to reduce total female number of plants in the population in order to decrease total seed production. Greenhouse studies using an adapted BBCH scale indicated that the lifecycles of female and male Palmer amaranth were not synchronous, but the differences in reproductive phases revealed patterns that can favor fertility and, therefore, seed production. Field studies with PRE herbicides and cover crop [absent, terminated two weeks before planting ("dead" at planting), or terminated on the planting day ("green" at planting)] suggested that the greater the biomass produced by cover crops, resulted in greater weed suppression early in the season. Cover crop alone, dead or green, provided a minimum of 74% of Palmer amaranth control across four site-year experiments. Combination of PREs with cover crops provided greater Palmer amaranth control at 28 days after treatment. Overall, the outcome of this research suggested that 1) P450-mediated metabolism of PPO-inhibitors confers resistance to this herbicide group; 2) differences in female and male reproductive phases can maximize fertilization and, therefore, increase seed production; 3) the use of cover crops provided greater control in early-season, and combinations of PREs and cover crops were more effective method to control Palmer amaranth.

Keywords: *Amaranthus palmeri* S. Watson, cover crop, cytochrome P450, development, emergence, phenology, pre-emergence.

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Dedication

This dissertation is dedicated to Angela Fatima dos Santos, who did not have the opportunity to complete her middle school studies but has always made all efforts in support for her children to pursue their dreams. Love you mom!

Chapter 1 - Literature Review

1.1. Literature Review

Palmer amaranth (*Amaranthus palmeri* S. Watson) is a C4, eudicot, dioecious summer annual weed, currently classified as the most troublesome weed in the United States (Van Wychen 2020). This species is known to be an outstanding competitor and to cause significant economic damage as consequence of yield reduction. Palmer amaranth has several biological characteristics that facilitate in its rapid adaptation to diverse environmental conditions.

Palmer amaranth is a highly prolific weed, with the ability to produce up to 600,000 seeds per plant in the absence of competition (Keeley et al. 1987). Populations from distinct geographic regions have been found to produce from 47 to 94,000 seeds per plant, with the flushes emerging earlier in the growing season resulting in greater number of seeds (Spaunhorst et al. 2018). As a C4 plant (Wang et al. 1992) with a more efficient photosynthetic rate compared to CAM and C3 plants, it shows a fast growth rate and high biomass accumulation. Palmer amaranth was found to produce 88 to 531 g of dry weight per plant growing in field conditions (Spaunhorst et al. 2018). When in competition with crops, Mahoney et al. (2021) found that females accumulated 195, 434, 1879 and 2014, whereas males produced 77, 258, 879, 934 g biomass per plant in competition with corn, soybean, peanut, and cotton, respectively, suggesting that females generally grow more than males even under competition.

Weeds can reduce yield by up to 52% in soybean (Soltani et al. 2017), 50% in corn (Soltani et al. 2016), 34% in winter wheat (Flessner et al. 2021) and 61% in grain sorghum (Dille et al. 2019) in North America. Palmer amaranth has showed to be a strong competitor, resulting in yield losses in soybean from 13 to 68% at 0.33 and 10 plants m^{-2}, respectively (Klingaman and Oliver 1994). In corn, 0.66 plants m^{-2} resulted in 11% yield reduction, but at 10.5 plants m^{-2} that

index was raised to 91%, when emerging with the crop (Massinga et al. 2001). Maximum yield loss indices are also high for Palmer amaranth in competition with cotton (59%) and peanut (68%), among other crops (Morgan et al. 2001; Burke et al. 2007).

Palmer amaranth became an issue in soybean and cotton fields with the occurrence of multiple herbicide resistance to ALS-inhibitors and glyphosate in several populations (Horak et al. 1995; Sprague et al. 1997; Culpepper et al. 2006). Protoporphyrinogen oxidase- (PPO-) inhibitor herbicides gained importance for managing glyphosate- and ALS-resistant Palmer amaranth in soybean and cotton fields, but later populations with resistance to PPO-inhibitors have been identified in several states in the US (Heap 2022).

Target- (TSR) and non-target-site (NTSR) mechanisms conferring resistance to PPO-inhibitors have been reported. The TSR mechanisms include a deletion of a glycine codon at the 210^{th} position (ΔG210) in the *PPO2* gene, as well as an arginine substitution for glycine or methionine at the 128^{th} position (R128G/M), and a glycine substitution for an alanine at the 399^{th} position (G399A) (Salas et al. 2016; Giacomini et al. 2017; Rangani et al. 2019). Metabolic resistance to PPO-inhibitors *via* cytochrome P450 and glutathione *s*-transferase activity has also been reported in Palmer amaranth (Varanasi et al. 2018).

Because Palmer amaranth is a dioecious species and, consequently, an obligate outcrosser, it can quickly spread and accumulate adaptive traits across populations, including herbicide resistance genes (Sosnoskie et al. 2012; Küpper et al. 2017; Singh et al. 2018; Chaudhari et al. 2020). Resistance to herbicides is a critical adaptation in weed species, as it limits the number of chemicals that can provide effective control, but it isn't the only type of adaptation observed in Palmer amaranth. Bravo et al. (2017) analyzed growth aspects of several populations grown in specific cropping systems for many years and found that the populations

differed in fresh and dry weight accumulation, plant height, and days to flowering according to the inherently unique growing conditions of the cropping system.

Understanding weed biology is critical to design effective practices to apply during more sensitive stages of weeds' life cycle (Norsworthy et al. 2012). To date, there is not much information available describing differences in growth and development parameters, considering gender differentiation in weeds. Recently, Montgomery et al. (2019, 2021) found the presence of a male-specific Y (MSY) chromosomal region of 1.3 Mb size and 121 predicted genes in palmer amaranth, with the possibility of having multiple agronomic factors (i.e., light, water, and nutrition levels) that could alter the expression patterns of this genomic region. If Palmer amaranth follows the XX and XY chromosomal model for sex-determination, it is possible that genes associated with growth, development and reproduction are present in the MSY region, and females and males could express different phenotypes. Studies modelling the differences between female and male plants could provide valuable information to optimize weed control practices.

Nonetheless, integrating multiple effective control options is crucial for the sustainability of food production, given the ability that weeds such as Palmer amaranth can adapt to selection pressure imposed by management. Despite being good competitors, there are several chemical, cultural, physical, and biological practices that can be used to maintain crop yield potential, i.e., the use of herbicides, crop rotation, narrow row-spacings, cover crops, flooding, and many others.

Cover crops are becoming widely adopted in the mid-west as they improve soil health parameters, such as organic matter content, increase water use efficiency by reducing the fallow period, provide a cover to the soil, which limits the occurrence of erosion, as well as increasing

soil aggregation (Obour et al. 2021). Cover crops can also be explored for weed suppression purposes (Teasdale and Mohler 2000; Norsworthy et al. 2007; Bachie and McGiffen 2013; Petrosino et al. 2015), which can occur due to competition, creating a physical barrier impeding weed emergence, or allelopathic activity (Barnes et al. 1987; Hutchinson and McGiffen 2000; Mirsky et al. 2013; Kunz et al. 2016). To maximize weed control with a cover crop in early season, it is often recommended to use residual herbicides. However, residual herbicides can have soil and foliar activity, and the interactions between residuals and cover crops terminated or planted green remain unknown.

A Palmer amaranth population from Kansas was recently documented to be resistant to six herbicide sites of action, including PPO-inhibitors (i.e., lactofen and fomesafen) (Shyam et al. 2021). The precise mechanism of PPO-inhibitor resistance in this population is unknown, which is one of the areas of investigation in this dissertation. Also, given the occurrence of a MSY region, which expression may be triggered by environmental factors (i.e., photoperiod), it's possible that female and male plants have differences in their life cycle (i.e., emergence, growth, development) that could be explored for more efficient control. Considering the importance of herbicides and the increase in adoption of cover crops for weed suppression, a question is raised regarding possible interactions between residual herbicides and cover crops, as well as impacts of cover crop on cash-crop yield.

Given the topics mentioned above, the objectives of this dissertation were to (1) characterize resistance level and mechanism(s) conferring resistance to PPO-inhibitor herbicides in a Palmer amaranth population from Kansas, (2) investigate the differences in emergence, growth, and development patterns in female and male plants in general, and (3) investigate the

effect of applying residual herbicides in association with cover crops on Palmer amaranth control and soybean yield.

1.2. References

Bachie O, McGiffen ME. (2013). Effects of summer cover cropping on weed population density and biomass in a subsequent broccoli crop. p. 31-36. In: International Conference on Sustainable Environment and Agriculture. IPCBEE 57:31-36.

Barnes JP, Putnam AR, Burke BA, Aasen AJ. (1987). Isolation and characterization of allelochemicals in rye herbage. *Phytochemistry*, 26:1385-1390.

Bravo W, Leon RG, Ferrell JA, Mulvaney MJ, Wood CW. (2017). Differentiation of life-history traits among Palmer amaranth populations (*Amaranthus palmeri*) and its relation to cropping systems and glyphosate sensitivity. *Weed Science*, 65:339-349.

Burke IC, Schroeder M, Thomas WE, Wilcut JW. (2007). Palmer Amaranth interference and seed production in peanut. *Weed Technology*, 21(2):367-371.

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 Technology*, 34, 447-453.

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 Science*, 54(4):620-626.

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 Technology*, 34:624-629.

Flessner ML, Burke IC, Dille JA, Everman WJ, VanGessel MJ, Tidemann B, Manuchehri MR, Soltani N, Sikkema PH. (2021). Potential wheat yield loss due to weeds in the United States and Canada. *Weed Technology*, 35: 916-923.

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 Management Science*, 73(8):1559-1563.

Horak MJ, Peterson DE. (1995). Biotypes of Palmer amaranth (*Amaranthus palmeri*) and common waterhemp (*Amaranthus rudis*) are resistant to imazethapyr and thifensulfuron. *Weed Technology*. 9:192-195.

Hutchinson CM, McGiffen ME. (2000). Cowpea cover crop mulch for weed control in desert pepper production. *HortScience*, 35:196-198.

Keeley PE, Carter CH, Thullen RJ. (1987). Influence of planting date on growth of Palmer amaranth (*Amaranthus palmeri*). *Weed Science*, 35(2):199-204.

Klingaman TE, Oliver LR. (1994). Palmer amaranth (*Amaranthus palmeri*) interference in soybeans (Glycine max). *Weed Science*, 42:523-527.

Kunz C, Sturm DJ, Sökefeld M, Gerhards R. (2016). Weed suppression and early sugar beet development under different cover crop mulches. *Plant Protection Science*, 52:1-7.

Küpper A, Borgato EA, Patterson EL, Goncalves Netto A, Nicolai M, Carvalho SJP, Nissen SJ, Gaines TA, Christoffoleti PJ. (2017). Multiple resistance to glyphosate and acetolactate

synthase inhibitors in Palmer amaranth (*Amaranthus palmeri*) identified in Brazil. *Weed Science*, 65, 317-326.

Mahoney DJ, Jordan DL, Hare AT, Leon RG, Roma-Burgos N, Vann MC, Jennings KM, Everman WJ, Cahoon CW. (2021). Palmer amaranth (*Amaranthus palmeri*) growth and seed production when in competition with peanut and other crops in North Carolina. *Agronomy*, 11:1734.

Massinga RA, Currie RS, Horak MJ, Boyer Jr J. (2001). Interference of Palmer amaranth in corn. *Weed Science*, 49(2):202-208.

Morgan GD, Baumann PA, Chandler JM. (2001). Competitive impact of Palmer amaranth (*Amaranthus palmeri*) on cotton (*Gossypium hirsutum*) development and yield. *Weed Technology*, 15:408-412.

Mirsky SB, Ryan MR, Teasdale JR, Curran WS, Reberg-Horton CS, Spargo JT, Wells MS, Keene CL, Moyer JM. (2013). Overcoming weed management challenges in cover crop–based organic rotational no-till soybean production in the eastern United States. *Weed Technology*, 27:193-203

Montgomery JS, Giacomini DA, Weigel D, Tranel PJ. (2021). Male-specific Y-chromosomal regions in waterhemp (*Amaranthus tuberculatus*) and Palmer amaranth (*Amaranthus palmeri*). *New Phytologist*, 229(6):3522-3533.

Montgomery JS, Sadeque A, Giacomini D, Brown P, Tranel PJ. (2019). Sex-specific markers for waterhemp (*Amaranthus tuberculatus*) and Palmer amaranth (*Amaranthus palmeri*). *Weed Science*, 67(4), 412-418.

Norsworthy, J.K., Malik, M.S., Jha, P. And Riley, M.B. (2007). Suppression of *Digitaria sanguinalis* and *Amaranthus palmeri* using autumn-sown glucosinolate-producing cover crops in organically grown bell pepper. *Weed Research*, 47: 425-432.

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 Science*, 60(sp1):31-62.

Obour AK, Simon LM, Holman JD, Carr PM, Schipanski M, Fonte S, Ghimire R, Nleya T, Blanco-Caqui H. (2021). Cover crops to improve soil health in the North American Great Plains. *Agronomy Journal*, 113:4590-4604.

Petrosino JS, Dille JA, Holman JD, Roozeboom KL. (2015). Kochia suppression with cover crops in southwestern Kansas. *Crop Forage Turfgrass Management*, 1(1).

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 Science*, 10:568.

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 Management Science*, 72:864-869.

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

Soltani N, Dille J, Burke I, Everman W, VanGessel M, Davis V, Sikkema P. (2016). Potential corn yield losses from weeds in north America. *Weed Technology*, 30(4), 979-984.

Soltani N, Dille J, Burke I, Everman W, VanGessel M, Davis V, Sikkema P. (2017). Perspectives on potential soybean yield losses from weeds in north America. *Weed Technology*, 31(1), 148-154.

Spaunhorst DJ, Devkota P, Johnson WG, Smeda RJ, Meyer CJ, Norsworthy JK (2018) Phenology of Five Palmer amaranth (*Amaranthus palmeri*) Populations Grown in Northern Indiana and Arkansas. *Weed Science*, 66:457-469.

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 Management Science*, 75:1131-1139.

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 Science*, 60:366-373.

Sprague CL, Stoller EW, Wax LM, Horak MJ. (1997). Palmer amaranth (*Amaranthus palmeri*) and common waterhemp (*Amaranthus rudis*) resistance to selected ALS-inhibiting herbicides. *Weed Science*, 45:192-197.

Teasdale JR, Mohler CL. (2000). The quantitative relationship between weed emergence and the physical properties of mulches. *Weed Science*, 48:385-392.

Van Wychen L. (2020). 2020 survey of the most common and troublesome weeds in grass crops, pasture, and turf in the United States and Canada. Available online at: https://wssa.net/wp-content/uploads/2020-Weed-Survey_grass-crops.xlsx (accessed October 19, 2022).

Varanasi V, Brabham C, Norsworthy JK. (2018). Confirmation and characterization of non–target site resistance to fomesafen in Palmer amaranth (*Amaranthus palmeri*). *Weed Science*, 66(6):702-709.

Wang JL, Klessig DF, Berry JO. (1992). Regulation of C4 gene expression in developing amaranth leaves. *Plant Cell*, 4:173-184.

Chapter 2 - Metabolic Resistance to Protoporphyrinogen Oxidase-Inhibitor Herbicides in a Six-Way Resistant Palmer amaranth Population from Kansas

2.1. Abstract

Palmer amaranth has evolved target- and non-target site-based resistance to protoporphyrinogen oxidase-inhibitor herbicides in the United States. Recently, a population (KCTR) from a long-term conservation tillage study in Kansas was found to be resistant to six herbicide sites of action, including PPO-inhibitors (e.g., lactofen and fomesafen). The objectives of this research were to characterize the level of resistance to lactofen, identify the mechanism(s) conferring resistance in this population, and assess its sensitivity to pre-emergence (PRE) applied treatments. Dose-response analysis confirmed 5.9- to 34.5-fold resistance to lactofen in KCTR. *PPO2* sequence alignment revealed the absence of known mutations conferring resistance to PPO-inhibitors in Palmer amaranth, and differential expression of the *PPO2* gene did not occur. A reverse-phase HPLC assay confirmed that KCTR plants metabolized more fomesafen and faster compared to susceptible plants. Further, treatment with a cytochrome P450-inhibitor followed by lactofen restored the sensitivity of KCTR to this herbicide. Also, KCTR was completely controlled by the field recommended dose of the PREs applied PPO-inhibitors fomesafen, flumioxazin, saflufenacil, sulfentrazone and oxadiazon. These results suggest that P450-mediated metabolism confers resistance to PPO-inhibitors in KCTR, rather than alterations in the *PPO2*, which were more commonly found in other Palmer amaranth populations. Research

is in progress to identify the fomesafen metabolites and to understand the genetic basis of resistance to PPO-inhibitor herbicides in KCTR Palmer amaranth.

Keywords: cytochrome P450 monooxygenases, cross-resistance, metabolism-based resistance, lactofen

2.2. Introduction

Palmer amaranth (*Amaranthus palmeri* S. Watson) is known to be a strong competitor resulting in crop yield losses of 79% in soybean (*Glycine max* L.), 91% in corn (*Zea mays* L.), 65% in cotton (*Gossypium hirsutum* L.), 50% in sorghum (*Sorghum bicolor* (L.) Moench) (Rowland et al. 1999; Massinga et al. 2001; Bensch et al. 2003; Moore et al. 2004). This species is highly prolific with the ability to produce more than 600,000 seeds per plant in the absence of competition (Keeley et al. 1987). If left uncontrolled or poorly controlled, it can become a predominant problem in fields in only a few seasons (Norsworthy et al. 2014).

To date, Palmer amaranth is reported to have evolved resistance to nine herbicide sites of action (SOA) in the United States (Sprague et al. 1997; Culpepper et al. 2006; Salas et al. 2016; Nakka et al. 2017a, b, c; Brabham et al. 2019; Carvalho-Moore et al. 2022; Priess et al. 2022; Shyam et al. 2022; Heap 2022). This weed is a dioecious species, and therefore an obligate outcrosser, facilitating spread of resistance genes (Sosnoskie et al. 2012), and multiple alleles can accumulate within and across populations (Bagavathiannan and Norsworthy 2016; Chahal et al. 2017; Schwartz-Lazarro et al. 2017; Singh et al. 2018; Porri et al. 2022). Despite being native in the US (Sauer 1957), Palmer amaranth is now present in other countries such as Brazil, Greece, Israel, Mexico, and Spain, showing that this species has become a global issue (Carvalho et al. 2015; Gonçalves Netto et al. 2016; Küpper et al. 2017; Kanatas et al. 2021; Torra et al. 2020).

With the occurrence of resistance to glyphosate and ALS-inhibitor herbicides in multiple weed populations (Wise et al. 2009; Sosnoskie et al. 2011; Nandula et al. 2012; Kohrt et al. 2017; Garetson et al. 2019; Aulakh et al 2021; Mausbach et al. 2021; Heap 2022), the use of protoporphyrinogen oxidase (PPO)-inhibitor herbicides (hereafter referred to as PPO-inhibitors) gained more importance as an alternative herbicide option, especially in soybean and cotton

cropping systems. However, Palmer amaranth has also evolved resistance to this herbicide SOA. Resistance to PPO-inhibitors has occurred in several populations of this species in the midsouth US, including in soybean and cotton fields located in Arkansas, Illinois, Mississippi, Missouri, and Tennessee (Salas-Perez et al. 2017; Copeland et al. 2018; Noguera et al. 2020; Varanasi et al. 2018a; Wu et al. 2020), but this issue is gaining importance in the midwestern US as well (Montgomery et al. 2021; Oliveira et al. 2021).

Target (TSR)- and non-target-site resistance (NTSR) mechanisms conferring resistance to PPO-inhibitors in several populations have been documented. TSR mechanisms include the following mutations: R128G/M, ΔG210, and G399A, in the *PPO2* gene, which reduce herbicide binding efficacy (Salas et al. 2016; Giacomini et al. 2017; Rangani et al. 2019). NTSR mechanisms include herbicide detoxification *via* the activity of cytochrome P450 monooxygenases (P450s) and glutathione *s*-transferases (GSTs) (Varanasi et al. 2018b; 2019). Both P450s and GSTs are large groups of enzymes involved in stress tolerance in living organisms, including plants, that often metabolize xenobiotic compounds such as herbicides, fungicides, and insecticides (Gaines et al. 2020; Pandian et al. 2020). A critical aspect of metabolic resistance is the possibility of a specific mechanism conferring resistance to multiple SOAs in the same weed population (Busi et al. 2017; Iwakami et al. 2019; Dimaano et al. 2020; Han et al. 2021).

A Palmer amaranth population from Kansas (labelled as KCTR) from a conservation tillage experimental field was recently found to be resistant to six SOAs, including PPO-inhibitors (Shyam et al. 2021). More specifically, 29% and 84% of individuals survived the field-recommended dose of commonly used PPO-inhibitors fomesafen and lactofen, respectively. The objectives of this research were to (1) determine the relative level of resistance of KCTR to

lactofen, (2) investigate the occurrence of TSR mechanisms in the *PPO2* gene, (3) analyze the metabolic profile of KCTR to ^{14}C-fomesafen in KCTR, (4) identify the effect of P450 enzymes in metabolizing PPO-inhibitors using a P450-inhibitor (malathion) in KCTR and (5) assess the sensitivity of this population to pre-emergence application of PPO-inhibitors.

2.3. Material and Methods

2.3.1. Plant materials

Seeds from KCTR Palmer amaranth plants from a long-term conservation tillage study that survived the field recommended dose of various herbicides, including PPO-inhibitors (Shyam et al. 2021), were used in this study. During the 2018 growing season, a few plants of KCTR that survived the application of the field-labeled dose of 2,4-D (560 g ae ha^{-1}) were collected 28 days after herbicide treatment, transplanted into pots, and moved into the greenhouse to allow them to mate and produce seeds.

KCTR-G1, which are progenies from those plants collected from the field, were screened with fomesafen (264 g ha^{-1}) and lactofen (219 g ha^{-1}) and showed 29% and 84% survival to each herbicide, respectively (Shyam et al. 2021). Survivors were grouped in the greenhouse and covered with a pollen exclusion tent to avoid undesired pollination to produce the second KCTR generation (KCTR-G2). Known susceptible populations to PPO-inhibitors from Kansas (KSS) and from Mississippi (MSS) were included in the studies for comparison.

2.3.2. Greenhouse dose-response experiments

Dose-response experiments were performed to assess the level of resistance of KCTR to lactofen relative to the susceptible biotypes KSS and MSS. Seeds of KCTR-G1, KCTR-G2, as

well as KSS and MSS were planted in individual plastic trays filled with potting soil (Pro-Mix® premium potting mix, Premier Tech Home and Garden Inc., Ontario, CA) for germination. Emerged seedlings were transplanted into individual pots (6 × 6 × 6.5 cm) when they reached the first true leaf-stage. Plants were grown in a greenhouse under a 15 h photoperiod, 30/23 ± 2 C day/night temperature, and watered daily.

