THESIS

INTEGRATED WEED MANAGEMENT: INSIGHTS FROM A WEED RESISTANCE SURVEY AND NON-CHEMICAL WEED SEED CONTROL IN THE CENTRAL GREAT PLAINS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Masters of Science

Colorado State University

Fort Collins, Colorado

Fall 2023

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ABSTRACT

INTEGRATED WEED MANAGEMENT: INSIGHTS FROM A WEED RESISTANCE SURVEY AND NON-CHEMICAL WEED SEED CONTROL IN THE CENTRAL GREAT PLAINS

With the impending release of genetically engineered sugar beet varieties with resistance to glyphosate, dicamba, and glufosinate, significant changes are expected in weed management practices, particularly with regards to in-crop weed control. Glyphosate is used during fallow and in-crop periods, while dicamba is commonly employed in fallow applications, specifically targeting glyphosate-resistant weed species. This study provides insights into the resistance status and frequency of resistance in problematic weed species to the three active ingredients in sugar beet systems across Colorado, Nebraska, and Wyoming. While numerous studies have highlighted the widespread prevalence of glyphosate-resistant kochia and Palmer amaranth across the United States, there is limited research focusing on these species within the context of a sugar beet system. Additionally, our findings reveal the first occurrence of glyphosate-resistant and dicamba-resistant Palmer amaranth populations in Colorado, and dicamba-resistant kochia populations within a sugar beet system. Furthermore, we report that all dicamba-resistant kochia populations tested in Colorado lack a known target-site resistance mechanism, suggesting the involvement of a novel resistance mechanism. Surveys assessing glufosinate resistance in the sugar beet system have not been conducted until now, and we provide valuable baseline information on the resistance frequency for this herbicide prior to an anticipated increase in glufosinate use.

To address the widespread issue of herbicide resistance in various crop systems, it is crucial to adopt alternative strategies that mitigate resistance evolution and maintain the long-term effectiveness of available herbicides. One promising approach is chaff lining, a harvest weed seed control method that has gained popularity in Australia due to its effectiveness in reducing populations of herbicide-resistant ryegrass, especially when combined with other weed control methods. However, the efficacy of chaff lining may be influenced by several factors, including crop and environmental factors, as reported in Australian literature. Scientific studies assessing the applicability and effectiveness of chaff lining in the United States are limited. Recognizing this research gap and aiming to explore the potential of chaff lining, our study investigated its applicability in field settings within the Central Great Plains region of the United States. Through our research, we provide insights into chaff lining efficacy of and highlight the potential inconsistencies that may arise in suppressing weed seeds using this method. Notably, we demonstrate that various factors, including location and environmental conditions, may be involved and impact the effectiveness of chaff lining as a weed management strategy. These findings underscore the importance of integrating chaff lining with other weed management methods to achieve effective and sustainable weed control. Chaff lining, like any other weed management strategy, should not be solely relied upon. Instead, it should be implemented as part of an integrated approach to ensure its long-term effectiveness.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the funds that made this research possible: the USDA National Institute of Food and Agriculture and the Western Sugar Joint Research Committee.

I would like to thank my advisor, Todd A. Gaines, for his unwavering support, availability, and motivation throughout my master's degree. I am also grateful to my friend Eric P. Westra for his teachings, assistance, and advice during my program.

To my family and friends in Brazil, who have always supported me in pursuing my dreams, I am very thankful. To my friends in the CSU weed research laboratory that were always supportive to me.

The authors acknowledge the Western Sugar Cooperative for their assistance in identifying field sites and collecting weed seeds. This research was supported in part by the Western Sugar Joint Research Committee and by the USDA National Institute of Food and Agriculture, Hatch project COL00783 to the Colorado State University Agricultural Experiment Station.

This work was supported by award USDA-NIFA Award 2019-70006-30438, and by USDA-NIFA Hatch project COL00783 to the Colorado State University Agricultural Experiment Station. The authors acknowledge Dr. Vipan Kumar and Dr. Misha Manuchehri for their contributions to conduct field work in Kansas and Oklahoma.

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INTRODUCTION

Weeds pose a significant threat to agronomic crop yields as they compete with desirable plants for essential resources such as water, nutrients, space, and light. While various strategies exist to manage weeds, herbicide applications are widely used for their control. The combination of genetically modified crops and herbicide applications has greatly facilitated post-emergence weed management. However, the excessive reliance on a single herbicide or a limited number of modes of action within crop systems has led to the proliferation of herbicide-resistant weed populations (Brunharo et al., 2022).