This experiment was conducted in a completely randomized design with 8 replicates and 7 doses per biotype in the first run. The resistant population, KCTR, was treated with 0.25, 0.5, 1, 2, 4 and 8X, and both KSS and MSS were treated with 0.0625, 0.125, 0.25, 0.5, 1 and 2X, with 1X being 219 g ha^{-1}, which is the field-labeled rate of lactofen to control Palmer amaranth (Lancaster et al. 2022, Anonymous 2015). Eight non-treated control replicates of each population were included for comparison. In a second experimental run, 16 and 32X doses were added to the resistant population for better assessment of the level of resistance. Crop oil concentrate at 1% v/v was added, following label instructions, to a spray volume proportional to 187 L ha^{-1}.

Herbicide doses were applied when plants were between 8 to 10 cm tall using an automated spray chamber (Research Track Sprayer, Generation III, De Vries Manufacturing, Hollandale, MN) equipped with an even flat-fan nozzle (Teejet Spraying Systems, Wheaton, IL) calibrated to deliver 187 L ha^{-1}.

Aboveground biomass was harvested 14 days after treatment (DAT), oven-dried at 60 C for 72 h, and weighed. A linear mixed effect model function (*lmer*) available in the *lme4* package (Bates et al. 2015) in R (RStudio Team 2022) was fit to the dry weight data, with replicates as a random effect, and normality of residuals and homoscedasticity of variances were verified. Subsequently, data were subjected to ANOVA ($\alpha = 0.05$) to compare runs, and data were combined if no significant differences were found.

A log-logistic regression analysis was performed to estimate the dose of herbicide required to reduce growth by 50% (GR_{50}) and by 90% (GR_{90}) using a three-parameter equation in the *drc* package in R (Knezevic et al. 2007; Ritz et al. 2015). The built-in function *modelFit* was used to verify the lack-of-fit test. The level of resistance was assessed by comparing the GR_{50} of KCTR-G1 and KCTR-G2 with GR_{50} of KSS and MSS. Relative dry weight (% of non-treated) was obtained by comparing dry weight from each treated replicate to their respective average dry weight of non-treated control for graphic visualization (Figure 2.1).

2.3.3. *PPO2* sequence and relative expression analyses

Approximately 100 mg of young leaf tissue from KCTR-G1 and KCTR-G2 plants were collected to assess the presence of any known mutations in the *PPO2* gene that confer resistance. Leaf tissue was collected from ten KCTR plants that survived lactofen application and three KSS. The samples were homogenized in liquid nitrogen using prechilled mortar and pestle before total RNA isolation. RNA was isolated using the TRIzol method (Thermo Fisher Scientific, Waltham, MA). RNA quality was assessed using agarose electrophoresis gel (1.5%), and concentration was verified using a NanoDrop 1000 (Thermo Fisher). One µg of RNA from each plant was used for complementary DNA (cDNA) synthesis using the First Strand Reverse-Transcriptase kit (Thermo Fisher), following the manufacturer's instructions.

Primers were designed to amplify approximately 1600 base pairs of the *PPO2* coding sequence, covering the 128, 210, and 399 positions, which are known to have mutations that confer resistance to PPO-inhibitors. Primers were designed using Primer3web (version 4.1.0, https://primer3.ut.ee), based on several *PPO2* sequences available in the GeneBank.

Polymerase chain reactions (PCR) were performed with a total volume of 20 µL, prepared as follows: 10 µL GoTaq® G2 MasterMix (Promega, WI, USA), 2 µL of each forward (5'-GGGGTACCCGGGTAAACTGATCTTAT-3') and reverse (5'-GGAATTCGAGCTCGCATGCTTACGCG-3') primers (5 µM), 4.5 µL molecular grade water (Thermo Fisher Scientific). and 1.5 µL cDNA. The reactions were performed with the following profile: 95 C for 5 m, and 39 cycles of 95 C for 30 s, 55 C for 30 s, 72 C for 1 m 30s, and final extension at 95 C for 10 m.

PCR products were purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific) and sent off for Sanger sequencing at the Genewiz facilities (South Plainfield, NJ). The forward primer from the PCR, with two other internal primers (5'-GCTACTGAGCTTTCTGATGAGCATG-3' and 5'-GTTATGATTACTGCATTCAAGAAGG-3') were used in the sequencing to cover the whole coding sequence of the *PPO2* gene. The KCTR and KSS sequences were aligned using Clustal Omega. Also, the accessions MF583744.1 (wild type), and MF583746 (ΔG210), KY882136.1 (R128G) and MK408971.1 (G399A) (resistant) available in the GeneBank were included for comparisons at each locus.

Overexpression of herbicide-targeted enzymes is often associated with resistance in weed species (Gaines et al. 2010; LaForest et al. 2017). An assay using real-time quantitative PCR was performed to QUANTIFY *PPO2* expression relative to β-tubulin in KCTR. A separate study investigated the occurrence of constitutive and induced expression by treating 12 biological replicates of KCTR, 4 of KSS and 8 of MSS with 219 g ha^{-1} of lactofen, and leaf tissue from each replicate was collected prior to and 3 h after the herbicide treatment.

RNA isolation and cDNA synthesis were performed following the same procedures listed above. The reactions were prepared using 1.5 µL of cDNA, 2 µL of F (5'-

AGGAAAAGGGTGGAGGAGAA-3') and R (5'-GACAAGGACAGCACCTCACA-3') primers (5 µM), 8 µL of PowerUp SYBR Green Master Mix (ThermoFisher Scientific), 0.5 µL of molecular grade water. *β-tubulin* primers sequences can be found in Koo et al. (2018). Reactions were performed with 50 C for 5 m, 95 C for 10 m and 39 cycles of 95 C for 30 s, 60 C for 1 m, with the melt curve included. *PPO2* expression was measured with three technical replicates were used for each biological sample and primer set, and the experiment was repeated. Two no-template control (NTC) reactions in triplicate were added to each plate. Expression was determined using the 2ΔCT method (Livak et al. 2001), where CT is the threshold cycle and ΔCT is $CT_{\text{target gene (}\textit{PPO2}\text{)}} - CT_{\text{reference gene (}\textit{β-tubulin}\text{)}}$. Biological replicates that did not have a consistent measurement across technical replicates or runs were not included in the analysis.

2.3.4. ^{14}C-fomesafen metabolism using HPLC

An excised leaf assay (Ma et al. 2013) was performed to determine if the metabolism of fomesafen confers resistance in KCTR. Based on Shyam et al. (2021), KCTR is resistant to both lactofen and fomesafen; the metabolism experiments were performed with fomesafen as this was the only ^{14}C PPO-inhibitor that was available for these studies. Resistant (KCTR) and susceptible (MSS) plants were grown in a greenhouse as previously described, and when plants reached approximately 15 cm height, they were moved to the lab and acclimatized for one day before the experiment started. The third, fourth, and fifth youngest leaves (with petioles) from each biological replicate were immersed in a 1.5 mL tube containing 1 mL solution of ^{14}C-fomesafen (specific activity of 4.1 MBq mg^{-1}) 50,000 disintegrations per minute (dpm). ^{14}C-fomesafen stock solution was initially diluted in acetonitrile and then added into distilled water to 50,000 dpm per leaf sample for herbicide uptake. The tubes were sealed with parafilm with a small hole

in which petioles of excised leaves were inserted, and 1 mL distilled water was added to the tube as needed to maintain photosynthetic activity in the leaves without interruptions until harvest.

Leaves from the same plant were collected at 24, 48, 72, and 96 h after herbicide treatment (HAT), frozen in liquid nitrogen, and kept at -80 C until the next step. Samples were then ground in liquid nitrogen using a mortar and pestle and placed in a Falcon tube with 10 mL of acetone (90%) for extraction and kept in an automatic shaker at 4 C for about 16 h. Subsequently, samples were centrifuged, supernatant from each sample was transferred to new falcon tubes, and vacuum dried at 45 C for 1.5 h until approximately 0.5 mL of solution was remaining with a rotary evaporator (Centrivap, Labconco, Kansas City, MO). Supernatant was transferred to 1.5 mL tubes and centrifuged at 15000 g for 10 mins, and radioactivity was measured from a 15 mL liquid scintillation containing 10 of µL of the supernatant.

Samples were normalized to 60 dpm mL^{-1} by diluting samples in acetonitrile, and 50 µL (3000 dpm) of the final solution were analyzed using reverse-phase high-performance liquid chromatography (HPLC) (Agilent technologies) employing a Zorbax SB-C18 column (4.6 x 250 mm, 5µm particle size; Agilent Technologies) to resolve the solutions into parent ^{14}C-fomesafen and metabolites. The mobile phases consisted of water with 0.1% trifluoroacetic acid (HPLC grade, ThermoFisher Scientific) (A) and acetonitrile with 0.1% trifluoroacetic acid (HPLC grade, ThermoFisher Scientific) (B). The procedure took a total of 24 mins. The first 20 min was a linear gradient from 0 to 100% of solvent B, and in the next 4 mins it was returned to 0% B. At 21 min, linear gradient was set from 100 to 80% solvent B, and then reduced to 60% B, 40% B and 0% B respectively, in the following 1 min of the run. The radioactivity was measured by a detector (EG&G Berthold, LB 509) employed with an admixture flow cell, Z-1000 (Berthold

Technologies GmbH) at a flow rate of 1 mL min^{-1} of scintillation fluid, Ultima-Flo AP cocktail (Perkin-Elmer).

The experiment was conducted as a completely randomized design with three replicates and repeated. Percent parent ^{14}C-fomesafen remaining in each sample was determined based on the peak areas as a percent of ^{14}C-fomesafen relative to the total extractable radioactivity. The metabolic profile of KCTR and MSS was demonstrated by chromatograms (Figure 2.3) and means of percent ^{14}C-fomesafen in KCTR and MSS as time progressed were compared by Tukey test ($\alpha = 0.05$) (Figure 2.4).

2.3.5. Dose-response with the P450-inhibitor malathion

Malathion, a P450-inhibitor, was used to test if the metabolism of PPO-inhibitors (as identified by the HPLC analysis) was mediated by P450 activity in KCTR in a separate dose-response study. The experimental design and treatments were similar as described before, but this time KCTR-G2 received the herbicides dose without and with malathion to assess the effect of this P450-inhibitor in altering the resistance in this population. The application of malathion at 2000 g ha^{-1} was done two hours prior to herbicide treatment, and the soil was drenched with additional 50 mL $plant^{-1}$ at 5 mM of malathion at 24 h after herbicide treatment instead of 48 h as described in previous research (i.e., Ma et al. 2013, Oliveira et al. 2017, Shyam et al. 2021, 2022). In this study, the soil drenched application of malathion was done 24 h after herbicide treatment because preliminary tests indicated that as 24 h was the best timing for application, as compared to 48 h, due to the fast action of PPO-inhibitors as compared to systemic herbicides (not shown).

2.3.6. Screening for resistance to pre-emergence applied PPO-inhibitor herbicides

Resistance in KCTR to pre-emergence (PRE) applied PPO-inhibitor herbicides was investigated using flumioxazin, fomesafen, oxadiazon, saflufenacil, and sulfentrazone at field-labeled rates for each product (Table 2.1). The experiment was performed in a greenhouse with growing conditions as described above, in a completely randomized design with four replicates, and repeated.

Table 2.1. Recommended PPO-inhibitor rates for pre-emergence applications.

Herbicide	Rate (g ai ha^{-1})
Flumioxazin	71
Fomesafen	263
Oxadiazon	3386
Saflufenacil	25
Sulfentrazone	280

Replicates constituted of pots (5 by 5 by 5 cm) filled with a field soil [silty clay loam (sand:silt:clay 16:54:30), 2.2% OM, pH 7.8] available at the KSU Department of Agronomy. Twenty-five viable seeds of KCTR-G2 and of MSS were placed in individual pots and slightly covered with the same soil. All pots were treated with a fungicide (Subdue Maxx, Syngenta Crop Protection ®) at the recommended dose to avoid seedling damping-off. The herbicide treatments were subsequently applied at the recommended rates (Table 2.1) using the same procedures as described in the previous section. Pots were moved back to the greenhouse and irrigated with 3 mm of water for herbicide incorporation, and watered daily until 28 DAT.

Seedling emergence (number) and visual control (percent relative to non-treated control) were recorded at 28 DAT. Aboveground biomass was harvested, dried, and weighed for analyses. Data were subjected to ANOVA using a linear mixed-effect model, with replicates as a

random effect, in R Studio. As no significant differences were found across experimental runs, data were pooled. Subsequently, means of seedling emergence, visual control, and dry weight were compared by Tukey (α=0.05).

2.4. Results and Discussion

2.4.1. Level of resistance to the PPO-inhibitor lactofen

The dose-response experiments indicated that the dose of lactofen required to reduce aboveground biomass growth by 50% was 51.1 g ha^{-1} in KCTR-G1 and 110.3 g ha^{-1} in KCTR-G2, whereas only 3.2 and 8.7 g ha^{-1} were needed to reduce KSS and MSS by 50%, respectively, showing a 5.9- to 15.9-fold resistance in the first and 12.7 to 34.5-fold resistance in the second KCTR generation. Also, GR_{90} was 608 g ha^{-1} in KCTR-G1, indicating that the recommended dose of lactofen (219 g ha^{-1}) was no longer effective in controlling this population.

Table 2.2. GR_{50} and GR_{90} (g ai ha^{-1}) and the level of resistance to lactofen in KCTR-G1 and -G2, and KSS and MSS Palmer amaranth populations.

Biotype	GR50 (±SE)	GR90 (±SE)	R/S (-fold)
KCTR-G1	51.1 (±7.8)	608 (±133.2)	5.9 to 15.9
KCTR-G2	110.3 (±38.9)	1296 (±342.1)	12.7 to 34.5
KSS	3.2 (±1.4)	92.7 (±25.3)	
MSS	8.7 (±2.5)	131.4 (±44.3)	

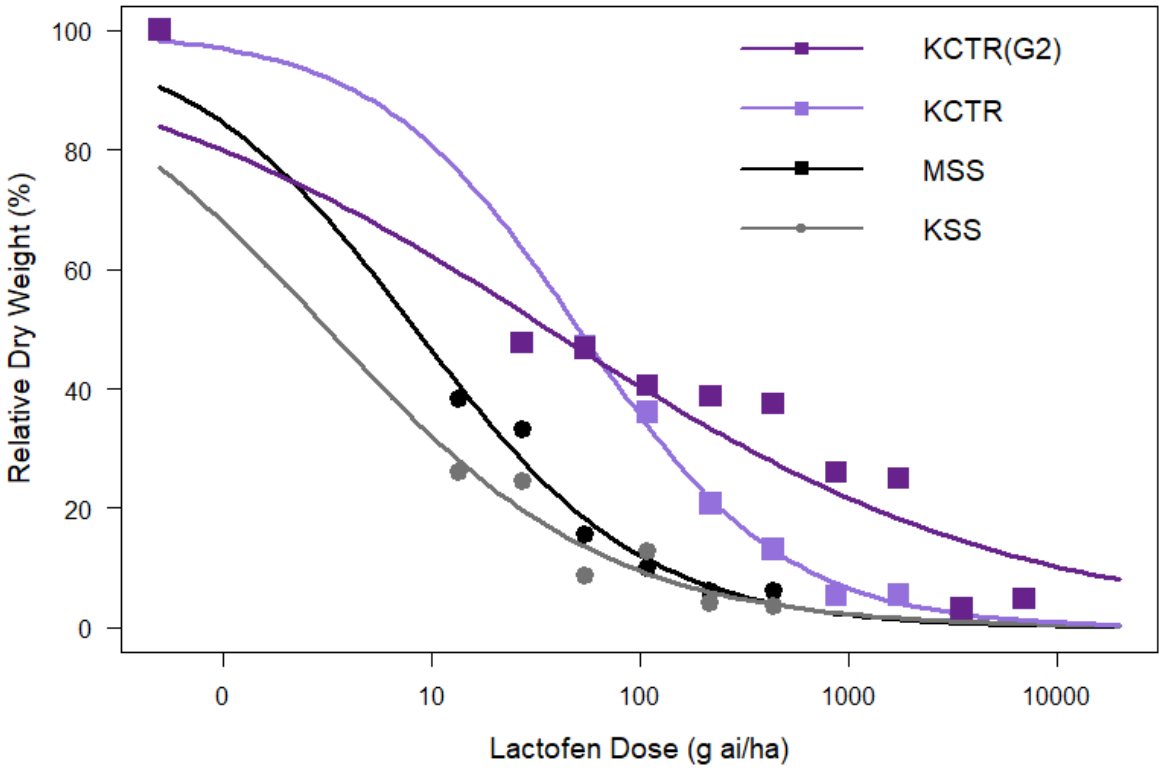

Figure 2.1. Dose-response with lactofen in KCTR-G1, KCTR-G2, KSS and MSS Palmer amaranth populations**.**

The GR_{50} of KCTR-G1 and -G2 were similar to others previously reported. Two Palmer amaranth populations from Arkansas had GR_{50} values of 77.8 and 81.8 g ai ha^{-1} of fomesafen (Salas et al. 2016; Varanasi et al. 2018). A different population from Kansas showed that the dose resulting in 50% reduction of adjusted dry weight was 1,100 g ha^{-1} lactofen (Montgomery et al. 2021). Some factors that can explain the differences between this KCTR population and previous reports with other populations in terms of GR_{50} values are the experimental conditions (i.e., growing conditions and/or plant height at treatment), and natural variability among ecotypes, which include differences in susceptible lines used in each experiment as well because the resistance level is relative (Burgos 2015).

The level of resistance to lactofen in KCTR population increased from 51.1 to 110.3 g ai ha^{-1} of lactofen across consecutive KCTR generations (Table 2). A similar response was documented in a population from Arkansas carrying the ΔG210 mutation, with the GR_{50} to fomesafen increasing from 81.8 to 167.8 and then 265 g ha^{-1} across consecutive generations. The increase in the GR_{50} between KCTR-G1 and -G2 occurred likely due to the elimination of susceptible alleles that were present in the progenies from the field, as resistant individuals were selected (1X survivors) prior to mating to generate KCTR-G2.

2.4.2. *PPO2* sequence and expression analyses

Sequence fragment analysis showed the absence of genetic alterations that cause resistance to PPO-inhibitors in KCTR (Figure 2). Additionally, a few polymorphisms were found but resulted in synonymous sequences (Supplemental material 1). The haplotypes of KCTR were also assessed for the known resistant loci but no heterozygous individuals were identified (not shown). The R128 G/M, ΔG210 and G399A mutations in *PPO2* were found in other studies investigating the occurrence and distribution of *PPO2* mutations across Palmer amaranth populations in the Mid-South US (Salas et al. 2017; Varanasi et al. 2017; Copeland et al. 2018; Noguera et al. 2020; Wu et al. 2020). Those mutations were also found in some populations from Nebraska and Kansas (Oliveira et al. 2020; Montgomery et al. 2021). Generally, the mutations in the *PPO2* mutations were identified in populations from fields with a history of use of PPO-inhibitors. The lack of mutations in KCTR was likely due to lack of selection pressure by PPO-inhibitors, as KCTR was identified in a field where there was continuous sorghum grown for 45+ years without the use of post-emergence application of PPO-inhibitors. The occurrence of gene flow could be possible, as Palmer amaranth is an obligate outcrossing species and as several

soybean fields with Palmer amaranth populations treated with PPO-inhibitors were nearby. However, historically populations from fields treated with PPO-inhibitors have shown the occurrence of *PPO2* mutations, which were not found in KCTR (Figure 2.2).

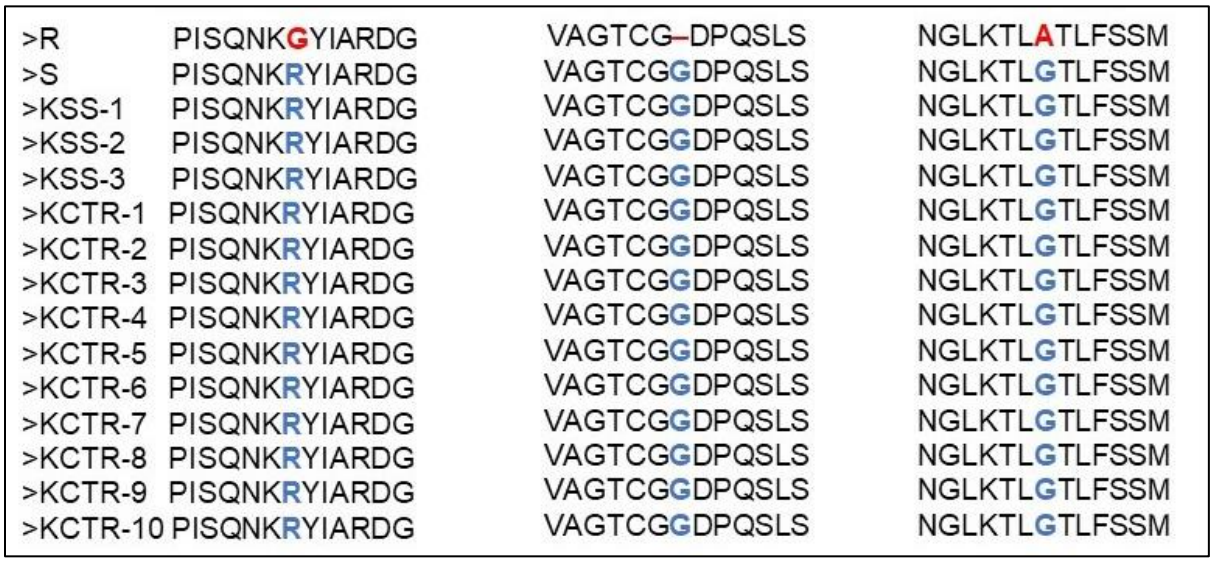

Figure 2.2. *PPO2* amino acid sequence fragment alignment of KCTR, KSS at the positions 128, 210, and 399. Resistant (R) and susceptible (S) were extracted from the GenBank.

Regarding the *PPO2* expression analyses, constitutive expression was similar across populations. The application of lactofen resulted in greater induced expression of the *PPO2* gene as compared to constitutive for KCTR, KSS and MSS individuals tested. The increased expression of the *PPO2* gene after herbicide treatment is likely to have occurred due to a compensation mechanism, in which the plants try to produce more enzymes to replace those inhibited by the herbicide. However, since no differences were found among KCTR, KSS and MSS, differential expression of the *PPO2* gene was not the mechanism of resistance in KCTR Palmer amaranth (Figure 2.3). To date, increased expression of target gene normally inhibited by

herbicides was found to be associated with resistance to glyphosate, ACCase- and ALS-inhibitors, more often happening because of gene duplication (Gaines et al. 2010; LaForest et al. 2017; Yu et al. 2020). Other reports did not find overexpression of *PPO* genes in PPO-inhibitor resistant Palmer amaranth either from Arkansas or Kansas (Varanasi et al. 2018, Montgomery et al. 2021), which was similar to present study in KCTR. The data suggested that resistance to PPO-inhibitors in KCTR is not related to alterations in *PPO2* gene, nor due to increased expression.

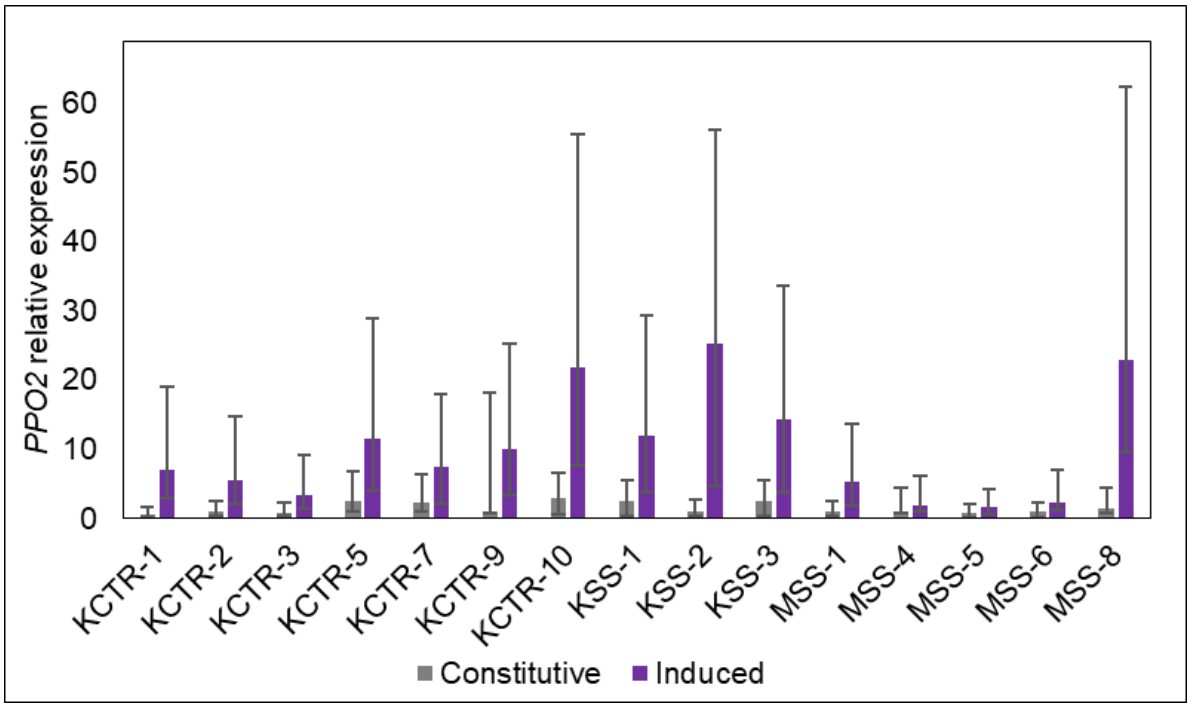

Figure 2.3. *PPO2* constitutive (before treatment, grey) and induced (3 h after treatment, purple) expression levels in KCTR, KSS and MSS Palmer amaranth. Bars represent means of three technical replicates, and experiment was repeated. Reactions without cDNA (no template control) were included in qPCR runs but were not shown.

2.4.3. ^{14}C-fomesafen metabolism

The metabolism of PPO-inhibitors has been documented as a mechanism of resistance in Palmer amaranth (Varanasi et al. 2018, 2019), but the metabolic profile of fomesafen has not been elucidated in weedy plants so far. In this study, ^{14}C-fomesafen parent compound (herbicidally active) was detected in a single peak with a retention time (RT) of about 18.5 minutes (Figure 2.4). Besides parent ^{14}C-fomesafen, three major metabolites were identified in soybean (RT of about 11.2, 12, and 12.9 min) and four major metabolites were identified in Palmer amaranth (RT of about 10.8, 12, 12.8, and 13.7 min). Soybean metabolized fomesafen faster than Palmer amaranth, with >93% of the parent compound being metabolized within 16 HAT, whereas no metabolites were observed in MSS or KCTR Palmer amaranth within 24 hours (Figure 2.4). As time progressed to 48, 71, and 96 HAT, KCTR metabolized more herbicide than MSS (Figure 2.4). Considering mean values of replicates, the interaction between population and HAT was significant ($P = 0.007$), as well as population ($P = 0.0367$) and HAT ($P < 0.001$). Based on the HPLC analysis, the ^{14}C-fomesafen at 24, 48, 72, and 96 HAT in KCTR were 82%, 72%, 41%, and 32%, whereas in MSS was 100%, 90%, 89%, and 92%, respectively (Figure 2.5). MSS showed the presence of metabolites that appeared to have similar RT to those observed in KCTR (Figure 2.4), but those metabolites occurred in much lower concentration and since the amount of parent ^{14}C-fomesafen remained constant over time, those peaks occurred likely due to a natural plant defense type of response (Figure 2.5).

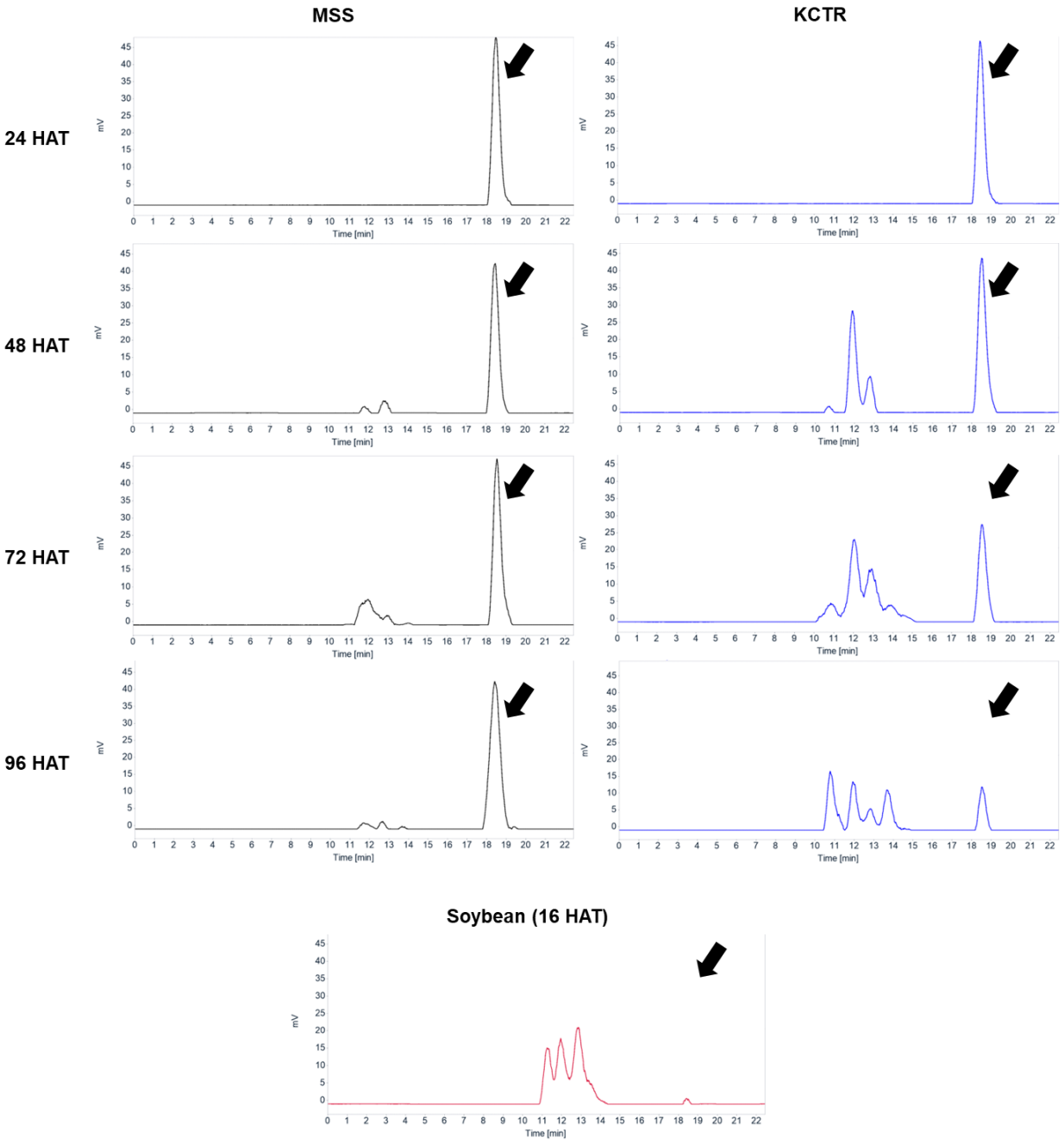

Figure 2.4. Metabolic profile of ^{14}C-fomesafen at 24, 48, 72 and 96 HAT using reverse-phase HPLC in MSS (left series) and KCTR (right series) Palmer amaranth and at 16 HAT in soybean (bottom). Black arrows indicate ^{14}C-fomesafen parent compound at a retention time of 18.5 mins and peaks identified before that retention time (<18.5 mins) indicate ^{14}C-fomesafen metabolites.

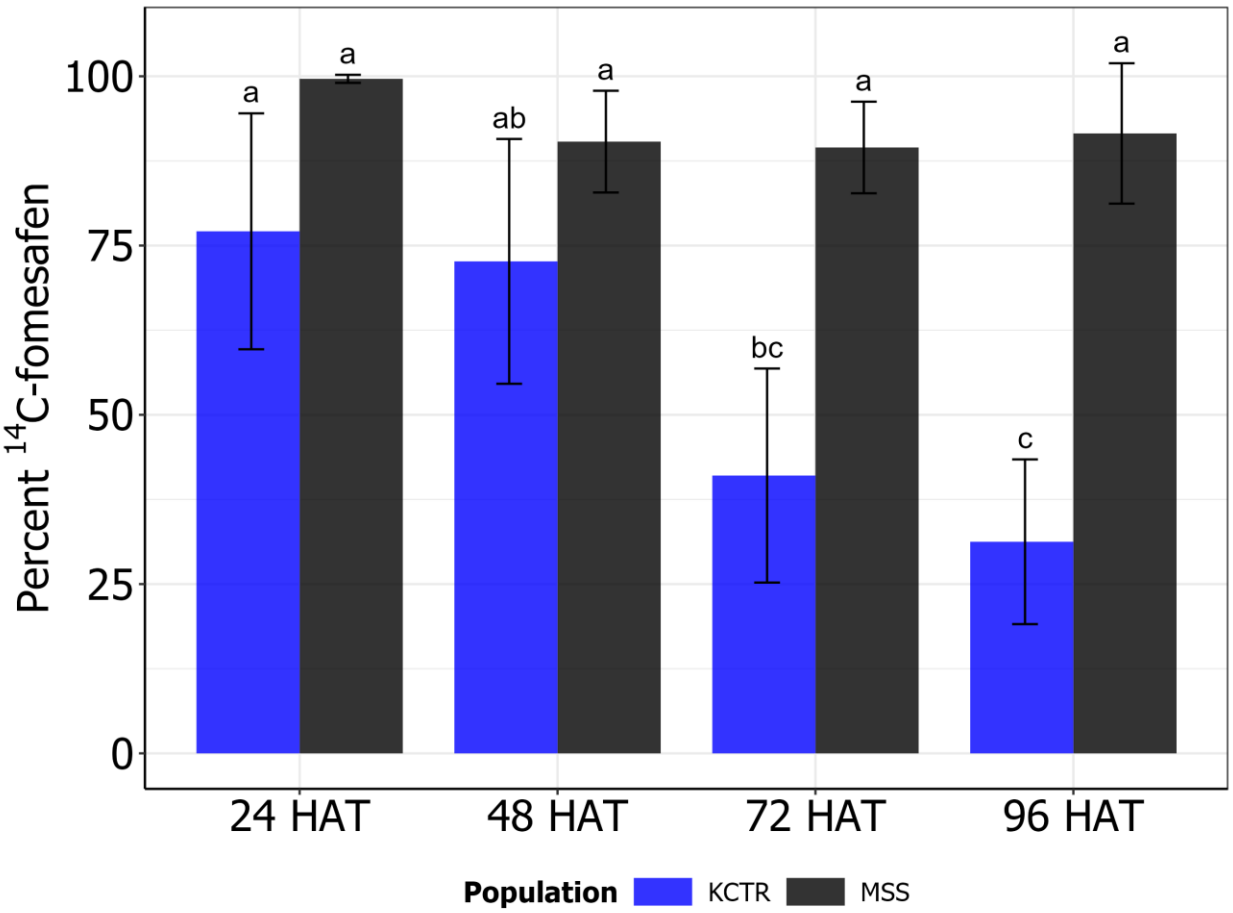

Figure 2.5. Percent 14C-fomesafen in KCTR and MSS Palmer amaranth at 24, 48, 72 and 96 HAT. Bars represent means of at least 5 replicates, and error bars represent standard error of the mean. Different letters across treatments indicate differences by Tukey ($\alpha = 0.05$).

Fomesafen is not the only herbicide that Palmer amaranth can metabolize. Another population from Kansas was found to be resistant to ALS-, PSII-, and HPPD-inhibitors due to metabolism, further confirming the involvement of P450 in conferring resistance to herbicides inhibiting the ALS and HPPD enzymes (Nakka et al. 2017a, b, c), and a population from Nebraska to HPPD-inhibitors (Küpper et al. 2018). Metabolism of 2,4-D was also characterized

in KCTR, and the results of this study suggest that KCTR finding that this population can metabolize ~20 to 30% more 2,4-D herbicide faster than a susceptible population (Shyam et al. 2022). Studies to unravel the metabolic profile of KCTR to other herbicides are currently ongoing.

Enhanced metabolism is also a common resistance mechanism found in *A. tuberculatus*, a related species. Malathion restored sensitivity to 2,4-D, mesotrione, and chlorimuron in a 6-way resistant population from Missouri (Shergill et al. 2018). In a similar way, *A. tuberculatus* populations from Nebraska were found resistant to tembotrione and 2,4-D via P450 activity (Oliveira et al. 2017; Figueiredo et al. 2018).

Herbicide detoxification is found in weed populations with resistance to multiple SOAs, as mechanisms conferring metabolic resistance are often associated with cross-resistance to multiple herbicide SOAs (Busi et al. 2017; Nakka et al. 2017abc; Iwakami et al. 2019; Dimaano et al. 2020; Han et al. 2021). The data suggest that the ability of KCTR to metabolize greater amounts of fomesafen more quickly than MSS is the main mechanism of resistance.

2.4.4. Dose-response with malathion

In the dose-response assay conducted to assess the effect of P450 inhibitor in the level of resistance in KCTR-G2, the addition of malathion decreased the GR_{50} from 110.3 to 4.6 g ha^{-1} of lactofen (Figure 2.6), restoring the sensitivity in KCTR-G2. This highlights the involvement of P450 enzymes in metabolizing these herbicides, and thereby imparting resistance in KCTR.

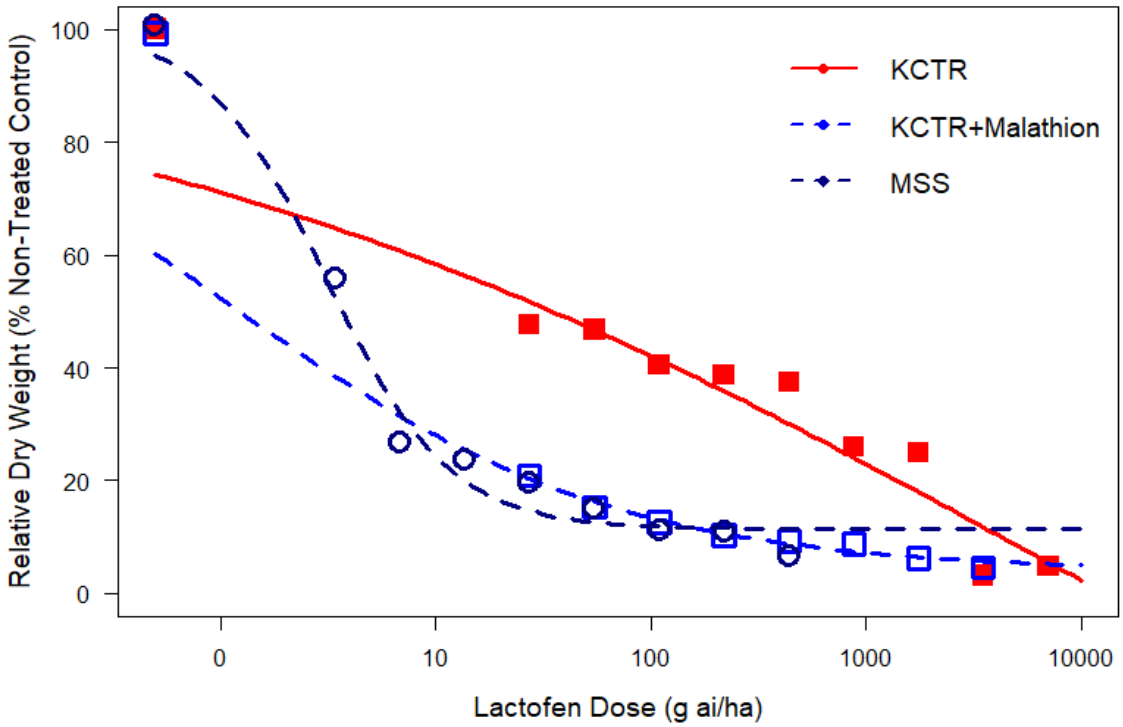

Figure 2.6. Assessment of the effect of the P450-inhibitor, malathion on the level of resistance to lactofen in KCTR Palmer amaranth.

Reversing resistance using P450- and GST-inhibitors is a common method used to characterize the role of the P450 or GST enzymes in metabolic resistance to herbicides in weed species (Ma et al. 2013). Reversal of resistance using P450 inhibitors was found in a PPO-inhibitor-resistant Palmer amaranth population from Arkansas not carrying mutations in *PPO2* gene, with the GR_{50} decreasing from 238 to 83 g ha^{-1} of fomesafen when malathion was added to herbicide treatment (Varanasi et al. 2019). Use of malathion also restored sensitivity to carfentrazone in *A. tuberculatus* lacking *PPO2* mutations (Obenland et al. 2019). P450s are associated with metabolic resistance to other SOAs in Palmer amaranth, including 2,4-D, ALS-,

and HPPD-inhibitors (Nakka et al. 2017a, b, c; Küpper et al. 2018; Shyam et al. 2022,). P450s were also found to metabolize several SOA in *A. tuberculatus*, other dicots, and monocot weeds, such as *Lolium multiflorum* and *Alopecurus myosurioides* (Ma et al. 2013, Giacomini et al. 2020; Oliveira et al. 2020; Strom et al. 2020; Yanniccari et al. 2020; Franco-Ortega et al. 2021).

2.4.5. Palmer amaranth sensitivity to PRE applied PPO-inhibitor herbicides

KCTR was fully controlled with flumioxazin, fomesafen, oxadiazon, saflufenacil, and sulfentrazone when applied as PRE, suggesting sensitivity of this population to soil-applied PPO-inhibitors. No seedling emergence and, consequently, no aboveground biomass were observed in pots treated with any herbicide tested, resulting in 100% control of both KCTR and MSS compared to the non-treated control. Umphres et al. (2017) found efficacy of PRE PPO-inhibitors (fomesafen, flumioxazin, saflufenacil and sulfentrazone) in resistant and susceptible populations, but resistant plants showed 5X less sensitivity to the treatment. In a different study comparing the sensitivity of a resistant population (homozygous with ΔG210 mutation) to fomesafen PRE and POST and flumioxazin (PRE only), data suggested that the dose of fomesafen required to control Palmer amaranth was greater in POST, compared with PRE application, and even greater in late-POST when compared with early-POST (Lillie et al. 2019). KCTR plants survived POST fomesafen, but PRE applications resulted in 100% control, suggesting that this population is less sensitive to POST applications of fomesafen as compared to PRE, similar to found by Lillie et al. (2019). However, the physiological basis of fomesafen activity, and of the other PPO-inhibitors, PRE *versus* POST applications is not yet elucidated. For management purposes, KCTR could be controlled with PRE applications of PPO-inhibitors, but it is important to diversify weed control practices to reduce selection pressure imposed by

herbicides of a single SOA. This should be combined with the use of multiple effective herbicide SOAs and integrated weed management practices for more and sustainable control.

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2.6. References

Anonymous 2015, Valent ® Cobra herbicide. Accessed on 10/12/2022 and available in: https://s3-us-west-1.amazonaws.com/agrian-cg-fs1-production/pdfs/Cobrar_Herbicide_Label1y.pdf

Aulakh J, Chahal P, Kumar V, Price A, Guillard K. (2021). Multiple herbicide-resistant Palmer amaranth (*Amaranthus palmeri*) in Connecticut: Confirmation and response to POST herbicides. *Weed Technology*, 35(3):457-463.

Bagavathiannan MV, Norsworthy JK. (2016). Multiple-herbicide resistance is widespread in roadside Palmer amaranth populations. *PLoS One*, 11(4):e0148748.

Bates D, Mächler M, Bolker B, Walker S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67(1):1-48.

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 Science*, 51:37-43.

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

Burgos NR. (2015). Whole-plant and seed bioassays for resistance confirmation. *Weed Science*, 63:152-165.

Busi R, Gaines TA, Powles S. (2017). Phorate can reverse P450 metabolism-based herbicide resistance in *Lolium rigidum*. *Pest Management Science*, 73:410-417.

Carvalho SJP, Goncalves Netto A, Nicolai M, Cavenaghi A, Lopez-Ovejero R, Christoffoleti P. (2015). Detection of glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) in agricultural areas of Mato Grosso, Brazil. *Planta Daninha*, 33:579-586.

Carvalho-Moore P, Norsworthy J, González-Torralva F, Hwang J, Patel J, Barber L, Butts TR, McElroy JS. (2022). Unraveling the mechanism of resistance in a glufosinate-resistant Palmer amaranth (*Amaranthus palmeri*) accession. *Weed Science*, 70(4):370-379.

Chahal PS, Jugulam M, Jhala AJ. (2019). Mechanism of atrazine resistance in atrazine- and HPPD-inhibitor-resistant Palmer amaranth (*Amaranthus palmeri* S. Watson) from Nebraska. *Canadian Journal of Plant Science*, 99:815-823.

Copeland J, Giacomini D, Tranel P, Montgomery G, Steckel L. (2018). Distribution of PPX2 mutations conferring PPO-inhibitor resistance in Palmer amaranth populations of Tennessee. *Weed Technology*, 32(5):592-596.