Herbicide resistance is a global concern, with over 500 cases of herbicide-resistant weed populations reported worldwide across various agronomic crop systems, including sugar beets (Heap, 2023; Westra et al., 2019). In the current sugar beet system, the selection pressure under glyphosate is intense since this is the most effective and commonly used option for post-emergence weed management (Kniss, 2018). Glyphosate-resistant (GR) kochia has become a major challenge due to the intensive use of glyphosate along with other problematic weed species such as Palmer amaranth, common lambsquarters, and redroot pigweed, requiring alternative techniques to provide an effective control of these species. A new genetically modified event that confers resistance to glyphosate, dicamba, and glufosinate is set to be released soon. It is crucial to gain an understanding of the current resistance status of economically significant weed species in crop systems, as early detection can aid in implementing proactive weed management strategies.

Evolution of herbicide resistance necessitates the exploration of alternative weed management approaches. In Australia, for example, growers have adopted a non-chemical and low-cost strategy known as chaff lining for controlling ryegrass populations with multiple herbicide resistance. This strategy involves harvesting weed seeds along with chaff during the main crop harvest and placing them on the soil surface. Studies have demonstrated the potential of chaff lining in reducing ryegrass emergence (Walsh et al., 2021). However, limited information is available regarding the efficacy of this strategy and the factors influencing its effectiveness in the United States.

In this study, we conducted a survey to assess herbicide resistance status (specifically glyphosate, dicamba, and glufosinate) in major weed species associated with sugar beet cultivation in Colorado, Nebraska, and Wyoming. Our aim is to provide valuable insights to growers and the scientific community regarding the resistance profiles of these herbicides. Additionally, we investigated the practical applicability of the chaff lining strategy and explored abiotic factors such as temperature and humidity that may influence its impact on weed seed emergence.

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CHAPTER 1: BASELINE RESISTANCE SURVEY IN BROADLEAF WEEDS IDENTIFIES GLYPHOSATE AND DICAMBA RESISTANCE PRIOR TO INTRODUCTION OF COMBINED GLYPHOSATE, DICAMBA, AND GLUFOSINATE RESISTANCE TRAITS IN SUGAR BEET

INTRODUCTION

The United States ranks fifth globally in sugar beet production with over 32 million metric tons in 2022 and the Central Great Plains region is one of the biggest sugar beet producers (USDA, 2023). Effective weed control strategies play a crucial role for the success of this crop. The slow-growing nature of sugar beet during its early stages renders it a poor competitor against weeds (Gerhards et al., 2017). Controlling weeds in pre and post sugar beet emergence is fundamental to maintain an appropriate yield and product quality (Bhadra et al., 2020). Before the advent of genetically engineered traits, weeds were a major hindrance in crop production due to the lack of effective herbicide options that often resulted in crop damage (Lueck et al., 2017).

Since the introduction of Roundup Ready sugar beet in 2008, glyphosate substantially facilitated weed management, and weeds were no longer the most problematic pests in sugar beet farms (Morishita, 2018). Glyphosate is extensively utilized as part of the current pre- and post-control program in sugar beet systems, and dicamba is often applied during the fallow season (Bhadra et al., 2020; Cioni and Maines, 2010). However, the utility of both herbicides has decreased in sugar beet farms, possibly due to herbicide selection pressure in fallow and in-crop management, and driven by the resistance evolution capability of certain weed species (Jhala et al., 2021).

Kochia, Palmer amaranth, common lambsquarters, and redroot pigweed are some of the major troublesome weeds in sugar beet systems that can result in significant yield loss when they are not properly managed (Soltani et al., 2018; Van Wychen, 2016). Kochia is a C4 summer-annual broadleaf weed species that is considered invasive and has garnered attention due to its persistence and ability to proliferate. This species is known to produce a staggering amount of seeds, with reports of over 100,000 seeds per square meter (Kumar and Jha, 2015). Furthermore, kochia is noted for its high tolerance to abiotic stressors such as low soil temperature, drought, soil salinity, and heat (Kumar et al., 2019b). This species possesses protogynous flowers, which promote outcrossing and gene flow, resulting in increased genetic diversity that may contribute to the rapid spread of herbicide resistance (Martin et al., 2020). Resistant kochia populations were identified to several herbicide groups, including glyphosate, dicamba, acetolactate synthase inhibitors, among others (Heap, 2023).

Common lambsquarters is an annual weed species that poses a significant challenge in sugar beet production (Bhadra et al., 2020). This weed is capable of wind and insect-mediated pollination and can self- or cross-pollinate. Additionally, its high reproductive capacity allows for a single plant to produce over 70,000 seeds, thereby contributing to its rapid spread and persistence in sugar beet farm. Common lambsquarters resistance was identified for photosystem II (PSII)and acetolactate synthase (ALS) inhibitors (Heap, 2023). Inconsistent glyphosate resistance has been reported and is frequently associated with a growth stage, reduced sensitivity or differential tolerance has been described (DeGreeff et al., 2018; Sivesind et al., 2011; Yerka et al., 2013).

Palmer amaranth and redroot pigweed are problematic amaranth species in sugar beet and both possess prolonged emergence period which presents a challenge for their management (Werle et al., 2014). Palmer amaranth is an annual, dioecious plant species that produces a substantial number of seeds (Ward et al., 2013). This species is an obligate outcrosser, exhibiting a high genetic diversity and pollen-mediated gene flow that contributes to herbicide resistance spread (Jhala et al., 2021; Sosnoskie et al., 2012). GR and dicamba-resistant (DR) Palmer amaranth populations have been reported in several U.S. states (Foster and Steckel, 2022; Kumar et al., 2020; Vieira et al., 2018), but not in Colorado. Redroot pigweed shares comparable botanical characteristics with Palmer amaranth and can cause analogous problems in sugar beet production (Jursík et al., 2008; Soltani et al., 2018). Nonetheless, this particular weed species is monoecious and has a greater occurrence in Colorado.

Target-site resistance (TSR) mechanism to glyphosate have been reported in kochia and Palmer amaranth species (Gaines et al., 2019; Patterson et al., 2019). In both species, increased *EPSPS* gene copy number has been reported and may cause high resistance factor depending on how many gene copies are replicated (Gaines et al., 2016; Giacomini et al., 2019; Godar et al., 2015; Wiersma et al., 2015). TSR mechanism to dicamba has been reported in kochia populations and dicamba efficacy was drastically reduced (LeClere et al., 2018). Understanding weed resistance mechanisms is crucial for implementing effective weed management strategies that enable the development of sustainable and focused practices, such as crop rotation or utilizing different traits to allow for the use of alternative chemicals (Brunharo et al., 2022).

There is ongoing development of a sugar beet event with a triple stack trait that will confer resistance to glyphosate, dicamba, and glufosinate. The introduction of novel herbicide options for post-emergence weed control in sugar beet is anticipated to improve weed management, particularly during the challenging early growth phase. Although new alternatives are expected to aid in post-emergence weed management, the use of these three active ingredients is not a new approach. Glufosinate is not a current herbicide in sugar beet system, but resistance to this herbicide has been reported in weed species in different crop systems (Carvalho-Moore et al., 2022; Priess et al., 2022a). A previous survey (Westra et al., 2019) conducted from 2011 to 2014 in Colorado reported resistance in kochia to glyphosate and dicamba. Currently, there is limited available information regarding the current resistance status of major weed species in sugar beet to glyphosate, dicamba, and glufosinate.

In this study, we conducted a resistance survey in 2020 and 2021, with the aim of addressing the concerns of growers and predicting the efficacy of the three herbicide resistance traits for weed management in the sugar beet growing areas of Colorado, Nebraska, and Wyoming. Our focus was on four weed species belonging to the *Amaranthaceae* family: kochia, Palmer amaranth, redroot pigweed, and common lambsquarters. The objectives of this study were two-fold: (1) to determine the geographical distribution of glyphosate, dicamba, and glufosinate resistance in the regions of Colorado, Nebraska, and Wyoming, and (2) to investigate whether resistance observed in kochia and Palmer amaranth populations was due to previously documented TSR mechanisms.