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 Science*, 54(4):620-626.

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 Molecular Biology*, 102(4-5):403-416.

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 Management Science*, 74(10):2356-2362.

Franco-Ortega S, Goldberg-Cavalleri A, Walker A, Brazier-Hicks M, Onkokesung N, Edwards R. (2021). Non-target site herbicide resistance is conferred by two distinct mechanisms in black-grass (*Alopecurus myosuroides*). *Frontiers in Plant Science*, 12:636652.

Gaines TA, Duke SO, Morran S, Rigon CAG, Tranel PJ, Küpper A, Dayan FE. (2020). Mechanisms of evolved herbicide resistance. *Journal of Biological Chemistry*, 295(30):10307-10330.

Gaines TA, Zhang W, Wang D, Bukun B, Chisolm 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*. *PNAS*, 107(3):1029-1034.

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 Management Science*, 73(8):1559-1563.

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 Biology and Evolution*, 12(12):2267-2278.

Gonçalves Netto A, Nicolai M, Carvalho SJP, Borgato EA, Christoffoleti PJ. (2016). Multiple resistance of *Amaranthus palmeri* to ALS and EPSPS inhibiting herbicides in the state of Mato Grosso, Brazil. *Planta Daninha*, 34:581-587.

Han H, Yu Q, Beffa R, González S, Maiwald F, Wang J, Powles SB. (2020). Cytochrome P450 CYP81A10v7 in *Lolium rigidum* confers metabolic resistance to herbicides across at least five modes of action. *Plant Journal*, 105(1):79-92.

Heap I (2020). The international herbicide-resistant weed database. (2022). Online. Sunday, September 25, 2022. Available www.weedscience.org

Iwakami S, Kamidate Y, Yamaguchi T, Ishizaka M, Endo M, Suda H, Nagai K, Sunohara Y, Toki S, Uchino A, Tominaga T, Matsumoto H. (2019) CYP81A P450s are involved in concomitant cross-resistance to acetolactate synthase and acetyl-CoA carboxylase herbicides in *Echinochloa phyllopogon*. *New Phytologist*, 221:2112-2122.

Kanatas P, Tataridas A, Dellaportas V, Travlos I. (2021). First report of *Amaranthus palmeri* S. Wats. in cotton, maize and sorghum in Greece and problems with its management. *Agronomy*, 11(9):1721.

Keeley PE, Carter CH, Thullen RJ. (1987). Influence of planting date on growth of Palmer amaranth (*Amaranthus palmeri*). *Weed Science*, 35(2):199-204.

Knezevic SZ, Streibig JC, Ritz C. (2007). Utilizing R software package for dose-response studies: the concept and data analysis. *Weed Technology*, 21(3):840-848.

Kohrt JR, Sprague CL. (2017). Response of a multiple-resistant Palmer amaranth (*Amaranthus palmeri*) population to four HPPD-inhibiting herbicides applied alone and with atrazine. *Weed Science*, 65(4):534-45.

Koo DH, 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*. PNAS, 115(13):3332-3337.

Küpper A, Borgato EA, Patterson EL, Goncalves Netto A, Nicolai M, Carvalho SJP, Nissen SJ, Gaines TA, Christoffoleti PJ. (2017). Multiple resistance to glyphosate and acetolactate synthase inhibitors in Palmer amaranth (*Amaranthus palmeri*) identified in Brazil. *Weed Science*, 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 Management Science*. 74(10):2325-2334.

Laforest M, Soufiane B, Simard MJ, Obeid K, Page E, Nurse RE. (2017), Acetyl-CoA carboxylase overexpression in herbicide-resistant large crabgrass (*Digitaria sanguinalis*). *Pest. Management Science*, 73:2227-2235.

Lancaster SH, Fick WH, Currie RS, Kumar V. (2022). Chemical weed control for field crops, pastures, rangelands and noncropland. Kansas State University Research and Extension. Report Progress 1169.

Livak KJ, Schmittgen TD. (2001). Analysis of relative gene expression data using real time quantitative PCR and the 2(-ΔΔC(t)) method. *Methods*, 25(4):402-408.

Ma R, Kaundun SS, Tranel PJ, Riggins CW, McGinnes 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 Physiology*, 163:363-377.

Massinga RA, Currie RS, Horak MJ, Boyer Jr J. (2001). Interference of Palmer amaranth in corn. *Weed Science*, 49(2):202-208.

Mausbach J, Irmak S, Sarangi D, Lindquist J, Jhala AJ. (2021). Control of acetolactate synthase inhibitor/glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) in isoxaflutole/glufosinate/glyphosate-resistant soybean. *Weed Technology*, 35(5):779-785.

Montgomery J, Giacomini D, Tranel PJ. (2021). Molecular confirmation of resistance to PPO inhibitors in *Amaranthus tuberculatus* and *Amaranthus palmeri*, and isolation of the G399A PPO2 substitution in *A. palmeri*. *Weed Technology*, 35(1):99-105.

Moore JW, Murray DS, Westerman RB. (2004). Palmer amaranth (*Amaranthus palmeri*) effects on the harvest and yield of grain sorghum (*Sorghum bicolor*). *Weed Technology*, 18(1):23-29.

Nakka S, Godar AS, Thompson CR, Peteron DE, Juglam M. (2017a). Rapid detoxification via glutathione *s*-transferase (GST)-conjugation confers high level of atrazine resistance in Palmer amaranth (*Amaranthus palmeri*). *Pest Management Science*, 73:2236-2243.

Nakka S, Godar AS, Wani PS, Thompson CR, Peterson DE, Roelofs J, et al. (2017b). Physiological and molecular characterization of hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor resistance in Palmer amaranth (*Amaranthus palmeri* S. Wats.). *Frontiers in Plant Science*, 8:555.

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 Science*, 65:681-689.

Nandula V, Reddy K, Koger C, Poston D, Rimando A, Duke S, Bond J, Ribeiro D. (2012). Multiple resistance to glyphosate and pyrithiobac in Palmer amaranth (*Amaranthus palmeri*) from Mississippi and response to flumiclorac. *Weed Science*, 60(2):179-188.

Noguera MM, Rangani G, Heiser J, Bararpour T, Steckel LE, Betz M, Porri A, Lerchl J, Zimmermann S, Nichols RL, Roma-Burgos N. (2021), Functional PPO2 mutations: co-occurrence in one plant or the same ppo2 allele of herbicide-resistant *Amaranthus palmeri* in the US mid-south. *Pest Management Science*, 77:1001-1012.

Norsworthy JK, Griffith G, Griffin T, Bagavathiannan M, Gbur E. (2014). In-field movement of glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) and its impact on cotton lint yield: evidence supporting a zero-threshold strategy. *Weed Science*, 62(2):237-249.

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 Science*, 60(sp1):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(4):e0215431.

Oliveira MC, Giacomini D, Arsenijevic N, Vieira G, Tranel PJ, Werle R. (2021). Distribution and validation of genotypic and phenotypic glyphosate and PPO-inhibitor resistance in Palmer amaranth (*Amaranthus palmeri*) from southwestern Nebraska. *Weed Technology*, 35(1):65-76.

Oliveira MC, Gaines TA, Dayan FE, Patterson EL, Jhala AJ, Knezevic SZ. (2017). Reversing resistance to tembotrione in an *Amaranthus tuberculatus* (var. *rudis*) population from Nebraska, USA with cytochrome P450 inhibitors. *Pest Management Science*, 74(10):2296-2305.

Pandian BA, Sathishraj R, Djanaguiraman M, Prasad PVV, Jugulam M. (2020). Role of cytochrome p450 enzymes in plant stress response. *Antioxidants*, 9(5):454.

Porri A, Noguera MM, Betz M, Sälinger D, Brändle F, Bowe SJ, Lerchl J, Meyer L, Knapp M, Roma-Burgos N. (2022). Can double PPO mutations exist in the same allele and are such mutants functional? *Pest Management Science*, 78(6):2258-2264.

Priess GL, Norsworthy JK, Godara N, Mauromoustakos A, Butt TR, Roberts TL, Barber T. (2022). Confirmation of glufosinate-resistant Palmer amaranth and response to other herbicides. *Weed Technology*, 36(3):368-372.

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. *Frontiers in Plant Science*, 10:568.

RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/.

Ritz C, Baty F, Streibig JC, Gerhard D. (2015). Dose-response analysis using R. *PLOS ONE*, 10(12):e0146021.

Rowland M, Murray D, Verhalen L. (1999). Full-season Palmer amaranth (*Amaranthus palmeri*) interference with cotton (*Gossypium hirsutum*). *Weed Science*, 47(3):305-309.

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 Management Science*, 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 Science*, 65(6):718-731.

Sauer JD. (1957). Recent migration and evolution of the dioecious amaranths. *Evolution*, 11:11-31.

Schwartz-Lazaro LM, Norsworthy JK, Scott RC, Barber T. (2017). Resistance of two Arkansas Palmer amaranth populations to multiple herbicide sites of action. *Crop Protection*, 96:158-163.

Shyam C, Peterson D, Jugulam M. (2022). Resistance to 2,4-D in Palmer amaranth (*Amaranthus palmeri*) from Kansas is mediated by enhanced metabolism. *Weed Science*, 70(4):390-400.

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

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

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 Management Science*, 75:1131-1139.

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 Science*, 60:366-373.

Sosnoskie LM, Kichler JM, Wallace RD, Culpepper AS. (2020). Multiple resistance in Palmer amaranth to glyphosate and pyrithiobac confirmed in Georgia. *Weed Science*, 59(3):321-325.

Sprague CL, Stoller EW, Wax LM, Horack MJ. (1997). Palmer amaranth (*Amaranthus palmeri*) and common waterhemp (*Amaranthus rudis*) resistance to selected ALS-inhibiting herbicides. *Weed Science*, 45:192-197.

Torra J, Royo-Esnal A, Romano Y, Osuna MD, León RG, Recasens J. (2020). *Amaranthus palmeri* a new invasive weed in Spain with herbicide resistant biotypes. *Agronomy*, 10:993.

Umphres AM, Steckel LE, Mueller TC. (2018). Control of protoporphyrinogen oxidase inhibiting herbicide resistant and susceptible Palmer amaranth (*Amaranthus palmeri*) with soil-applied protoporphyrinogen oxidase-inhibiting herbicides. *Weed Technology*, 32(1):95-100.

Varanasi V, Brabham C, Korres N, Norsworthy JK. (2019). Nontarget site resistance in Palmer amaranth [*Amaranthus palmeri* (S.) Wats.] confers cross-resistance to protoporphyrinogen oxidase-inhibiting herbicides. *Weed Technology*, 33(2):349-354.

Varanasi V, Brabham C, Norsworthy JK. (2018a). Confirmation and characterization of non–target site resistance to fomesafen in Palmer amaranth (*Amaranthus palmeri*). *Weed Science*, 66(6):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 Science*, 66(2):149-158.

Wu C, Goldsmith M, Pawlak J, Feng P, Smith S, Navarro S, Perez-Jones A. (2020). Differences in efficacy, resistance mechanism and target protein interaction between two PPO inhibitors in Palmer amaranth (*Amaranthus palmeri*). *Weed Science*, 68(2):105-115.

Wise A, Grey T, Prostko E, Vencill W, Webster T. (2009). Establishing the geographical distribution and level of acetolactate synthase resistance of Palmer amaranth (*Amaranthus palmeri*) accessions in Georgia. *Weed Technology*, 23(2):214-220.

Yanniccari M, Gigón R and Larsen A. (2020). Cytochrome P450 herbicide metabolism as the main mechanism of cross-resistance to ACCase- and ALS-inhibitors in *Lolium* spp. populations from Argentina: a molecular approach in characterization and detection. *Frontiers in Plant Science*, 11:600301.

Yu J, McCullough PE, McElroy SJ, Jespersen D, Shilling DG. (2020). Gene expression and target-site mutations are associated with resistance to ALS inhibitors in annual sedge (*Cyperus compressus*) biotypes from Georgia. *Weed Science*, 68:460-466.

Chapter 3 - Comparative emergence, phenology, and development of female and male Palmer amaranth

3.1. Abstract

Palmer amaranth (*Amaranthus palmeri*) is a summer annual, highly prolific dioecious C4 weed with a fast growth rate. Differences in female and male life history were investigated to explore gender as an opportunity for improving control of this species. We conducted replicated studies in a controlled environment to compare the emergence patterns of three populations: KS-1, KS-2, and MS-1. A separate phenology study compared growth, development, and reproduction in female and male plants using the MS-1 population. Development was characterized using an adapted BBCH scale for Palmer amaranth. Growth characterization included female and male height, inflorescence length, GDD to visible inflorescence, and GDD to anthesis (flowers open) across genders. Data indicated that different populations required different numbers of GDD for 90% emergence, with females requiring 150 and 160 GDD in KS-1 and MS-1, respectively, whereas males required 144 and 190 GDD, respectively. In contrast, males in KS-2 reached 90% emergence with 150 GDD, while females required 190 GDD, so anticipated emergence of female seedlings was not consistent across populations. A linear regression analysis from the phenology study using a BBCH scale indicated that female and male development overlap during their life cycle. However, using an adapted BBCH scale considering unique stages of Palmer amaranth phenology, female and male development differed after reproductive stages, suggesting that it is important to adapt the BBCH scale to each species' life cycle. Analysis of plant height indicated that female and male Palmer amaranth continued to grow after the flowering stage was initiated, suggesting an indeterminate growth habit, with the female plants

being taller than males at senescence. In the first study, males flowered (305 GDD) ahead of females (381 GDD), whereas in the second study, both genders flowered at 414 GDD. Anthesis occurred with 566 GDD in males and 599 GDD in females in the first study, and 566 GDD with females and 626 GDD in males in the second experimental run, showing inconsistencies across runs. Indeterminate vegetative (height) and inflorescence growth could be a characteristic that favors fertility in Palmer amaranth. Inconsistencies across genders among populations make it difficult to explore gender for controlling this species. Management decisions could incorporate knowledge of reproductive differences of female *versus* male to proactively reduce seed production. Future research will investigate population dynamics of Palmer amaranth considering gender manipulation.

Keywords: *Amaranthus palmeri* S. Watson, control, growing degree days, prediction, reproduction

3.2. Introduction

Palmer amaranth (*Amaranthus palmeri* S. Watson) is classified as the most troublesome weed in the United States (Van Wychen 2020). It is currently resistant to nine herbicide sites of action (Heap 2022), posing a threat to multiple cropping systems across the US. The evolution of resistance to multiple herbicides is a key aspect that make Palmer amaranth such a troublesome weed. Nonetheless, many biological characteristics also enable the aggressive nature of this weed species.

One of the important biological characteristics of Palmer amaranth is that it has a wide window of emergence. Germination can occur when temperatures reach a minimum of 10 C (Steckel et al. 2004). However, as a summer annual species (Sauer 1957), higher temperatures can result in greater germination rates. Steckel et al. (2004) observed germination rates of 4, 36, 50, 56, 61 and 71% at 10, 15, 20, 25, 30 and 35 C, respectively, and that alternating temperature regimes increased germination rates. In field scenarios, Palmer amaranth can display 10% emergence with 125 growing degree days (GDD) to 90% emergence with 445 GDD, according to a study conducted in the Central Plains (Liu et al. 2021).

Palmer amaranth is a eudicot C4 plant (Wang et al. 1992), with a more efficient photosynthetic pathway compared to CAM and C3 plants and displays fast growth rate and high biomass accumulation. Height is a characteristic that facilitates Palmer amaranth to outcompete crops because of more sunlight available when growing above the crop canopy. In a study conducted in California, plants were taller when they emerged on March 1 (257 cm) compared to emerged on August 1 (136 cm) (Keeley et al. 1987). Spaunhorst et al. (2018) compared growth of multiple populations emerging early-, mid-, and late-season and found similar results,

suggesting a positive response of increments in height of Palmer amaranth growing under longer days.

High biomass accumulation is another characteristic that demonstrates the ability of Palmer amaranth to utilize environmental resources. Dry weight of populations from distinct geographic regions varied from 88 to 258 g plant^{-1} at senescence, with a population from Indiana producing 126 g plant^{-1}, and a population from Mississippi, 252 g plant^{-1} (Spaunhorst et al. 2018), suggesting differential growth rates among ecotypes.

Palmer amaranth can drastically decrease crop yield, as it is a strong competitor. Massinga et al. (2001) found that flushes emerging with the crop caused 80% yield loss, and when they emerged when corn was in V4-V7 reduced yield by 35% at 10.5 plants m^{-2}. In soybean, densities ranging from 0.33 to 3.33 plants m^{-2} increased yield loss from 17% to 64% (Klingaman and Oliver 1994). This suggests that keeping the field clean early in the season is critical for crop establishment and performance, but also suggests that even at low densities Palmer amaranth can cause significant losses.

Another characteristic that favors Palmer amaranth spread and establishment is high seed production. Keeley et al. (1987) found that, in the absence of competition, one single female plant can produce from 200,000 to 600,000 seeds when emerging from March to June, and 115,000 to 80,000 seeds if emerging from July through September. This suggests that Palmer amaranth grows more under longer days, indicating that period of emergence and, consequently, photoperiod play an important role on the development and reproduction of this species.

Crop-weed competition can affect weed performance and reproduction, and crop rotation has been explored as a cultural control method. Burke et al. (2007) found that Palmer amaranth can produce up to 1.2 billion seeds ha^{-1} at a density of 5.2 plants m^{-1} of peanut crop row.

Mahoney et al. (2021) observed that average seed production was about 550,000 seeds plant^{-1} in competition with cotton, 445,000 seeds plant^{-1} with peanut, 175,000 seeds plant^{-1} with soybean, and 50,000 seeds plant^{-1} with corn, suggesting that the density and architecture of crop canopy can suppress weed growth and reproduction, but Palmer amaranth is still able to produce a high number of seeds.

Adaptation in weeds can be observed not only as response of selection pressure imposed by herbicides, but also growth and morphological traits can be selected in response to crop-weed competition and management practices. The impacts of herbicide-resistance traits have been extensively investigated in weed species. In Palmer amaranth, glyphosate resistance was not found to be associated with changes in growth aspects or fitness costs, or with seed production and longevity (Sosnoskie et al. 2013; Giacomini et al. 2014; Vila-Aiub et al. 2014; Webster and Grey 2015; Bravo et al. 2017). A study performed with Palmer amaranth populations from Florida and Georgia found that fresh and dry weight, height, and days to flowering were different across populations with different rotation history and crop canopy structure (Bravo et al. 2017). In that study, specifically, gender and herbicide were not found to be associated with adaptation of those growth and morphological traits.

Even being a highly prolific species, Palmer amaranth seeds do not stay viable in the soil seedbank for long periods. Korres et al (2018) found that seed viability dropped to 80 to 85% after 12 months, 5% to 10% after 24 months and about 5% or less after 36 months. As a sexually propagated annual species, Palmer amaranth is reproduced strictly by seed, therefore reducing soil seedbank replenishment could be an effective strategy to significantly decrease the infestation of this species in the long-term.

Recently, the critical period for seed control, that is the period of the growing season during which weed control can minimize weed seed production by targeting the adoption of practices during phenological stages, would be crucial for seed development (Gueddes and Davis 2021). Because Palmer amaranth is a dioecious species, differences in phenology between female and male stages of development could potentially be incorporated in management decisions aiming to reduce seed production. The genetic basis of dioecy in Palmer amaranth is caused by the presence of a male-specific-Y chromosomal region with 1.3 Mb and 121 predicted gene models, with the males to be the heterogametic gender and likely to display female inflorescence in cases where the male-specific-Y chromosomal region is not fully expressed (Montgomery et al. 2019, 2021).

For this purpose, phenology models based on thermal requirements to progress through stages of the life cycle could possibly determine the optimum time for control of Palmer amaranth. Therefore, the objectives of this research were to (1) study the emergence profile of female and male seedlings, (2) identify differences in phenological stages, and (3) compare growth aspects of female and male Palmer amaranth plants in a greenhouse setting.

3.3. Materials and methods

3.3.1. Plant materials

Three Palmer amaranth populations were used in these studies, KS-1, KS-2, and MS-1, to verify if results were reproducible and to understand possible differences among populations. KS-1 and MS-1 are originally from Kansas and Mississippi, respectively, and are susceptible to herbicides, whereas KS-2 is also from Kansas but is resistant to multiple herbicide sites of action (Shyam et al. 2021).

3.3.2. Emergence of female and male Palmer amaranth

A study was performed to determine if female and male individuals require different thermal units for emergence, which could provide a window for management opportunity to shift population ratios to more male-to-female ratios, and, therefore, reduce seed production. KS-1, KS-2 and MS-1 seeds were individually sown in plastic pots (6 × 6 × 6.5 cm) filled with potting soil (Pro-Mix® premium potting mix, Premier Tech Home and Garden Inc., Ontario, CA) at the soil surface, slightly covered, and placed in a growth chamber at 30 C constant with 12/12 hours day/night (600 µmol m^{-2} s^{-1} photon lux density at pot surface) for three weeks. Thirty degrees C temperature was chosen based on high germination rates for Palmer amaranth (Steckel et al. 2004) and 12 h day length was used considering the possibility that neither females or males were favored by long or short days. Pots were watered on a daily basis, so water was not a limiting factor. The experiment was performed in randomized complete block design with 85 replicates from each population per run, and repeated six times, with each block constituted of a tray containing 32 replicates of a single population. Ultimately, the total number of seedlings that provided data for analysis were 119 for KS-1, 327 for KS-2 and 200 for MS-1. That variation occurred because of different germination rates among populations.

The start date for each experimental run was recorded and seedling emergence was documented when the fully expanded cotyledon stage was observed in each replicate on a daily basis for three weeks. Plants were then moved to a greenhouse (32/23 C d/n and 16 h day length) until flowering when gender was documented (Table 3.1). The growing degree days (GDD) required for emergence was calculated with a base temperature (T_b) of 16.6 C for Palmer amaranth (Steinmaus et al. 2000). Cumulative emergence (percent) for female and male KS-1,

KS-2, and MS-1 populations were analyzed as a response to GDD, and comparisons between genders and populations were made based on GDD required to reach 90% emergence.

Table 3.1. Total number of females, males, total plant number, and male-to-female ratios of KS-1, KS-2, and MS-1 Palmer amaranth populations in the emergence study.

Population	Male	Female	Total	Male to female ratio
KS-1	73	46	119	1.59:1
KS-2	172	155	327	1.1:1
MS-1	121	79	200	1.5:1

3.3.3. Phenology of female and male Palmer amaranth

A greenhouse study was performed to investigate differences in the phenology of female and male plants that could be incorporated into management decisions. Pots (1 L) were filled with potting soil and kept in a greenhouse with the same conditions as described above. Three seeds of MS-1 were placed in the center of each pot and slightly covered, and pots were spaced 15 cm radius apart to allow plants to grow. Seedlings were thinned when needed, leaving the first one to emerge in each replicate. The experiment was performed under a completely randomized design, with 108 replicates, and repeated. The first experimental run was completed with 101 replicates (57 males and 44 females) and the second with 97 replicates (49 males and 48 females) due to death of or lack of flowering in a few individuals. Also, the first experiment started on 11/24/2021 and was completed on 03/11/2022, and the second experiment ran from 02/11/2022 through 05/11/2022.