MATERIALS AND METHODS

1.1 Sample collection

Seeds were collected during the autumn of 2020 in Wyoming and Nebraska, and in 2021 in Colorado. The locations of sugar beet farms were obtained from the Western Sugar Cooperative, and all growers were contacted prior to the collection. A total of thirty-seven sugar beet fields were visited. The collection was carried out by driving transects, ensuring a minimum distance of eight kilometers between each cropping area. To minimize data collection bias, sugar beet farms along the border and on side roads were specifically targeted, regardless of whether there were reports of herbicide failure in those areas. At each collection site, ten to fifteen plants of each weed species were threshed to harvest the seeds, which were then combined to form a population sample. The latitude and longitude of each area were recorded and georeferenced using a portable GPS device (Trimble Geo XH 2005 series: Trimble Boulder, Boulder, CO). Weed populations from Nebraska and Wyoming were sent by mail from the Western Sugar Cooperative to Colorado State University, and the latitude and longitude was recorded for each sample. In Nebraska, kochia, Palmer amaranth, and common lambsquarters were surveyed, while in Wyoming, only kochia was surveyed and Colorado the four species were tested.

2.2 Greenhouse bioassay

To obtain a representative sample from each collection site, seeds of kochia, common lambsquarters, redroot pigweed, and Palmer amaranth were separated from dried shoots. This was done using sieves of various sizes in conjunction with a seed blower to achieve a homogenized progeny sampling. Individual seeds from each population were then planted in a plug tray with cells measuring 1.3 cm by 1.3 cm by 2.5 cm (American Clay Works, Denver CO). Seedlings were grown to a height of 3 cm. Subsequently, they were transplanted into pots measuring 3.8 cm by 3.8 cm by 5.8 cm (American Clay Works, Denver, CO). For each herbicide, a total of 96 plants (32 individuals per herbicide) were screened per collection site, when they reached a height of 5-7 centimeters. Glyphosate, dicamba, and glufosinate were the herbicides tested, and a known susceptible line was planted alongside for comparison during the screening process. Throughout the experiment, all plants were maintained in a greenhouse with a temperature range of 22 to 26 °C and a photoperiod regimen of 10-14 h of light per day. Additionally, they were irrigated daily to ensure they remained at field capacity of soil-less media.

2.3 Herbicide Applications

Each collected population was individually subjected to screening for glyphosate, dicamba, and glufosinate. Weed species were treated with glyphosate (RoundUp Weathermax®, 840 g ae ha^{-1}, Bayer CropScience LP, 800. North Lindbergh Blvd, St. Louis, MO 63167, USA) adding ammonium sulfate (AMS) at a concentration of 20 g L^{-1}; dicamba (Engenia®, 280 g ae ha^{-1}, BASF Corporation, 100 Park Avenue, Florham Park, NJ 07932, USA) with nonionic surfactant (Induce®) at a concentration of 0.25% v/v, and glufosinate (Liberty®, 590 g ai ha^{-1}, BASF Corporation, 26 Davis Drive, Research Triangle Park) along with a nonionic surfactant at a concentration of 0.25% v/v. Herbicide applications were carried out using a laboratory chamber single-nozzle sprayer (DeVries Generation III Research Sprayer, MN, Hollandale), calibrated to deliver 187 L ha^{-1}. Discriminating rates for kochia, common lambsquarters, and Palmer amaranth were selected based on the recommended dosage specified on each herbicide label. Phenotype characterization was conducted by comparing each treated population to a known susceptible reference and an untreated population. Individual plants that survived were visually assessed, and their status was categorized as resistant if they remained alive after a 4-week period, regardless of whether they exhibited herbicide injury or not. Survival frequency (%) was calculated by dividing the number of survivors at each herbicide rate by the total number of screened plants. Phenotype classification followed previously established categories (Owen et al., 2007), where populations with <1% survivors were categorized as susceptible, those with 1% to 19% survivors were classified as having low frequency, and populations with >20% survivors were classified as resistant. Collection sites were geographically referenced, and a map was created using QGIS software version 3.28.3, employing the WGS84 (EPSG:4326) coordinate system.