The phenological stages for each female and male Palmer amaranth plant was documented every other day using a BBCH (Biologische Bundesanstalt, Bundessortenamt and

CHemical industry) scale (Table 1) (Hess et al. 1997). Plant height (cm) (measured from the soil surface to the base of the inflorescence) and the length of the inflorescence (cm) (measured from the base to the tip) of the main stem were taken in a similar fashion to assess more growth aspects of this species considering the possibility there could be differences across genders. Gender was recorded when flowers became visible.

The BBCH scale had a few limitations and needed to be modified for this study. Because Palmer amaranth is an annual and dioecious species, some stages [i.e., stage 4: vegetative propagation, stage 7: development of fruit (in males)] from the BBCH scale were absent in the species or in one of the genders. Additionally, the formation of side shoots (branching, stage 2) is a facultative phenomenon not always observed in individual plants. Also, after statistical analyses the mean and variance measurements will account for comparisons and data interpretation; because mean values could result in stages absent in this species or in one of the genders (i.e., mean of 5 and 3 is 4, which refers to vegetative propagation, which is absent in Palmer amaranth), resulting in misleading conclusions. Facing this issue, it is proposed herein the adoption of Dille's rule, which states: when studying phenology in weed (and crop) species, the BBCH scale should be adapted to your targeted species' life cycle.

Table 3.2. BBCH scale (left) (Hess et al. 1997) and adapted BBCH scale for female and male Palmer amaranth (right), following Dille's rule for adaptation of the BBCH scale for the species of interest.

Stage	Description
0	Germination
1	Leaf development
2	Formation of side shoots
3	Stem elongation
4	Vegetative propagation
5	Inflorescence emergence
6	Flowering
7	Development of fruit
8	Maturity of fruit and seed
9	Senescence

Female	Male	Palmer amaranth stages
0	0	Germination
1	1	Emergence
2	2	Leaf development and stem elongation
3	3	Inflorescence development
4	4	Flowering (anthesis)
5	-	Development of fruit
6	-	Maturity of fruit and seed
7	5	Senescence

For more complete description of what was observed at each stage in these experiments: stage 0 - germination was documented from the beginning of the experiments until cotyledons emerged, 1 - emergence was documented when the cotyledons were fully expanded; 2 - leaf development and stem elongation were recorded as progressed, 3 - inflorescence development occurs when reproductive organs were developing, 4 - flowering (anthesis) when pistils were exposed (female) or anthers (male) were releasing pollen, stage 5 - in females, the development of soft fruit when seeds (green, white or brown) were observed and in males, plants were senescing, 6 - maturity of fruit when black seeds (hard) were present, and 7 - female plants were senescing when plant tissue became brown. Branching was observed in all replicates in the first experimental run, along with flowering, but as it was not a dominant stage, it was not included in

the adapted BBCH scale for female and male Palmer amaranth; branching was not observed in any plant in the second experiment.

For data analyses, phenological stages using the BBCH scale and the adapted BBCH scale for female and male Palmer amaranth were subjected to ANOVA type 3 ($\alpha = 0.05$) using a linear mixed effect model (*lmer*) available in the *lme4* package in R Studio. Replicates were treated as random effects, and variable responses with other explanatory variables (GDD to reach each stage, gender and experimental run), as well as two- and three-way interactions were treated as fixed effects; Experimental runs were treated as fixed effects because plants reached senescence with 1200 GDD in the first run, whereas in the second, senescence occurred with 950 GDD.

Linear regression was used to describe BBCH and adapted BBCH for female and male Palmer amaranth as a response to GDD to better describe progress through phenological stages as thermal units were accumulated, and to further demonstrate the importance of adopting Dille's rule. Linear regressions were fit with the *lm* function in R Studio, with BBCH and adapted BBCH as response of GDD for both genders.

Female and male plant height (cm) and inflorescence length (cm) were also described as a response to GDD to assess more information about female and male Palmer amaranth growth and reproductive habits. The average GDD to reach each BBCH female and male stages were compared using the Mann-Whitney U test ($\alpha = 0.05$), a test for mean comparison that does not make assumptions of normality of residuals and homoscedasticity of variances. Also, plant height (cm) and inflorescence length (cm) at senescence were compared using Fisher's protected LSD test ($\alpha = 0.05$) to identify differences between genders.

3.4. Results and discussion

3.4.1. Emergence of female and male Palmer amaranth

Overall, KS-1, KS-2, and MS-1 reached 10% emergence within 30 GDD, but no differences between female and male were observed for that emergence index (Figure 3.1). To reach 50% emergence, KS-1 and MS-1 females needed 80 GDD, whereas males needed 100 GDD; however, both female and male needed 80 GDD for 50% emergence in KS-2 (Figure 3.1). At least 90% emergence was observed with 150 GDD, however populations and genders showed differences in GDD required to reach 90% emergence (Figure 3.1). In KS-1, 90% emergence was observed with 150 GDD, and no differences were found between female and male Palmer amaranth. In KS-2, males required 150 GDD, whereas females needed 190 GDD. For MS-1, females needed 160 GDD to display 90% emergence, whereas males needed > 190 GDD to reach the same emergence index (Figure 3.1). In a controlled environment study, Palmer amaranth emergence of three different populations, California, Kansas, and Texas, were not different between female and male individuals, but differed by population in terms of how fast emergence occurred (Mesgaran et al. 2021). Emergence of Palmer amaranth was found to reach 10%, 50% and 90% at 77, 278 and 593 GDD in field conditions in North Carolina (Reinhardt Piskackova et al. 2020), which was different than current findings, suggesting differences due to population, or environment, or population and environment, but no distinction was between female and male seedling emergence, similar to this study.

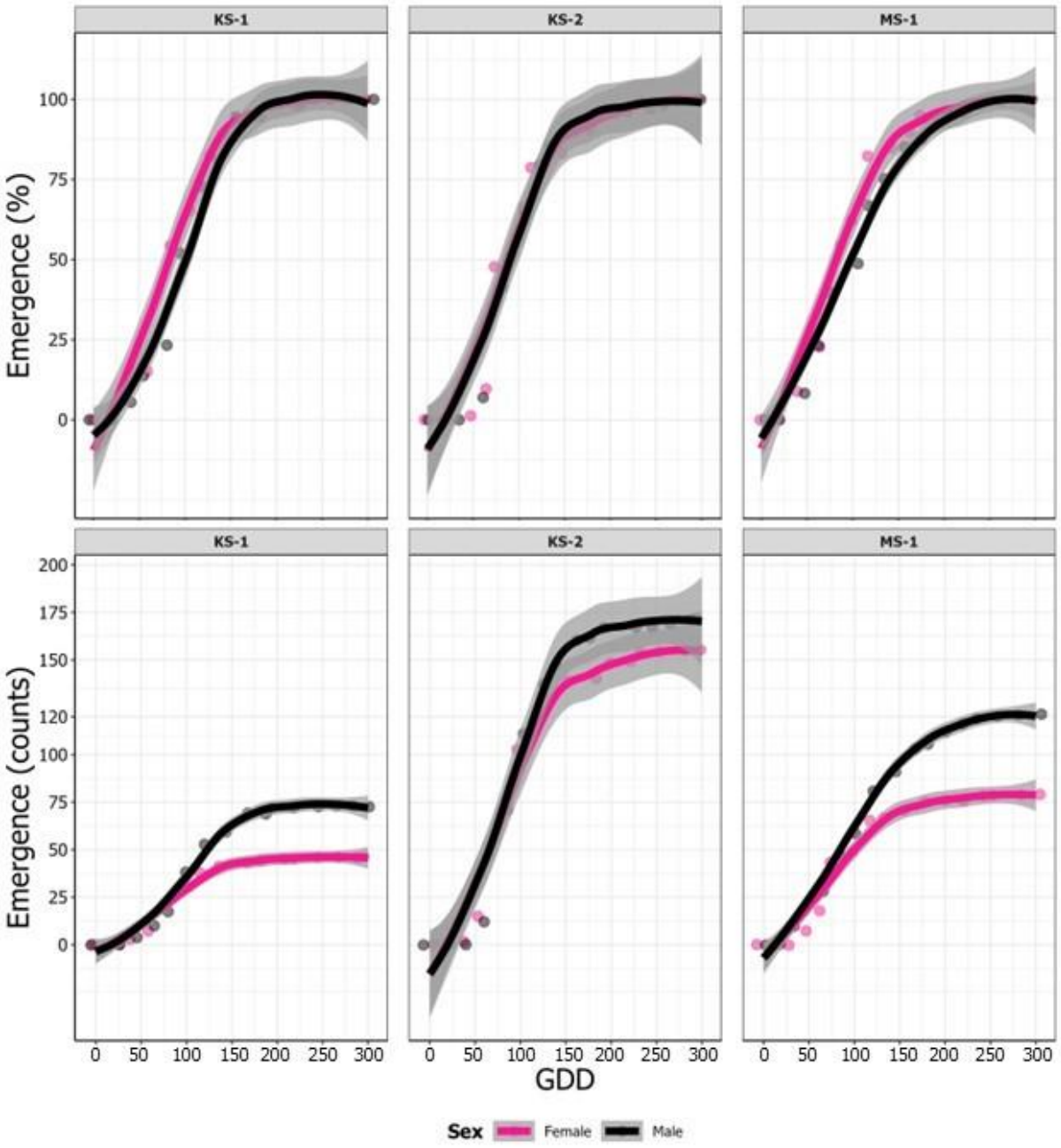

Figure 3.1. Female and male Palmer amaranth percent (top) and total (bottom) emergence of KS-1, KS-2, and MS-1 populations as response of GDD. Pink and black lines represent females and males, respectively.

From a different perspective, when GDD was greater than 90, the number of males emerged in KS-1, KS-2 and MS-1 populations was greater than females (Figure 3.1), likely due to greater male-to-female ratios in those three populations (Table 3.1). Considering the number of individuals emerged, after 90 GDD, all three populations had greater number of males than females, so controlling emerged Palmer amaranth after 90 GDD could potentially decrease the number of males in the population.

Anticipated female emergence could provide an opportunity to control females and shift population ratios to more male individuals, and therefore reduce seed production due to limited number of females. However, that characteristic was not consistent across populations tested in this study (Figure 3.1). Also, in MS-1, males needed only about 30 GDD more than females to reach 90% emergence, which in practicality corresponds to about two days during the growing season, challenging the adoption of practices that could decrease the number of females present in the population in such short time.

As this study was performed in a controlled environment for three weeks, and growing conditions were optimum, it does not capture the effect of multiple emergence flushes that occur in field scenarios. Some replicates did not emerge, which could happen in field conditions, and the influence of remaining seed in the soil seed bank and impact on emergence patterns and gender dynamics throughout growing seasons is unknown. Also, the sample size could have an impact on data analysis and interpretation, i.e., altering male-to-female ratios, and not displaying population patterns with precision due to limited number of replicates. However, in this study the total number of replicates were 46, 155, and 79 females and 73, 172 and 121 males in KS-1, KS-2 and MS-1, respectively (Table 3.1), which should be a good representation of emergence patterns in a population. Other components to consider are genotype by environment interactions

in areas where Palmer amaranth occurs, which could influence how female and male individuals respond to natural growing conditions. These conditions were not captured in this study because temperature was constant at 30 C with 12 h day length and no lack of water. In contrast, Steckel et al. (2004) found that fluctuating temperatures increased germination rate of Palmer amaranth (and other *Amaranthus* species) compared to constant temperature.

There is a need to consider the existence of ecotypes, as well as the occurrence of genotype by environment interactions, that could change emergence patterns. Considering the total number of individuals, controlling flushes of Palmer amaranth repeatedly after 90 GDD could decrease the total number of both females and males. However, the genetic basis of maleness in Palmer amaranth is not yet well understood, and it is possible that uncontrolled plants could reproduce and recover the ratio of male-to-female to optimize for maximum seed production, as the male-Y chromosomal region is (likely) dominant (Montgomery et al. 2019, 2021).

3.4.2. Phenology of female and male Palmer amaranth

BBCH phenological stages of Palmer amaranth as a response to GDD were different across experimental runs ($P < 0.001$) and genders ($P < 0.001$). Palmer amaranth reached senescence with 1220 and 950 GDD in the first and second experimental runs, respectively (Figure 3.2). A linear regression analysis based on the BBCH scale (Hess et al. 1997) did not reveal any differences in the phenological stages as a response to GDD between female and male Palmer amaranth plants, considering a 95% confidence interval, indicating that phenological stages occurred at similar time in females and males in both experiments as heat units were accumulated (Figure 3.2). Applying Dille's rule for adapted BBCH scale, gender ($P < 0.001$) and

experimental runs ($P < 0.001$) were different. Additionally, a linear regression based on the adapted BBCH scale revealed that phenology of female and male plants were different in the first and second experimental runs (Figure 3.2), indicating that the adapted BBCH scale was important for data analyses and interpretation.

Table 3.3. Linear regression parameters of BBCH and adapted BBCH phenological stages for female and male Palmer amaranth as a response to GDD in two experimental runs.

BBCH scale	Run	Gender	Intercept	Coefficient	R^2
	Experiment 1	Female	1.6	0.0065	0.94
		Male	2.1	0.0055	0.86
	Experiment 2	Female	0.2	0.0094	0.99
		Male	0.3	0.0089	0.98
Adapted BBCH scale	Experiment 1	Female	1.1	0.0049	0.96
		Male	1.7	0.0033	0.83
	Experiment 2	Female	0.3	0.0068	0.98
		Male	0.95	0.005	0.95

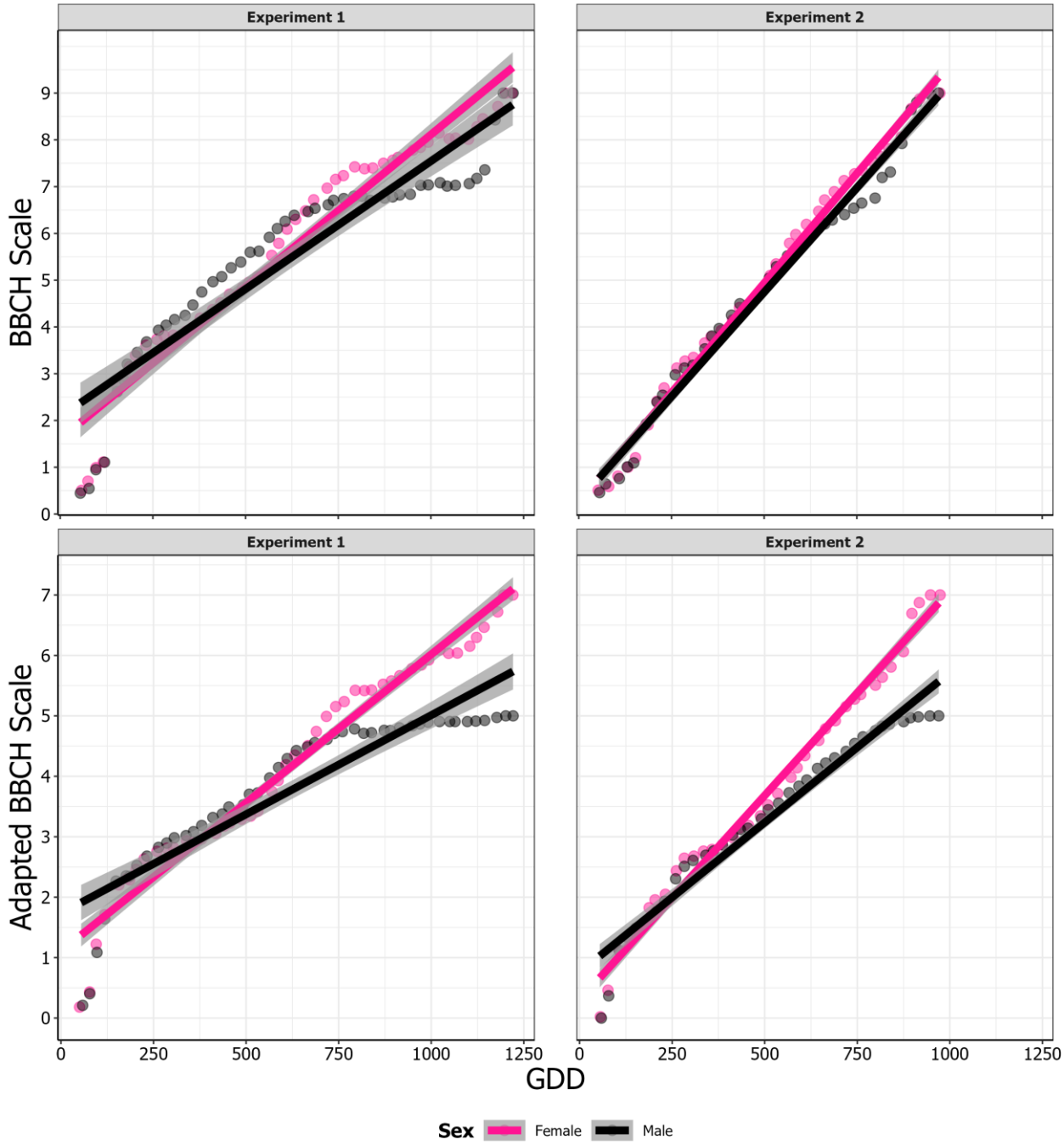

Figure 3.2. BBCH (A and B) and adapted BBCH (C and D) phenology scale for female and male Palmer amaranth. A and C represent the first and B and D represent the second run, respectively. Dots indicate means for female (pink) and male (black) individuals as GDD were accumulated, and solid lines represent a linear regression for each gender.

Palmer amaranth plant height continued to increase even after inflorescence development was initiated. Analysis of plant height through accumulated thermal units revealed that female and male Palmer amaranth kept growing during and after flowering stage (381 to 415 GDD in females and 305 to 415 GDD in males) (Figure 3.3AB). This suggests that Palmer amaranth has an indeterminate growth habit, indicating its ability to continue to invest energy towards growth after flowering.

Plant height was different between genders at senescence in both runs ($P < 0.001$), and Fisher's protected LSD test indicated that females were 87.7 cm (± 2.8) and 131.3 cm (± 3.8) at senescence, in the first and in the second run respectively, and were taller than males which were 68.1 cm (± 3.1) and 113.3 cm (± 2.7) at senescence (Table 3.4). As height was measured from the soil surface to the base of the inflorescence in the main stem, it is possible that females were taller to facilitate pollination, as they can expose pistils above the crop and weed canopy leaving inflorescence free of physical barriers. Palmer amaranth can grow up to 252 cm tall (Spaunhorst et al. 2018) but in this study they grew less than that because it was performed in controlled environment and pot size might have limited plant development. Also, Spaunhorst et al. (2018) did not find differences in plant height between female and male individuals, in contrast to our findings.

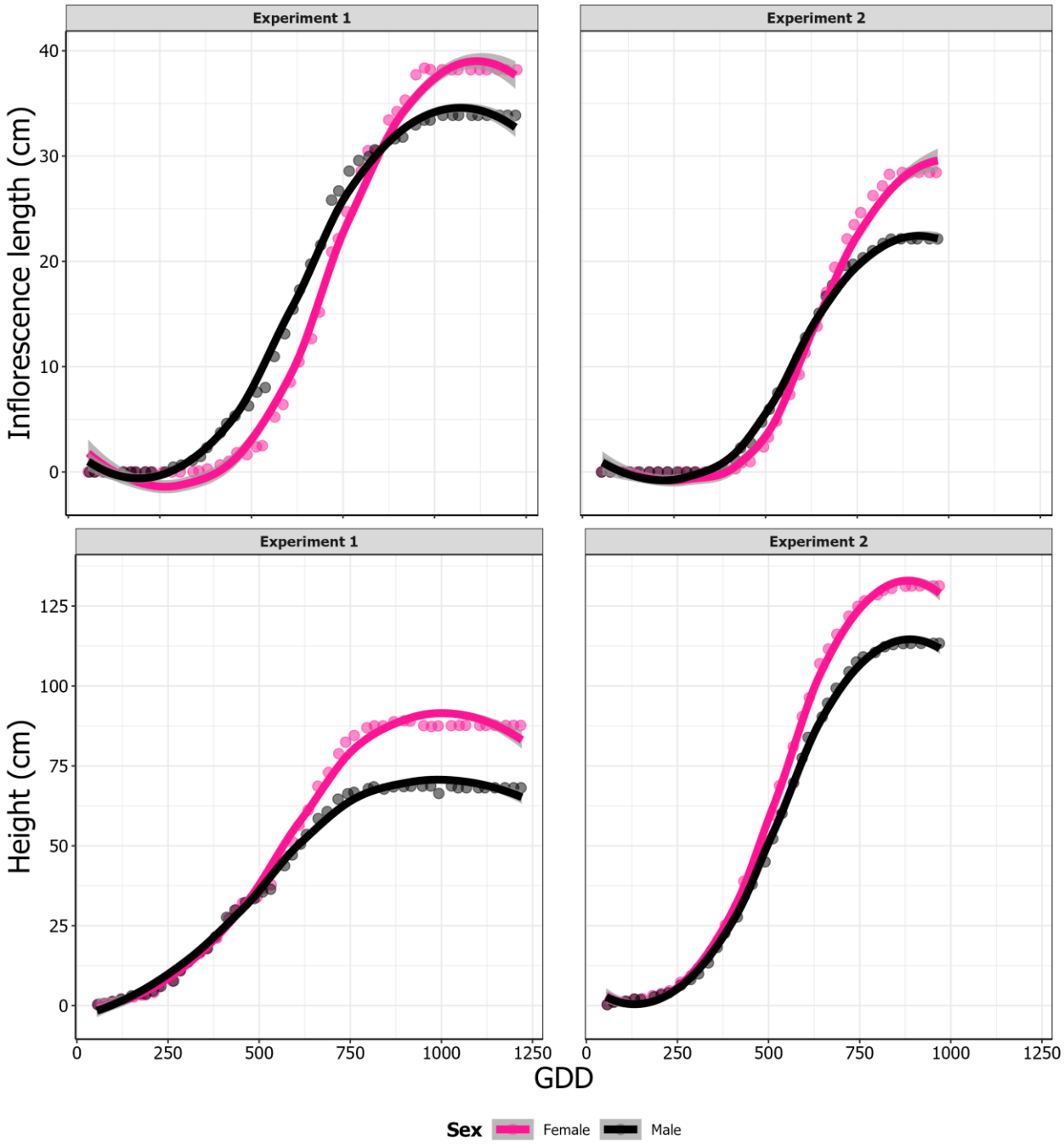

Figure 3.3. Plant height (cm) (bottom) and length of the inflorescence in the main stem (top) as response of GDD in the first and second experimental runs, respectively. Points represent mean values for female (pink) and males (black), and error bars represent standard error of the mean.

Table 3.4. Plant height (cm), inflorescence length (cm), GDD to inflorescence development and GDD to anthesis in female and male Palmer amaranth in two experimental runs. Different letters within the same variable and across genders indicate differences. Different case letters within variables across experiments indicate differences across experiments. Means of plant height and inflorescence length were compared with Fisher's protected LSD test ($\alpha = 0.05$). GDD to inflorescence development and GDD to anthesis were compared using the Mann-Whitney U test ($\alpha = 0.05$).

Run	Total n	Variable	n	Female $\bar{X} \pm$ SE	n	Male $\bar{X} \pm$ SE
1	101	Height (cm)	44	87.7 ± 2.8 A	57	68.1 ± 3.1 B
1	101	Inflorescence length (cm)	44	38.2 ± 1.8 A	57	33.9 ± 1.1 B
1	101	GDD to inflorescence development	44	381.5 ± 23.4 B	57	305.2 ± 24.3 A
1	101	GDD to anthesis	44	599.5 ± 17.7 B	57	566.8 ± 36.8 A
2	97	Height (cm)	48	131.3 ± 3.8 a	49	113.3 ± 2.7 b
2	97	Inflorescence length (cm)	48	28.4 ± 1.9 a	49	22.1 ± 1.6 b
2	97	GDD to inflorescence development	48	414.2 ± 18.8 a	49	414.2 ± 22.9 a
2	97	GDD to anthesis	48	566.8 ± 18.8 a	49	626.8 ± 24.3 b

Male plants were predicted to require fewer GDDs to inflorescence development (305.2 GDD) than female plants (381.5 GDD) in the first run, and initiated pollen at 566.8 GDD, which was 33 GDD before female pistils were receptive (599.5 GDD) (Table 3.4). However, in the second experiment, inflorescence development occurred at 414 GDD in both genders, but females became receptive with 566.8 GDD, whereas males-initiated pollination at 626.8 GDD (Table 3.4). It is possible that females became receptive before pollen was released to maximize fertility in the second run. It is also possible that delayed anthesis in females occurs for the same reason. Inflorescence development and anthesis were not consistent across experiments, which

made it difficult to make conclusions based on these studies. Previous research found that males initiate inflorescence development ahead of females, but that anthesis in females and males was synchronic (Mesgaran et al. 2021), which partially supports the findings of this study.

Regarding reproductive aspects, female and male individuals displayed continuous growth of inflorescence length on the main stem in response to accumulating GDD in both experiments (Figure 3.3). Inflorescences were initiated at 360 and 380 GDD in females, and 285 and 360 GDD in males, in the first and second experimental runs, respectively (Figure 3.3). Inflorescences kept growing until 970 and 870 GDD in females and until 1025 and 795 GDD in males, in the first and second experimental runs, respectively (Figure 3.3). This suggests that, besides indeterminate growth, Palmer amaranth also has indeterminate flowering, perhaps carrying the ability to thrive and keep on investing energy towards growing reproductive organs even after periods of environmental stresses. Spaunhorst et al. (2018) found that inflorescence development started after 400 GDD in multiple populations of Palmer amaranth from Arkansas, Indiana, Missouri, Mississippi, and Nebraska for early-emerged plants, with the GDD decreasing if flush was mid- or late-season emerged.

Overall, inflorescence length at senescence was greater in females than males in both experimental runs ($P < 0.05$ and $P < 0.05$). In terms of proportion of inflorescence length, females were 12.7% and 28.5% greater than males in the first and second run. As the experimental units were uniform within replicates and across runs, data suggested that females either were more efficient at directing resources to reproductive organs compared to males, or that females required fewer resources than males at reproductive stages. Differences in the length of female *versus* male inflorescence had not been documented in previous studies (Spaunhorst et al. 2018, Mahoney et al. 2021; Mesgaran et al. 2021; Reinhardt Piskackova et al. 2021).

The use of thermal models allows prediction of the phases of female and male Palmer amaranth life cycle. For management, decisions could incorporate the knowledge of thermal units required to reach flowering stages, and the adoption of control practices beforehand. In this study, female Palmer amaranth inflorescence developed with 380 to 414 GDD in the first and second run (Table 3.4), giving a window of about 12 to 14 days for control before inflorescence development. Attention needs to be given to lack of control during periods of interference and potential yield losses as consequence of crop-weed competition. However, controlling Palmer amaranth up to 414 GDD is likely to avoid soil seed bank replenishment, and reduce the occurrence of this species in the long term.

3.4. References

Bai G, Kolb FL, Shaner G, Domier LL. (1999). Amplified fragment length polymorphism markers linked to a major quantitative trait locus controlling scab resistance in wheat. *Phytopathology*, 89(4):343-8.

Bravo W, Leon RG, Ferrell JA, Mulvaney MJ, Wood CW. (2017). Differentiation of life-history traits among Palmer amaranth populations (*Amaranthus palmeri*) and its relation to cropping systems and glyphosate sensitivity. *Weed Science*, 65:339-349.

Giacomini D, Westra P, Ward SM. (2014). Impact of genetic background in fitness cost studies: an example from glyphosate-resistant Palmer amaranth. *Weed Science*, 62:29-37.

Geddes CM, Davis AS. (2021). The critical period for weed seed control: A proposed framework to limit weed seed return. *Weed Research*, 61:282-287.

Hess M, Barralis G, Bleiholder H, Buhr L, Eggers T, Hack H, Stauss R. (1997). Use of the extended BBCH scale – general for the descriptions of the growth stages of mono; and dicotyledonous weed species. *Weed Research*, 37:433-441.

Korres NE, Norsworthy JK, Young BG, Reynolds DB, Johnson WG, Conley SP, Smeda RJ, Mueller TC, Spaunhorst DJ, Gage KL, Loux M, Kruger GR, Bagavathiannan MV. (2018). Seedbank persistence of Palmer amaranth (*Amaranthus palmeri*) and waterhemp (*Amaranthus tuberculatus*) across diverse geographical regions in the United States. *Weed Science*, 66:446-456.

Liu R, Kumar V, Jha P, Stahlman PW. (2022). Emergence pattern and periodicity of Palmer amaranth (Amaranthus palmeri) populations from southcentral Great Plains. *Weed Technology*, 36:110-117.

Mahoney DJ, Jordan DL, Hare AT, Leon RG, Roma-Burgos N, Vann MC, Jennings KM, Everman WJ, Cahoon CW. (2021). Palmer amaranth (*Amaranthus palmeri*) growth and seed production when in competition with peanut and other crops in North Carolina. *Agronomy*, 11:1734.

Mesgaran MB, Matzafri M, Ohadi S. (2021). Sex dimorphism in dioecious Palmer amaranth (*Amaranthus palmeri*) in response to water stress. *Planta*, 254:17.

Montgomery JS, Giacomini DA, Weigel D, Tranel PJ. (2021). Male-specific Y-chromosomal regions in waterhemp (A*maranthus tuberculatus*) and Palmer amaranth (*Amaranthus palmeri*). *New Phytologist*, 229(6):3522-3533.

Montgomery JS, Sadeque A, Giacomini D, Brown P, Tranel PJ. (2019). Sex-specific markers for waterhemp (*Amaranthus tuberculatus*) and Palmer amaranth (*Amaranthus palmeri*). *Weed Science*, 67(4), 412-418.

Reinhardt Piskackova TA, Reberg-Horton SC, Richardson RJ, Jennings K, Franca L, Young BG, Leon RG. (2021). Windows of action for controlling palmer amaranth (*Amaranthus palmeri*) using emergence and phenology models. *Weed Research*, 61:188-198.

Spaunhorst DJ, Devkota P, Johnson WG, Smeda RJ, Meyer CJ, Norsworthy JK (2018) Phenology of Five Palmer amaranth (*Amaranthus palmeri*) Populations Grown in Northern Indiana and Arkansas. *Weed Science*, 66:457-469.

Sosnoskie L, Webster T, Culpepper A. (2013). Glyphosate resistance does not affect Palmer amaranth (*Amaranthus palmeri*) seedbank longevity. *Weed* Science, 61(2):283-288.

Steckel LE, Sprague CL, Stoller EW. (2004). Temperature effects on germination of nine *Amaranthus* species. *Weed Science*, 52:217-221.

Van Wychen L. (2020). 2020 survey of the most common and troublesome weeds in grass crops, pasture, and turf in the United States and Canada. Available online at: https://wssa.net/wp-content/uploads/2020-Weed-Survey_grass-crops.xlsx (accessed October 19, 2022).

Vila-Aiub MM, Goh SS, Gaines TA, Han H, Busi R, Yu Q, Powles SB. (2014). No fitness cost of glyphosate resistance endowed by massive *EPSPS* gene amplification in *Amaranthus palmeri*. *Planta*, 239:793-801.

Chapter 4 - Effect of pre-emergence herbicides and cover crop scenarios on Palmer amaranth control and soybean yield

4.1. Abstract

Due to the potential that weeds have to adapt to management practices, multiple control strategies should be integrated in weed control programs to increase the success of weed control in the long-term. Herbicides are important tool due to their benefit/cost relationship, and the adoption of cover crops for weed suppression is increasing, however, the interactions between the application of residual herbicides and cover crops is not well explained. This study investigated the effect of dead cover crop (DCC, terminated two weeks prior to planting), or green planting (GCC, terminated on the planting day) on the efficacy of residual herbicides, in comparison with soil applications and no cover crop (NCC), in Manhattan and Salina, KS, in the 2019-2020 and 2020-2021 growing seasons. Delaying cover crop termination resulted in greater cover crop biomass and greater weed suppression in early season. Overall, the residual herbicides combined with DCC or GCC provided good Palmer amaranth control that was greater than some herbicides or DCC or GCC alone, depending on the site and year. Herbicides did not affect soybean yield but cover crop treatments did. Yield in Manhattan was similar across cover crop treatments in both years. In 2019-2020 in Salina, there was a trend of lower yield as cover crop termination timing was delayed. In the second year, at both sites the yield was similar across cover crop treatments, and lower than in the first year, likely due to environmental conditions. Results from this study suggested that combining cover crop with residual herbicides did not negatively affect herbicide performance compared to soil applications. Also, in some cases, the yield of the cash crop was negatively affected by cover crop. For management,

integrating multiple effective practices is critical for sustainability, but the environmental conditions should be taken into consideration when managing cover crop to avoid impacts on cash crop yield.

Keywords: *Amaranthus palmeri*, cash crop, integrated weed management, residual, weed suppression

4.2. Introduction

Weeds are one of the most critical factors threatening crop production. As an example, the occurrence of weeds in competition with multiple crops can decrease yield up to 52% in soybean (Soltani et al. 2017), 50% in corn (Soltani et al. 2016), 34% in winter wheat (Flessner et al. 2021) and 61% in grain sorghum (Dille et al. 2019) in North America. Despite being good competitors, there are several chemical, cultural, physical, and biological practices that can be used to maintain crop yield potential, i.e., the use of herbicides, crop rotation, narrow row-spacings, cover crop, flooding, and many others.

Weeds can adapt to selection pressure, including control practices. The most frequent example of adaptation is the occurrence of herbicide-resistant weeds. Using some important summer annual weeds that are frequent in the mid-west US as examples, kochia (*Bassia scoparia* L.) has evolved resistance to four herbicide sites of action, Palmer amaranth (*Amaranthus palmeri* S. Watson) to nine, and common waterhemp (*A. tuberculatus* (Moq.) Sauer) to eight (Heap et al. 2019). The ability that herbicide-resistant weeds have to withstand the application of herbicides and the lack of other control practices allows them to succeed.

Herbicide resistance is not the only response of weeds to selection pressure. A study conducted in Florida investigated the influence of life-history (i.e., crop and herbicide history) on adaptive traits in Palmer amaranth (i.e., biomass accumulation, height, days to flowering, canopy width). Key findings include that the use of glyphosate and the occurrence of resistance among populations studied did not explain those morphological differences, but these were associated with cropping system components, such as crop rotation and crop canopy structure (Bravo et al. 2017). One of the best management practices recommended is to use a diversified approach toward weed management with the objective to reduce weed competition, seed production, and

the number of weed seeds in the soil seedbank (Norsworthy et al. 2012), as diversification is the only way to avoid (or delay) weed adaptation.

Palmer amaranth is a summer annual, eudicot, C4, highly prolific weed (Sauer 1950, 1955, 1957). With the ability to produce thousands of seeds per plant, it can become a predominant problem in fields if poorly controlled or left uncontrolled (Keeley et al. 1987; Burke et al. 2007; Norsworthy et al. 2014; Miranda et al. 2021). Another characteristic that makes its management difficult is that this species displays a wide window of emergence, with multiple flushes occurring during the growing season (Keeley et al. 1987, Liu et al. 2021). Therefore, it is crucial that farmers adopt multiple effective control practices to reduce weed emergence.

Cover crops are becoming widely adopted in the mid-west because they can improve soil health parameters and provide weed suppression (Teasdale and Mohler 2000; Norsworthy et al. 2007, Bachie and McGiffen 2013; Petrosino et al. 2015). Weed suppression from cover crops can occur due to competition, physical barrier impeding weed emergence, or allelopathic activity (Barnes et al. 1987; Hutchinson and McGiffen 2000; Mirsky et al. 2013; Kunz et al. 2016). Some cover crop management factors that can maximize its effectiveness for weed suppression include the species (or mixture) chosen, seeding rate, planting date, fertilization, and termination method and timing, all directly influencing the amount of biomass produced (Keene et al. 2017; Palhano et al. 2018; Chapagain et al. 2020; Koehler-Cole et al. 2020).

Documented weed suppression provided by cover crops has varied from 0 to almost 100% control, likely due to environmental factors, weed species present in the field, as well as cover crop species (or mixture) (Galloway and Weston 1996; Teasdale 1996; Hayden et al. 2012). To maximize weed control with cover crop in early season, it is often recommended to use residual herbicides. However, residual herbicides [pre-emergence herbicides (PREs)] can

have soil and foliar activity, and the interactions between residuals and cover crop scenarios, considering the timing of termination relative to planting the cash crop, remain unknown.

The objectives of this study were to (1) investigate the effect of cover crop dry biomass production on weed density in early-season, (2) assess the efficacy of herbicides on Palmer amaranth control as affected by three different cover crop scenarios including none, terminated two weeks prior to planting, and terminated at planting, and (3) assess cover crop effects on soybean yield.

4.3. Materials and methods

4.3.1. Field locations, description, cover crops used, and preparation

A four site-year experiment was performed to assess the performance of PREs as affected by different cover crop scenarios. Field experiments were conducted at the Department of Agronomy Ashland Bottoms Research Field, Kansas State University, near Manhattan, KS, and at Came Farms, Inc. near Salina, KS. The soil series at the Manhattan location was a moderately wet Reading silt loam (2.6% OM, 5.8 pH, 13%) in the 2021 and 2022 growing seasons, and the fields in Salina were a Hord silt loam (3.2% OM and 6.8 pH) in 2020 and a Solomon silty clay (3.4% OM and 6.9 pH) in 2021.

Cover crop establishment was designed to fit the local field environment and generate as much biomass as possible prior to termination and planting soybean. For the 2019-20 growing season, the field near Manhattan was prepared with a tillage disc, followed by drilling a cover crop mixture of triticale (× *Triticosecale* Wittmack) + Austrian winter pea (*Pisum sativum* L.) (100 + 67 kg ha^{-1}) in November 2019. In Salina, winter wheat (*Triticum aestivum* L. "Monument") was drilled at 85 kg ha^{-1} in September 2019. For the 2020-21 growing season,

spring oat (*Avena sativa* L.) was drilled at 100 kg ha^{-1} in a no-till field with sorghum residue in February 2021 in Manhattan, and a 1:1 mixture by weight of triticale (×*Triticosecale*) + ryegrass (*Lolium multiflorum* L.) was drilled at 70 kg ha^{-1} in September 2020 in Salina.

4.3.2. Experimental design and treatments

The treatments were established as a randomized complete block design with four replicates in a split-plot arrangement, with PRE herbicide treatments as the main plot (9 m by 20 m) and cover crop treatments as the subplots (3 m by 20 m). Three cover crops were established: dead cover crop (DCC), which was terminated two weeks before planting, green cover crop (GCC), which was terminated on the planting day, and no cover-crop (NCC) treatment which was terminated upon cover crop emergence. Termination was done with a tank mixture of glyphosate + dicamba (867 + 280 g ha^{-1}) and ammonium-sulfate at 2% v/v using a CO_2 pressurized back-pack sprayer with a 3-m wide boom equipped with six TTI8002 nozzles (Teejet Technologies, Wheaton, IL) calibrated to deliver 187 L ha^{-1} at 4.8 km h^{-1}.

The PRE herbicide treatments were applied at the time of soybean planting. Herbicides used in this study were selected based on common recommendations for burndown treatments as well as weed control in soybean, and doses were adjusted for both sites based on soil texture and organic matter content, following label instructions (Table 4.1). Glyphosate + dicamba (867 + 260 g ai ha^{-1}) and AMS (2% v/v) were added to the PRE spray mixture equally across NCC, DCC and GCC, so the green cover would be terminated at the planting date, and any possible herbicide interactions would occur across the three cover crop treatments and PREs.

Table 4.1. Herbicide treatments applied at time of soybean planting on no cover crop (NCC), dead cover crop (DCC) and green cover crop (GCC), and type of activity, ratio of the concentration of herbicide between water and octanol (Log K_{ow}), and WSSA group, at Manhattan and Salina, KS in 2019-2020 and 2020-2021 growing seasons.

Herbicide treatment	Manhattan	Salina	Anticipated herbicidal activity	Log K_{ow}	WSSA Group
	g ai ha^{-1}				
Non-treated control	-	-	-	-	-
Flumioxazin	29	36	soil and foliar	2.55	14
Metribuzin	227	227	soil and foliar	1.7	5
S-metolachlor	723	723	soil	3.13	15
Saflufenacil	10	10	soil and foliar	2.6	14
Sulfentrazone	113	142	soil and foliar	0.99	14

Soybean was planted in Manhattan in 2019-2020 and in 2020-2021 was AG36XF1 at 321,000 seeds $hectare^{-1}$ in 76 cm row spacing, and soybean was planted on 06/09/2020 and 06/04/2021. For Salina location, in 2019-2020 the soybean variety was P35A91BX and in 2020-2021 was P39A58X, also at 321,000 seeds $hectare^{-1}$, but in a 38.1 cm row, and planted on 05/18/2020 and 06/17/2021.

4.3.3. Data collection and analyses

Cover crop biomass samples were collected from one 0.25 m^2 quadrat from all DCC and GCC subplots to assess biomass production of sites and years at time of soybean planting. Weed counts (0.25 m^2 quadrat) was collected only in Manhattan 2020, as no emergence had yet occurred at other site-years. Cover crop biomass was oven dried at 60 C for seven days before

being weighed. Weed control was visually assessed 28 days after herbicide treatment (DAT), and therefore, after soybean planting. Weed density (counts m^{-2}), height (mean of three representative plants per subplot) and biomass (g m^{-2}) were also recorded at 28 DAT for analysis. Soybean yield data were obtained by harvesting the center two rows using a plot combine at Manhattan, and by clipping 1 m length of center two rows and threshing seed from samples. Moisture content from grain yield was corrected to 13%, and yield was displayed in kg ha^{-1} for comparisons.

Cover crop dry weight (g m^{-2}) was analyzed with a linear mixed effect model (*lme*) available in the *lme4* package in R Studio with replicates as random effects. "Year" was modeled as a fixed effect because the cover crop species planted in each year differed by location. Data were subsequently subjected to ANOVA ($\alpha = 0.05$), and normality of residuals and homogeneity of variances were verified. The ANOVA ($\alpha = 0.05$) was used to assess differences in biomass produced across sites, years, and different termination timings (NCC, DCC or GCC), and subsequently the means were compared by Tukey ($\alpha = 0.05$). Further, a linear regression was performed to assess the relationship between weed density in the 2019-2020 growing season for the Manhattan location and cover crop biomass for that site-year.

Palmer amaranth control (percent) at 28 DAT and soybean yield (kg ha^{-1}) were also analyzed using a linear mixed effect model as previously described, with "herbicide" as main plot, "cover crop" as subplot, and two-, three- and four-way interactions with site and year as fixed effects and replicates as random effect. Once differences were identified, means were compared using Tukey test ($\alpha = 0.05$).

4.4. Results and discussion

4.4.1. Cover crop biomass and weed suppression

Cover crop biomass had a three-way interaction among cover crop termination timing, sites, and years ($P < 0.001$). More biomass was produced in both sites in 2019-20 than in 2020-21. Triticale + peas resulted in 653 g m^{-2} of biomass dry weight in DCC and 999 g m^{-2} in GCC in Manhattan, whereas winter wheat resulted in 299 g m^{-2} in DCC and 515 g m^{-2} in GCC in Salina, during the 2019-20 growing season (Figure 1). In 2020-21, triticale + ryegrass accumulated 952 g m^{-2} in DCC and 1383 g m^{-2} in GCC in Salina, whereas spring oat produced 299 g m^{-2} in DCC and 515 g m^{-2} in GCC in Manhattan. The differences across sites in 2020 could be explained by the shorter stature of wheat, compared with triticale + peas, and by the capacity that triticale has to grow, in comparison with other cover crops. In 2021, the spring oats were planted late, and there was a period with lack of rainfall associated with warmer temperature, that made it difficult for the oat to grow more. In both sites and years, delaying cover crop termination (GCC) instead of terminating two weeks prior to planting (DCC) resulted in greater biomass, which is desired for greater weed suppression.