2.4 Laboratory assays

Glyphosate and dicamba herbicide resistance mechanisms were investigated in populations that were categorized as resistant (over 20% of survival) from the preliminary greenhouse screening. Increased *EPSPS* copy number was assessed for glyphosate resistance for each collection site. Young tissue material (100 mg) was collected from three randomly selected survivors and put into a 2 mL Eppendorf tub in liquid nitrogen and stored at -20 C when not in use. Samples were homogenized using a TissueLyser II (Qiagen). Genomic DNA extraction from each sample was conducted using a DNA extraction kit (Zymo quick DNA extraction). Genomic DNA was eluted in 50 µL of nuclease free water, and concentration and quality were verified using a NanoDROP 1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA). Relative *EPSPS* copy number was determined using quantitative polymerase chain reaction (qPCR) on the genomic DNA. Primers were as described previously (Gaines et al., 2021). *ALS* gene was utilized as a single copy reference gene. Each qPCR reaction was 20 µL, including 10 µL of PerfeCTa *SYBR*® green Supermix (Quanta Biosciences), 1.2 µL of the forward and reverse primers [5 µM final concentration], and 5 µL gDNA (10 ng). Reactions were performed in a BioRad CFX Connect Real-Time for all samples. The temperature for each of the reactions was utilized as follows: denaturation step was held at 95 C for 3 min, followed by 30 cycles of denaturation at 95 C for 30 s, and annealing/extension step at 72 for 30 sec, fluorescence measurements taken after each cycle. Melt curve analysis was conducted to determine the number of PCR products formed in each reaction where temperature was increased from 65 C to 95 C in 0.5 C increments. Melt-curve analysis using both *EPSPS* and *ALS* primers revealed only a single PCR product, confirming that the PCR amplifications were specific to the intended genes, and indicating the reliability and accuracy of the PCR reaction. Relative *EPSPS* gene copy number was determined using the $2^{\Delta Ct}$ ($\Delta C_t = C_t^{ALS} - C_t^{EPSPS}$) (Schmittgen and Livak, 2008). This method was applied for kochia and Palmer amaranth using three biological replicates (survival) and two technical replicates. Mean and standard error of the mean of the relative *EPSPS* copy number was calculated for each population. To establish a reference for comparison of experimental results and to verify the accuracy of the assay, a known population (Res) that is resistant to glyphosate was included, one known susceptible and known resistant populations were used as negative and positive control, respectively.

Dicamba TSR mechanism was investigated in populations of kochia classified as resistant in the previous greenhouse screening. *AUX/IAA16* (GenBank: MF376149.1) gene was sequenced to verify the presence of the $G_{73}N$ substitution in the degron region previously reported (LeClere et al., 2018). RNA extraction was carried out using a Zymo Quick RNA extraction kit following the manufacturer's recommendations. Extracted RNA was checked for quality and quantity using Nanodrop, employing the same methodology used for glyphosate previously described. Subsequently, cDNA was synthesized from the RNA product using a ProtoScript® II kit with random primers. PCR detection was performed using 1 µL of cDNA, 12.5 µL of EconoTaq® PLUS & PLUS GREEN 2× Master Mixes (Lucigen, USA), 2 µL of the forward primer, 2 µL of the reverse primer, and 6.5 µL of water, resulting in a total volume of 25 µL for each sample. PCR products were visualized on a 1.0% agarose gel stained with RedSafeTM Nucleic Acid Staining Solution (iNiTron, Korea). Palmer amaranth populations categorized as DRwere not investigated in this study for resistance mechanisms.

RESULTS AND DISCUSSION

3.1 Glyphosate status

A total of 37 sugar beet fields were surveyed across eastern Colorado to assess the presence of weeds. In total, 36 of the surveyed fields were infested with kochia (Figure 1.1), 5 fields had Palmer amaranth (Supplementary Figure 1), 23 fields had common lambsquarters (Supplementary Figure 2), and 18 fields had redroot pigweed (Supplementary Figure 3). In Nebraska, 50 collection sites had kochia (Figure 1.2), 6 fields had Palmer amaranth (Supplementary Figure 4) and 11 fields had common lambsquarters (Supplementary Figure 5). In Wyoming only kochia was present in a total of 12 fields (Figure 1.3) surveyed. Among these weed species, kochia was likely the most problematic in Colorado, Nebraska, and Wyoming in sugar beet farms since this weed species was present and widespread in most of the survey collection areas.