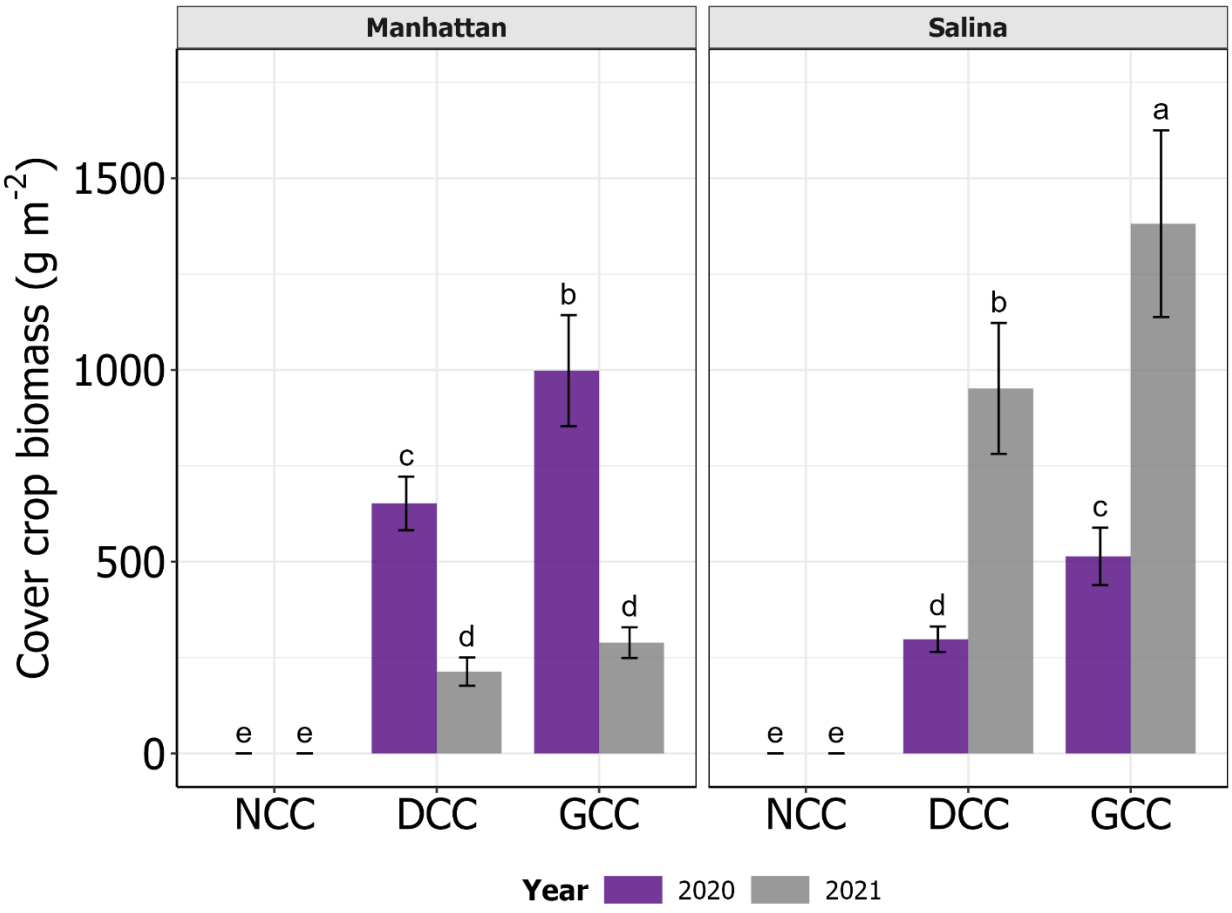

Figure 4.1. Dry biomass produced by cover crops terminated two weeks prior to planting (DCC) and at planting (GCC) in Manhattan and Salina during the growing seasons of 2019-2020 and 2020-2021. Bars represent cover crop biomass (g m^{-2}) across cover crop scenarios in both sites for each year. Different letters across cover crop treatment within site and year indicate differences by Tukey ($\alpha = 0.05$).

A linear regression analyzing Palmer amaranth weed emergence as a response to cover crop dry biomass found that greater biomass increased weed suppression in early-season (Figure 4.2). As dry biomass increased by approximately three units, weed emergence decreased by one. The low fit index ($R^2 = 0.36$) for the linear regression could be explained by variability in the

field, as weeds often occur in patches. Also, the linear regression might not the best way to describe weed emergence as response of dry biomass, as theoretically the weed counts could be negative, however, for this dataset the linear model was the best fit as compared to others, including exponential.

Delaying cover crop termination timing resulted in increased cover crop biomass and greater weed biomass reduction for multiple weed species, including waterhemp (*Amaranthus tuberculatus* (Moq.) Sauer), horseweed, and grasses in Indiana (Hodgskiss et al. 2021). Similarly, Smith (2021) also found an inversely proportional relationship between cover crop biomass and weed suppression in corn. These results agree with the findings of this study and are also supported by a meta-analysis of fifty other peer-reviewed research articles (Osipitan et al. 2019).

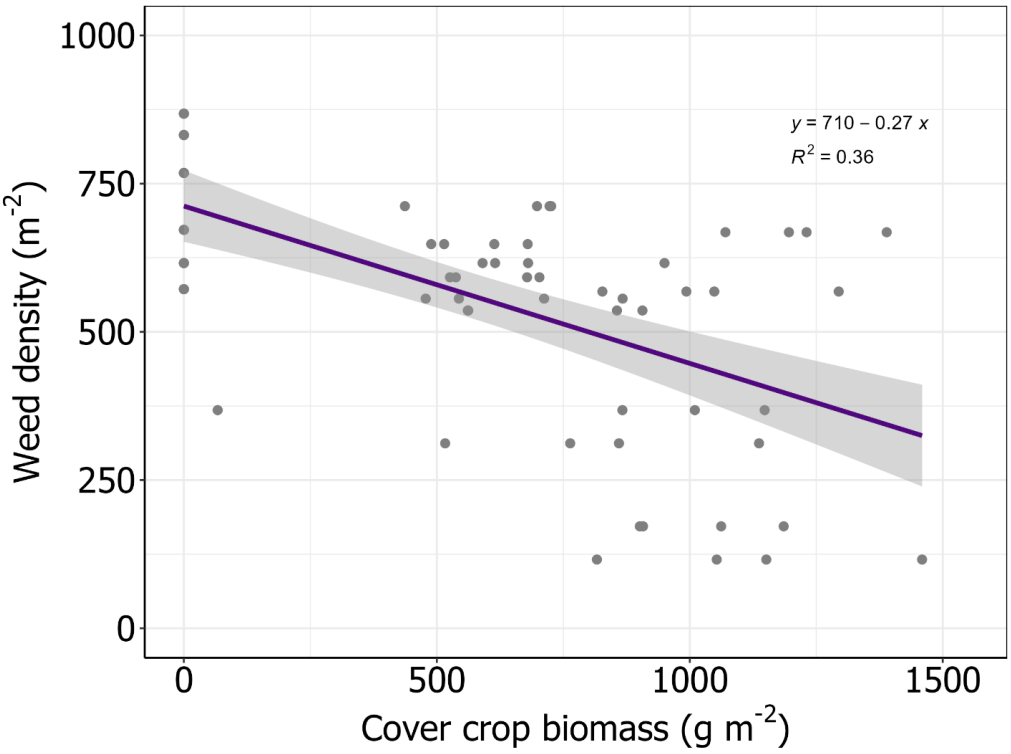

Figure 4.2. Palmer amaranth density (plants m^{-2}) at soybean planting as influenced by cover crop biomass (g m^{-2}) in Manhattan, KS, 2020.

4.4.2. Palmer amaranth control in response to herbicide and cover crop treatments

Weed control was affected by a three-way interaction among year, site, herbicide, and cover crop treatments ($P < 0.01$). In the 2019-2020 growing season in Manhattan, saflufenacil alone provided the poorest control, followed by s-metolachlor and metribuzin alone, among all other herbicides, DCC, GCC or combinations of herbicides with DCC or GCC (Table 4.2). All other herbicides used in NCC or in combination with DCC or GCC resulted in greater levels of Palmer amaranth control. The use of DCC or GCC resulted in satisfactory Palmer amaranth control, in the same magnitude as other PREs alone, and combinations of PREs+DCC or PREs+GCC. Additionally, combinations of herbicides with DCC or GCC did not affect herbicide performance, regardless their type of activity. For the 2020-2021 growing season, no differences were observed among DCC, GCC or herbicides alone or in combinations with cover crop. The second year had an intense dry and hot period (Figure 4.3) that likely resulted in less weed emergence. Also, the lack of rainfall after herbicide application may have limited residual activation (Figure 4.3), limiting distinctions normally observed across the treatments used in this study.

Table 4.2. Palmer amaranth control (%) as affected by PRE and cover crop treatments in Manhattan and Salina, KS in 2020 and 2021. Values represent average control for herbicide and cover crop scenario. Different letters across combinations of herbicide and cover crop treatments within site-year indicate differences by Tukey ($\alpha = 0.05$).

		Manhattan				Salina			
Herbicide	Cover Crop	2019-2020		2020-2021		2019-2020		2020-2021	
Non-treated control	NCC	0	F	0	B	0	C	0	E
	DCC	88	BCD	94	A	98	A	74	D
	GCC	91	ABC	97	A	98	A	84	CD
Flumioxazin	NCC	97	AB	100	A	95	A	94	ABC
	DCC	98	AB	100	A	99	A	95	AB
	GCC	98	AB	100	A	100	A	95	ABC
Metribuzin	NCC	84	CD	99	A	95	A	89	ABC
	DCC	97	AB	100	A	98	A	89	ABC
	GCC	96	AB	100	A	99	A	91	ABC
S-metolachlor	NCC	78	D	100	A	94	A	89	ABC
	DCC	91	ABC	100	A	98	A	87	BC
	GCC	94	ABC	99	A	97	A	93	ABC
Saflufenacil	NCC	39	E	96	A	81	B	92	ABC
	DCC	91	ABC	97	A	98	A	96	AB
	GCC	92	ABC	99	A	99	A	97	AB
Sulfentrazone	NCC	95	AB	100	A	93	A	96	AB
	DCC	100	A	100	A	97	A	98	A
	GCC	98	AB	100	A	99	A	98	A

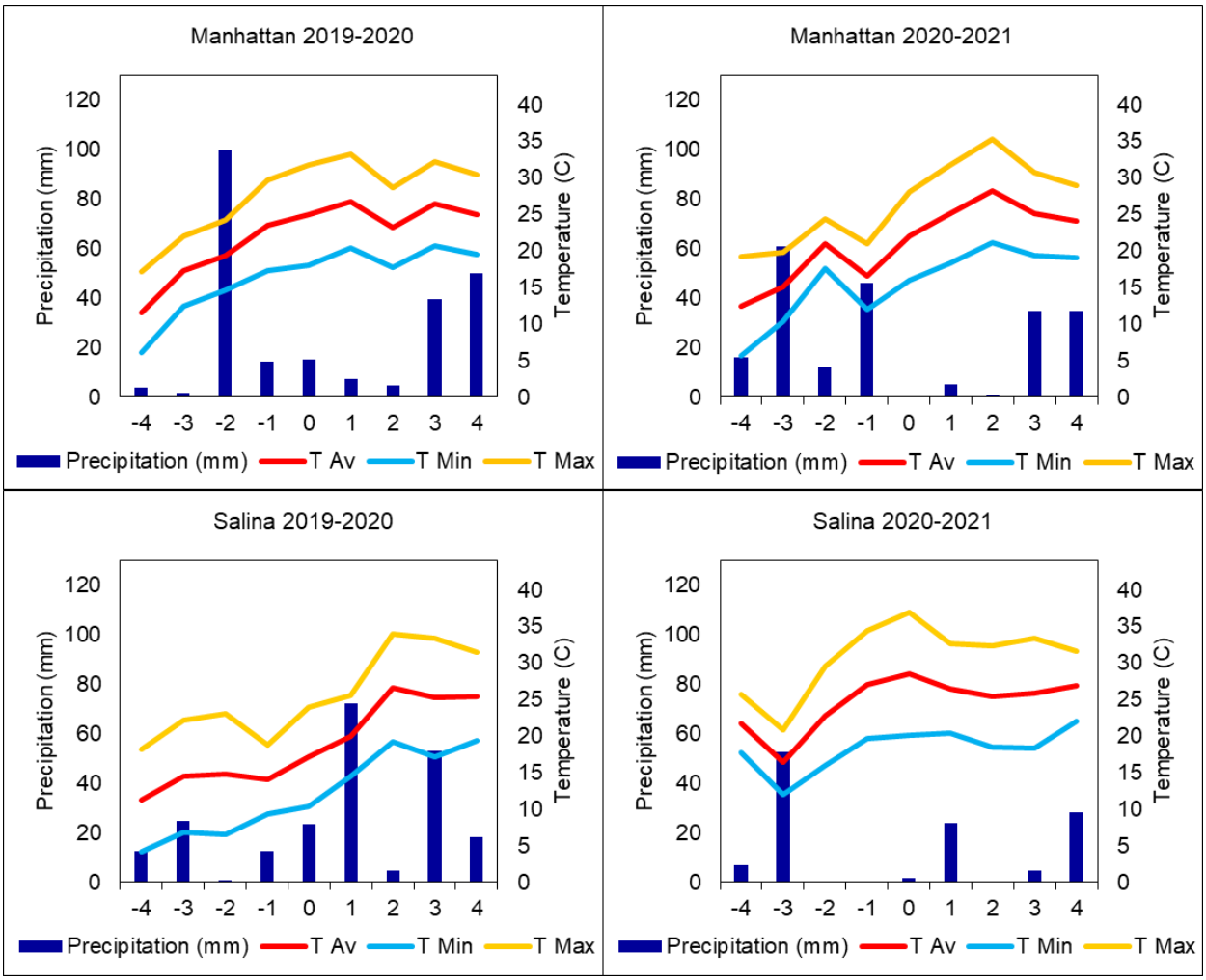

Figure 4.3. Weekly accumulated precipitation (mm), average (Av), minimum (Min) and maximum (Max) temperatures (T) (C) in four site-year studies in Manhattan and Salina in the growing seasons of 2019-2020 and 2020-2021. Numbers represent periods of weeks before (negative) and after (positive) the week when soybean was planted and PRE treatments were applied (0).

For the Salina location in 2019-2020, saflufenacil alone provided the least control (81%), but DCC, GCC, any other herbicide, or combinations of herbicide and cover crop provided satisfactory control and did not differ among each other (Table 2). In 2020-2021, DCC alone

provided the lowest control (74%), and was not different than GCC alone (84%) (Table 2). Sulfentrazone + DCC and sulfentrazone + GCC provided greater control (99% and 98%) and were not different than sulfentrazone alone (96%). S-metolachlor + DCC resulted in 87% control, which was similar than s-metolachlor alone and s-metolachlor + GCC, but lower than the best treatments. Other herbicides alone or in combinations with DCC or GCC provided control similar to the best treatments (Table 2).

Combinations of cover crop and residual herbicides were previously reported to increase Palmer amaranth control, as compared to cover crop alone. Perkins et al. (2020) found that metribuzin, flumioxazin, and s-metolachlor, among other herbicides, with cover crop delayed Palmer amaranth emergence and decreased density, as compared to cover crop only, which was similar to these results

4.4.3. Soybean yield in response to herbicide and cover crop treatments

Herbicide treatments did not affect soybean yield ($P = 0.079$), but there was an interaction among cover crop treatments, sites, and years ($P < 0.05$). Overall, the greatest yield was observed in Manhattan in 2020, with 5,780, 6,040 and 6,040 kg ha^{-1} for NCC, DCC and GCC, respectively, which were not different from each other (Figure 4.5). In the first year in Salina, the best yield estimate was 1810 kg ha^{-1} with NCC, followed by DCC (1390 kg ha^{-1}), which was greater than GCC (1170 kg ha^{-1}), suggesting that the longer the cover crop stayed alive, the more the yield of the cash-crop was affected. Similarly, yield in GCC (2810 kg ha^{-1}) was lowest in Manhattan during the second growing season, as compared to yields in NCC (3190 kg ha^{-1}) or DCC (3210 kg ha^{-1}), however, soybean yield was not different across cover crop

scenarios (Figure 4.5). In the second growing season in Salina, the yields were not different across cover crop treatments and were the lowest observed in this study (840 to 890 kg ha^{-1}).

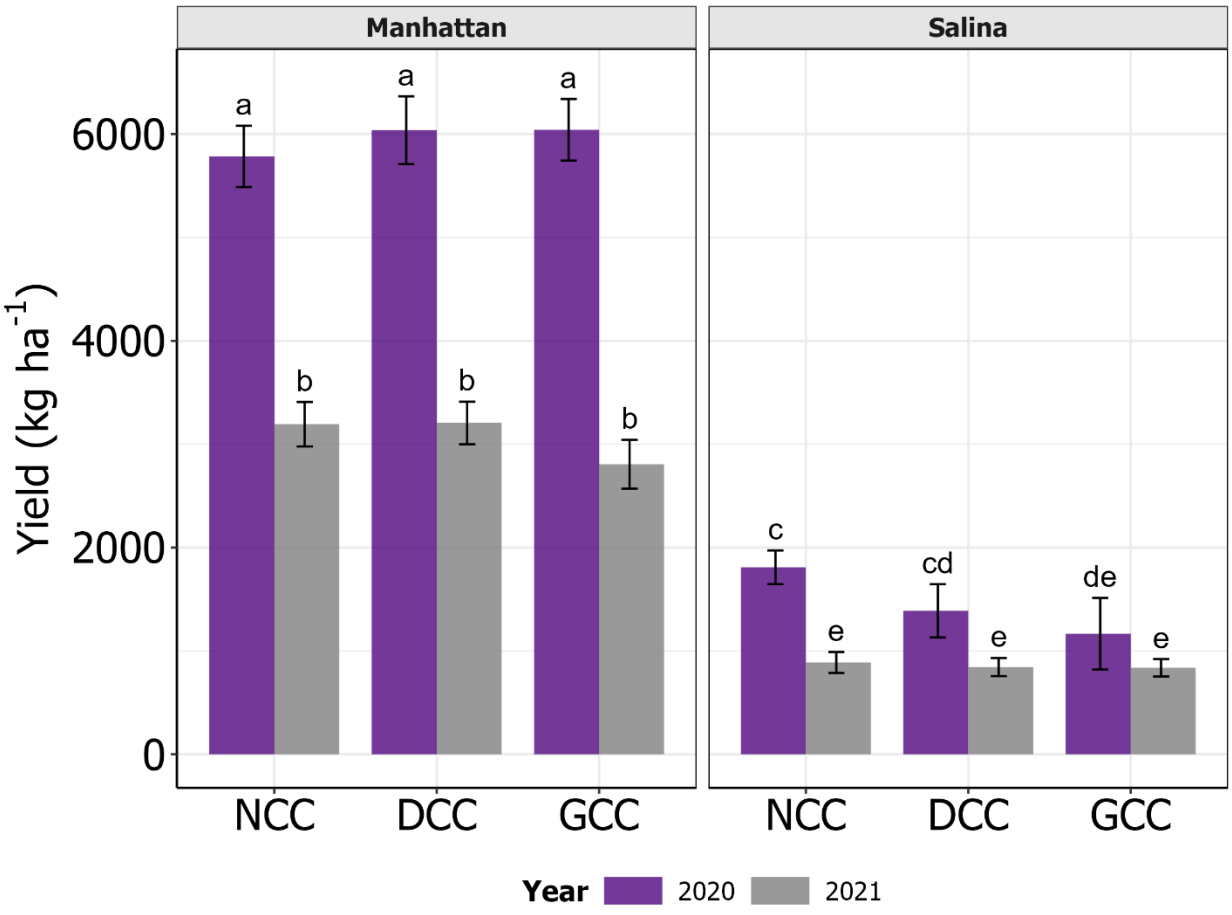

Figure 4.4. Soybean yield (kg ha^{-1}) as affected by cover crop use and termination timings in a four site-year study in Kansas. Bars represent main yield across herbicides and replicates within cover crop treatments for each site and year. Different letters across cover crop treatments indicate differences by Tukey test ($\alpha = 0.05$).

A question about how cash-crop yield can be affected if adopting cover crop is often raised. Previously, soybean yield was also affected by up to 41% when delaying cover crop

termination timing to at planting or two weeks after planting, as compared to the terminated two weeks before planting (Hodgskiss et al. 2021). In a different study, the presence of a legume cover crop increased horseweed suppression relative to cover crop absent, resulting in a difference of 196 versus 615 kg ha^{-1}, respectively, when horseweed was present or absent (Pitman et al. 2019).

In this study, the adoption of cover crop resulted in greater weed suppression in early season, as compared to treatments without cover crop, demonstrating the benefits of cover crop on weed management. Hodgkiss et al. (2021) found that even spring residual program was necessary to maximize horseweed control (*Conyza canadensis* (L.) Cronquist). Cereal rye was found to reduce Palmer amaranth emergence 75%, prior to POST applications, effectively reducing the selection pressure imposed by POST application, and that two effective herbicide sites of action with proper rainfall reducing Palmer amaranth densities by 99% (Hand et al. 2021). Often the effect of adding residual herbicides to cover crop is questioned due to the possibility that the herbicide may not reach the soil (Teasdale et al. 2003). However, in this study demonstrated that combination of residual herbicides with cover crops are more effective than cover crops alone.

There was a trend of reducing soybean yield as cover crop biomass increased in one out of four site-years. However, that did not happen in the situation which the greatest amount of cover crop biomass was obtained among all studies, suggesting that other factors (i.e., precipitation, precipitation distribution, temperature, periods of drought, crop establishment) need to be accounted. Also, it is important to consider the system as a whole when analyzing the impacts of using cover crop on cash-crop yield. For example, it is substantial to account for benefits of using cover crop, such as how greater weed suppression warranties yield protection,

as demonstrated by Pitman et al. (2018), instead of only considering how cover crops could be taken up resources that should be kept to the cash-crop.

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4.6. References

Bachie O, McGiffen ME. (2013). Effects of summer cover cropping on weed population density and biomass in a subsequent broccoli crop. p. 31-36. In: *International Conference on Sustainable Environment and Agriculture*. IPCBEE 57:31-36.

Barnes JP, Putnam AR, Burke BA, Aasen AJ. (1987). Isolation and characterization of allelochemicals in rye herbage. *Phytochemistry*, 26:1385-1390.

Bravo W, Leon RG, Ferrell JA, Mulvaney MJ, Wood CW. (2017). Differentiation of life-history traits among Palmer amaranth populations (*Amaranthus palmeri*) and its relation to cropping systems and glyphosate sensitivity. *Weed Science*, 65(3):339-349.

Burke IC, Schroeder M, Thomas WE, Wilcut JW. (2007). Palmer Amaranth Interference and Seed Production in Peanut. *Weed Technology*, 21(2):367-371.

Chapagain T, Lee EA, Raizada MN. (2020). The potential of multi-species mixtures to diversify cover crop benefits. *Sustainability*, 12:2058.

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 Technology*, 34:624-629.

Flessner ML, Burke IC, Dille JA, Everman WJ, VanGessel MJ, Tidemann B, Manuchehri MR, Soltani N, Sikkema PH. (2021). Potential wheat yield loss due to weeds in the United States and Canada. *Weed Technology*, 35:916-923.

Galloway BA, Weston LA. (1996). Influence of cover crop and herbicide treatment on weed control and yield in no-till sweet corn (*Zea mays* L.) and pumpkin (*Cucurbita maxima* Duch). *Weed Technology*, 10:341-346.

Hand LC, Randell TM, Nichols RL, Steckel LE, Basinger NT, Culpepper AS. (2021). Cover crops and residual herbicides reduce selection pressure for Palmer amaranth resistance to dicamba applied postemergence in cotton. *Agronomy Journal*, 113:5373-5382.

Hayden ZD, Brainard DC, Henshaw B, Ngouajio M. (2012). Winter annual weed suppression in rye-vetch cover crop mixtures. *Weed Technology*, 26:818-825.

Heap I. (2022). The International Herbicide-Resistant Weed Database. Online. Sunday, November 6, 2022. Available www.weedscience.org

Hodgskiss CL, Young BG, Armstrong SD, Johnson WG. (2022). Utilizing cover crops for weed suppression within buffer areas of 2,4-D-resistant soybean. *Weed Technology*, 36:118-129.

Hutchinson CM, McGiffen ME. (2000). Cowpea cover crop mulch for weed control in desert pepper production. *HortScience*, 35:196-198.

Keeley PE, Carter CH, Thullen RJ. (1987). Influence of planting date on growth of Palmer amaranth (*Amaranthus palmeri*). *Weed Science,* 35(2):199-204.

Keene CL, Curran WS, Wallace JM, Ryan MR, Mirsky SB, VanGessel MJ, Barbercheck ME. (2017). Cover crop termination timing is critical in organic rotational no-till systems. *Organic, Agriculture and Agroecology,* 109(1):272-282.

Koehler-Cole K, Elmore RW, Blanco-Canqui H, Francis CA, Shapiro CA, Proctor CA, Ruis SJ, Heeren DM, Irmak S, Ferguson RB. (2020). Cover crop productivity and subsequent soybean yield in the western Corn Belt. *Agronomy Journal,* 112:2649-2663.

Kunz C, Sturm DJ, Sökefeld M, Gerhards R. (2016). Weed suppression and early sugar beet development under different cover crop mulches. *Plant Protection Science,* 52:1-7.

Liu R, Kumar V, Jha P, Stahlman PW. (2022). Emergence pattern and periodicity of Palmer amaranth (*Amaranthus palmeri*) populations from southcentral Great Plains. *Weed Technology,* 36:110-117.

Miranda JW, Jhala AJ, Bradshaw J, Lawrence NC. (2021) Palmer amaranth (*Amaranthus palmeri*) interference and seed production in dry edible bean. *Weed Technology,* 35(6):995-1006.

Mirsky SB, Ryan MR, Teasdale JR, Curran WS, Reberg-Horton CS, Spargo JT, Wells MS, Keene CL, Moyer JM. (2013). Overcoming weed management challenges in cover crop–based organic rotational no-till soybean production in the eastern United States. *Weed Technology,* 27:193-203

Norsworthy JK, Ward SM, Shw 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 Science*, 60(sp1), 31-62.

Norsworthy JK, Malik MS, Jha P, Riley MB. (2007). Suppression of *Digitaria sanguinalis* and *Amaranthus palmeri* using autumn-sown glucosinolate-producing cover crops in organically grown bell pepper. *Weed Research,* 47:425-432.

Osipitan OA, Dille JA, Assefa Y, Radicetti Em, Ayeni A, Knezevic SZ. (2019). Impact of cover crop on management on level of weed suppression: a meta-analysis. *Crop Science*, 59:833-842.

Palhano MG, Norsworthy JK, Barber T. (2018). Evaluation of chemical termination options for cover crops. *Weed Technology*, 32:227-235.

Perkins CM, Gage KL, Norsworthy JK, Young BG, Bradley KW, Bish MD, Hager A, Steckel LE. (2021). Efficacy of residual herbicides influenced by cover-crop residue for control of *Amaranthus palmeri* and *A. tuberculatus* in soybean. *Weed Technology*, 35:77-81.

Petrosino JS, Dille JA, Holman JD, Roozeboom KL. (2015). Kochia suppression with cover crops in southwestern Kansas. *Crop Forage Turfgrass Manage*, 1(1).

Pittman KB, Barney JN, Flessner ML. (2019). Horseweed (*Conyza canadensis*) suppression from cover crop mixtures and fall-applied residual herbicides. *Weed Technology*, 33:303-311.

Sauer JD. (1950). The grain amaranths: a survey of their history and classification. *Ann. Missouri Bot. Gard*. 37:561–632.

Sauer JD. (1955). Revision of the dioecious amaranths. *Madrono,* 13:5-46.

Sauer JD. (1957). Recent migration and evolution of the dioecious amaranths. *Evolution,* 11:11-31.

Soltani N, Dille J, Burke I, Everman W, VanGessel M, Davis V, Sikkema P. (2016). Potential corn yield losses from weeds in north America. *Weed Technology*, 30(4), 979-984.

Soltani N, Dille J, Burke I, Everman W, VanGessel M, Davis V, Sikkema P. (2017). Perspectives on potential soybean yield losses from weeds in north America. *Weed Technology*, 31(1), 148-154.

Teasdale JR. (1996). Contribution of cover crops to weed management in sustainable agricultural systems. *Journal of Production Agriculture*, 9:475-479.

Teasdale JR, Mohler CL. (2000). The quantitative relationship between weed emergence and the physical properties of mulches. *Weed Science*, 48:385-392.

Teasdale JR, Shelton DR, Sadeghi AM, Isensee AR. (2003). Influence of hairy vetch residue on atrazine and metolachlor solution concentration and weed emergence. *Weed Science*, 51:628-634.

Chapter 5 - Conclusions and Future Directions

5.1. Conclusions and Future Directions

Palmer amaranth is an adapted weed that displays fast growth rate, ability to accumulate high amounts of biomass, produce seeds and spread adaptative genes across populations relatively fast as compared to other species (Keeley et al. 1987, Sosnoskie et al. 2012). Understanding biology and how this species can adapt is critical to better design more sustainable practices. And considering the potential that it has to adapt and evolve resistance to herbicides (Sprague et al. 1997; Culpepper et al. 2006; Salas et al. 2016; Nakka et al. 2017a, b, c; Brabham et al. 2019; Carvalho-Moore et al. 2022; Priess et al. 2022; Shyam et al. 2022; Heap 2022) and also to other selection pressure imposed by crop management (Bravo et al. 2017), integrating multiple control practices is key for the sustainability of agricultural practices.

This study demonstrated that a Palmer amaranth population from a long-term conservation tillage study in Kansas (KCTR) has a resistance to PPO-inhibitor herbicide, lactofen, at a level ranging from 5 to 35-fold depending on what generation (G1 or G2) was compared to the susceptible populations used in the study (KSS or MSS). Importantly, the GR_{50} of lactofen was between 51.1 to 110.3 g ha^{-1} across consecutive KCTR generations, comparable to other Palmer amaranth populations previously reported in the midsouth and midwestern US (Salas et al. 2016, Varanasi et al. 2018a, Montgomery et al. 2021a). Interestingly, KCTR did not carry alterations in the *PPO2* gene, the predominant mechanism reported across numerous resistant populations in the US (Salas-Perez et al. 2017; Copeland et al. 2018; Varanasi et al. 2018b; Noguera et al. 2020; Wu et al. 2020). Similar to previous reports by Varanasi et al. (2018a) and Montgomery et al. (2021a), no difference in the expression of the *PPO2* gene

between KCTR or MSS was found. Thus, the resistance to PPO-inhibitors in KCTR Palmer amaranth was not conferred by alterations in the target site of these herbicides (Chapter 2).

The analysis of ^{14}C-fomesafen metabolism using HPLC suggests that >93% of parent compound was remained in MSS across all the time points tested; whereas in KCTR it reduced to 77%, 72%, 41% and 31%, respectively, at 24, 48, 72 and 96 HA. Therefore, this study suggested that KCTR can metabolize more fomesafen faster than MSS. Further, the addition of malathion, a P450-inhibitor, restored the sensitivity of KCTR to lactofen, suggesting that the metabolism of PPO-inhibitors is mediated by P450 activity in this population (Chapter 2).

KCTR and MSS Palmer amaranth plants were completely controlled by PRE application of all PPO-inhibitors tested in this research (Chapter 2), suggesting that fomesafen, flumioxazin, saflufenacil, sulfentrazone, and oxadiazon could be viable options to control this population in field scenarios. However, it is important to emphasize the adoption of integrated weed management practices to reduce the risks of evolution of herbicide resistance (Norsworthy et al. 2012).

It is important to mention that KCTR has a unique resistance profile, with the ability to withstand applications of six SOAs, including 5-enolpyruvylshikimate 3-phosphate- (EPSPS-), acetolactate synthase- (ALS-), 4-Hydroxyphenylpyruvate dioxygenase- (HPPD-), photosystem II- (PSII-), synthetic auxins, and PPO-inhibitors, with the predominant mechanism being metabolic resistance (Shyam et al. 2021). Resistance to these SOA in Palmer amaranth have previously been reported (Sprague et al. 1997; Culpepper et al. 2006; Salas et al. 2016; Nakka et al. 2017a, b, c; Brabham et al. 2019; Carvalho-Moore et al. 2022; Priess et al. 2022; Shyam et al. 2022; Heap 2022), but not in a single population. The herbicides mostly used for weed control in that specific field were PRE applications of s-metolachlor, mesotrione, and atrazine, and POST

applications of atrazine and 2,4-D. Because this population comes from a field in 45+ years of continuous sorghum, in which PPO-inhibitors were not used for weed control in-season, it is possible that the predominance of metabolic resistance to other SOAs may have predisposed KCTR plants to evolve metabolic resistance to PPO-inhibitors, even without direct selection. On the other hand, it is also possible that the PPO-inhibitor resistance trait is unique and may be linked to gene(s) conferring resistance to other SOAs. Studies to identify the genetic basis of PPO-inhibitor resistance are currently ongoing to provide more information regarding the resistance trait.

In summary, KCTR Palmer amaranth was historically exposed to selection pressure from herbicides, as conservation agriculture practices were used (i.e., reduced tillage). This research highlights that in conservation agriculture, weed management relies heavily on chemical control. And as metabolic-based resistance can occur, genes of herbicide detoxification conferring metabolic resistance to multiple herbicides SOA can evolve, and resistance traits can appear in unexpected patterns, as resistance to PPO-inhibitors without using this group of herbicides.

In terms of differential emergence pattern of Palmer amaranth, female plants showed 90% emergence before males in KS-1 and MS-1 populations, suggesting that if Palmer amaranth was controlled at that thermal time, population ratios could be shifted to more male individuals, therefore resulting lower in seed production. However, that pattern was not consistent across all populations investigated in this study. Also, KS-1 and MS-1 populations reached 90% emergence with 160 GDD, which is relatively a short period (about 6 days), which in practically does not provide a good window for actions, as Palmer amaranth displays fast growth. Also, it is important to consider that there are genotype by environment interactions occurring in field

conditions, that were not captured in this study, as experiments were performed in controlled environment.

Analyzing progress of phenological stages with accumulating GDD, no differences between female and male life cycles were initially identified. Considering that not all plant species contain the same stages (i.e., vegetative propagation), and the dioecious nature of this species, in which male individuals will not have all reproductive stages as females due to the absence of fruit development, an adapted phenological BBCH scale was proposed to better describe female and male Palmer amaranth life cycles through regression analysis. It was then possible to observe that males required fewer heat units to complete their life cycle in the first experimental run. However, in the second experimental run females and males reached senescence with similar accumulated GDDs. Further, male plants developed inflorescence and opened their flowers ahead of females in one experimental run. However, in the second experimental run, both genders developed inflorescence with 414 GDDs, but females became receptive before males pollinated, suggesting inconsistencies in results, possibly due to the influence of growing conditions on the duration of their life cycle.

Female and male individuals continued to increase in height even after inflorescence was initiated, demonstrating that Palmer amaranth has indeterminate growth habit (Oliveira et al. 2022). Inflorescence of female and male plants increased in size as heat units were accumulated, indicating also indeterminate inflorescence development, which could potentially provide more resilience to this species. At senescence, females were taller than males and had longer inflorescences in both experiments. With a previous hypothesis that females and males were different and given the occurrence of the MSY chromosomal region (Montgomery et al. 2019, 2021b), it was suspected that genes associated with height and inflorescence length, which are

(likely to be) quantitative traits, located in the MSY chromosomal region would be responsible for that. However, as females were taller and had longer inflorescences than males, it is likely that those traits are not in the MSY locus, or that genes in the MSY region somehow suppress the expression of those traits. As females were consistently taller than males, it's been further hypothesized that females are taller to expose their inflorescence above crop (and weed) canopy to have fewer physical barriers to facilitate fertilization, as pollen in Palmer amaranth is mostly wind dispersed.

Palmer amaranth differentiated into female and male plants as an evolutionary process (Montgomery et al. 2019, 2021b), but little was known about differential development across genders. This research demonstrated that female and male life cycles were not synchronic, different than previous findings (Mesgaran et al. 2021), but that their anticipated inflorescence development and anthesis favor reproduction and, therefore, species perpetuation.

When using a cover crop, growers should consider managing their key objectives; if it is weed suppression in early-season, one way to achieve it is delaying the termination timing of the cover crop. As demonstrated by this study, in the majority of the experiments GCC produced more biomass than DCC, which was terminated two weeks earlier. The exception occurred with Manhattan in the 2020-2021 growing season, in which spring oat was used as cover crop, and the later planting date associated with a long drought period limited its development, resulting in similar biomass amounts in GDD as compared with DCC.

Herbicide applications and cover crops were thought to potentially be antagonistic (Teasdale et al. 2003), as some residuals have foliar activity and could interact with the cover crop and be less available in the soil for weed control. This study demonstrated that the combination of residual herbicide with a cover crop, regardless of termination timing, did not

affect herbicide performance, as supported by other research (Pitman et al. 2018; Hand et al. 2021; Hodgkiss et al. 2021). It is important to consider that Kansas environments overall have limited precipitation, and cover crop species can produce more biomass in other regions with greater precipitation. Therefore, the influence of geographies on cover crop biomass, and the possibility that greater biomass accumulation could limit the residual activity of PREs, should not be ignored to have best benefit from both cover crops and herbicides.

In time, it is important to consider the effect of using cover crop on subsequent cash crop. In this study, soybean yield was not affected by cover crop use in three out of four studies. Overall, the data suggests that if good precipitation happens during the growing season, soybean yield will not be affected by cover crop use, whether it was terminated two weeks prior to planting, or at panting. From a different perspective, if adverse climatic conditions are expected in growing season, it is not recommended that growers reduce the number and diversification in weed control practices aiming to save cash crop yield, but design effective practices for their system with the expectations that they will have instead.

This research demonstrated that Palmer amaranth has ability to adapt to selection pressure, and sometimes adaptative traits display unexpected patterns, as metabolic resistance to PPO-inhibitors in a field which those chemistries were not used. As a consequence, chemical options available to control this species become limited. Also, differences were found in vegetative and reproductive growth parameters in female *versus* male plants, suggesting adaptations that maximize reproduction. Even with such characteristics, it is possible to effectively combine control tools for integrated weed management, resulting in more successful and sustainable control.

5.2. References

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

Bravo W, Leon RG, Ferrell JA, Mulvaney MJ, Wood CW. (2017). Differentiation of life-history traits among Palmer amaranth populations (*Amaranthus palmeri*) and its relation to cropping systems and glyphosate sensitivity. *Weed Science*, 65:339-349.

Carvalho-Moore P, Norsworthy J, González-Torralva F, Hwang J, Patel J, Barber L, Butts TR, McElroy JS. (2022). Unraveling the mechanism of resistance in a glufosinate-resistant Palmer amaranth (*Amaranthus palmeri*) accession. *Weed Science*, 70(4):370-379.

Copeland J, Giacomini D, Tranel P, Montgomery G, Steckel L. (2018). Distribution of PPX2 mutations conferring PPO-inhibitor resistance in Palmer amaranth populations of Tennessee. *Weed Technology*, 32(5):592-596.

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 Science*, 54(4):620-626.

Hand LC, Randell TM, Nichols RL, Steckel LE, Basinger NT, Culpepper AS. (2021). Cover crops and residual herbicides reduce selection pressure for Palmer amaranth resistance to dicamba applied postemergence in cotton. *Agronomy Journal*, 113:5373-5382.

Heap I (2020). The international herbicide-resistant weed database. (2022). Online. Sunday, September 25, 2022. Available www.weedscience.org

Hodgskiss CL, Young BG, Armstrong SD, Johnson WG. (2022). Utilizing cover crops for weed suppression within buffer areas of 2,4-D-resistant soybean. *Weed Technology*, 36:118-129.

Keeley PE, Carter CH, Thullen RJ. (1987). Influence of planting date on growth of Palmer amaranth (*Amaranthus palmeri*). *Weed Science*, 35(2):199-204.

Mesgaran MB, Matzafri M, Ohadi S. (2021). Sex dimorphism in dioecious Palmer amaranth (*Amaranthus palmeri*) in response to water stress. *Planta*, 254:17.

Montgomery J, Giacomini D, Tranel PJ. (2021a). Molecular confirmation of resistance to PPO inhibitors in *Amaranthus tuberculatus* and *Amaranthus palmeri*, and isolation of the G399A PPO2 substitution in *A. palmeri*. *Weed Technology*, 35(1):99-105.

Montgomery JS, Giacomini DA, Weigel D, Tranel PJ. (2021). Male-specific Y-chromosomal regions in waterhemp (A*maranthus tuberculatus*) and Palmer amaranth (*Amaranthus palmeri*). *New Phytologist*, 229(6):3522-3533.

Montgomery JS, Sadeque A, Giacomini D, Brown P, Tranel PJ. (2019). Sex-specific markers for waterhemp (*Amaranthus tuberculatus*) and Palmer amaranth (*Amaranthus palmeri*). *Weed Science*, 67(4), 412-418.

Nakka S, Godar AS, Thompson CR, Peteron DE, Juglam M. (2017a). Rapid detoxification via glutathione *s*-transferase (GST)-conjugation confers high level of atrazine resistance in Palmer amaranth (*Amaranthus palmeri*). *Pest Management Science*, 73:2236-2243.

Nakka S, Godar AS, Wani PS, Thompson CR, Peterson DE, Roelofs J, et al. (2017b). Physiological and molecular characterization of hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor resistance in Palmer amaranth (*Amaranthus palmeri* S. Wats.). *Frontiers in Plant Science*, 8:555.

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 Science*, 65:681-689.

Noguera MM, Rangani G, Heiser J, Bararpour T, Steckel LE, Betz M, Porri A, Lerchl J, Zimmermann S, Nichols RL, Roma-Burgos N. (2021), Functional PPO2 mutations: co-occurrence in one plant or the same ppo2 allele of herbicide-resistant *Amaranthus palmeri* in the US mid-south. *Pest Management Science*, 77:1001-1012.

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 Science*, 60(sp1):31-62.

Oliveira MC, Jhala AJ, Bernards ML, Proctor CA, Stepanovic S, Werle R. (2022). Palmer Amaranth (Amaranthus palmeri) adaptation to US midwest agroecosystems. *Frontiers in Agronomy*, 4:887629.

Pittman KB, Barney JN, Flessner ML. (2019). Horseweed (*Conyza canadensis*) suppression from cover crop mixtures and fall-applied residual herbicides. *Weed Technology*, 33:303-311.

Priess GL, Norsworthy JK, Godara N, Mauromoustakos A, Butt TR, Roberts TL, Barber T. (2022). Confirmation of glufosinate-resistant Palmer amaranth and response to other herbicides. *Weed Technology*, 36(3):368-372.

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 Management Science*, 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 Science*, 65(6):718-731.

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

Shyam C, Peterson D, Jugulam M. (2022). Resistance to 2,4-D in Palmer amaranth (*Amaranthus palmeri*) from Kansas is mediated by enhanced metabolism. *Weed Science*, 70(4):390-400.

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 Science*, 60:366-373.

Sprague CL, Stoller EW, Wax LM, Horak MJ. (1997). Palmer amaranth (*Amaranthus palmeri*) and common waterhemp (*Amaranthus rudis*) resistance to selected ALS-inhibiting herbicides. *Weed Science*, 45:192-197.

Teasdale JR, Shelton DR, Sadeghi AM, Isensee AR. (2003). Influence of hairy vetch residue on atrazine and metolachlor solution concentration and weed emergence. *Weed Science*, 51:628-634.

Varanasi V, Brabham C, Norsworthy JK. (2018a). Confirmation and characterization of non–target site resistance to fomesafen in Palmer amaranth (*Amaranthus palmeri*). *Weed Science*, 66(6):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 Science*, 66(2):149-158.

Wu C, Goldsmith M, Pawlak J, Feng P, Smith S, Navarro S, Perez-Jones A. (2020). Differences in efficacy, resistance mechanism and target protein interaction between two PPO inhibitors in Palmer amaranth (*Amaranthus palmeri*). *Weed Science*, 68(2):105-115.

Appendix A - List of abbreviations

ΔG210: Deletion of a glycine at the 210th position

2,4-D: 2,4-Dichlorophenoxyacetic acid

ACCase: Acetyl CoA carboxylase

ALS: Acetolactate synthase

ANOVA: Analysis of variance

BBCH: Biologische Bundesanstalt, Bundessortenamt and CHemical industry

Bq: Becquerel

C3: C3 photosynthetic pathway

C4: C4 photosynthetic pathway

CAM: Crassulacean acid metabolism

cDNA: complementary DNA

CT: Threshold cycle

CO_2: Carbon dioxide

DAT: Days after treatment

DCC: Dead cover crop

Dpm: Desintegrations per minute

EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase

G399A: Glycine substitution by an alanine at the 399th position

GCC: Green cover crop

GDD: Growing degree days

GR_{50}: Growth reduction by 50%

GR_{90}: Growth reduction by 90%

GS: Glutamine synthetase

GST: Glutathione *s*-transferase

P450: Cytochrome P450 monoxygenase

POST: Post emergence (herbicide)

PPO: Protoporphyrinogen IX oxidase

PPO2: Isoform 2 of the protoporphyrinogen IX oxidase gene

PRE: Pre-emergence (herbicide)

PSII: Photosystem II

HAT: Hours after treatment

HPLC: High performance liquid chromatography

HPPD: 4-Hydroxyphenylpyruvate dioxygenase

KCTR: Palmer amaranth population from a long-term Conservation Tillage field in Kansas with

KSS: Kansas Palmer amaranth susceptible

KS-1: Kansas Palmer amaranth population 1

KS-2: Kansas Palmer amaranth population 2

lmer: Linear mixed-effect model

lme4: Linear mixed-effect models

Mb: Mega base pairs

MS-1: Mississippi Palmer amaranth population 1

MSS: Mississippi Palmer amaranth susceptible

Resistance to multiple herbicides sites of action

MSY: Male-specific Y chromosomal region

NCC: No cover crop

NTC: Non-template control

NTSR: Non-target-site resistance

PCR: Polymerase chain reaction

R128G/M: Arginine substitution by glycine or methionine at the 128th position

RNA: Ribonucleic acid

SOA: Site of action

TSR: Target-site resistance

TTI: Turbo Teejet Induction

T Av: Average temperature

T Min: Minimum temperature

T Max: Maximum temperature

USA: United States of America

v/v: Volume/volume

WSSA: Weed science society of America