Screening of kochia accessions in Colorado revealed that around 75% of the collected samples were classified as GR, while 19% exhibited low resistance and 6% were susceptible. The occurrence rate of glyphosate survival among kochia populations varied from 0% to 100%. For Nebraska, 86% of populations were classified as resistant, 12% as low resistance and only 2% as susceptible and Wyoming 33% was categorized as resistant, 42% low resistance, and 25% as susceptible (Figure 1.3A). Continued glyphosate selection pressure in sugar beets fields may cause low resistance kochia populations to rapidly evolve into resistant populations. This might be compounded by the difficulty faced by growers and agronomists in detecting a few resistant individuals in the field, as pesticide regulators typically set the threshold for herbicide control at 80%. Moreover, kochia intrinsic ability to outcross, facilitated by its protogynous nature, may accelerate this process by allowing gene flow quickly among populations. This could lead to a rapid spread of glyphosate resistance across generations.

Since GR sugar beet was commercialized, Colorado, Wyoming, and Nebraska had 85 to 90 % trait adoption in the first year (Khan, 2010). After fifteen years of utilizing this technology, weed management strategies continue to heavily rely on glyphosate through in-crop and fallow applications, making it the primary method for controlling weeds in sugar beet systems (Kniss, 2018; Morishita, 2018). This reliance on glyphosate is likely contributing to the evolution of resistance over time by selecting for resistant populations. Evolution of GR populations of kochia is a significant issue in North America, which has been recorded in multiple states across the United States and in Canada (Heap, 2023). For instance, a survey on kochia conducted in 2014 in 96 populations primarily from the wheat-fallow system in eastern Colorado showed that 23% of accessions were GR (Westra et al., 2019). By comparison, a survey conducted in 2018 in Manitoba identified a resistance rate of 59% in 315 sites, where the highest frequency of GR kochia was identified in GR crops such as soybean and corn (Geddes et al., 2021). Likewise, our study uncovered a noteworthy proportion of GR populations (Figure 1.4). Specifically, we observed the presence of GR populations in Colorado, Nebraska, and Wyoming at frequencies of 94%, 98%, and 75% respectively, considering both low-resistant and resistant populations.

In general, kochia glyphosate survival exhibited minimal to no damage following treatment at the field rate. Copy number variation assay targeting *EPSPS* genes revealed that all surviving individuals from Colorado and Nebraska exhibited a higher number of *EPSPS* gene copies (more than 3) compared to a known susceptible population (Sus) (0.8~1.5 copies) (Figure 1.5). This finding provides an explanation for the observed resistance phenotype, although there could be additional underlying mechanisms involved. For instance, one kochia population from Wyoming was classified as resistant, but individual survivors did not show an increased copy number (Figure 1.6). The resistance mechanism in this particular population remains unknown, and there are no reports of other resistance mechanisms in kochia apart from *EPSPS* gene amplification. Previous studies have shown a correlation between an increase in *EPSPS* copies and a reduction in glyphosate efficacy, which corresponds to an increase in resistance levels (Gaines et al., 2016; Godar et al., 2015). This mechanism of resistance has been observed in various weed species, such as *Amaranthus palmeri*, *Helianthus annuus*, and *Salsola tragus*, where none was controlled by glyphosate (Gaines et al., 2011; Singh et al., 2020; Yanniccari et al., 2023). The presence of multiple copies of this gene results in an increased amount of the target enzyme, which ultimately reduces the effectiveness of glyphosate at the field rate (Wiersma et al., 2015). Nuclear inheritance of resistance plays a role in the dissipation of increased gene copy number across generations, which may be an important factor to consider that contributes to the evolution of glyphosate resistance in kochia (Jugulam et al., 2014). This implies that a susceptible plant can produce resistant offspring if it gets pollinated by a resistant plant. In addition, evolution of resistance may be facilitated by seed and pollen gene flow, along with the natural protogynous characteristics of kochia that enable cross-pollination. Additionally, kochia's ability to function as a tumbleweed and disperse seeds over long distances facilitates the spread of herbicide resistance in this species (Beckie et al., 2016). Geddes et al. (2021) observed a drastic reduction in glyphosate efficacy in controlling kochia over the years mainly in areas with GR crop where they identified 78% and 70% of GR kochia population in soybean and corn areas, respectively. The same authors observed an increase in glyphosate resistance ranging from 1% to 59% in just five years. A survey conducted among stakeholders in Nebraska revealed that glyphosate was the primary post-emergence herbicide used in GR corn and soybean crops, and kochia was one of the top five weeds considered most challenging to manage statewide (Sarangi and Jhala, 2018). The nearly exclusive reliance on glyphosate for in-crop post-emergence weed control in GR soybean fields in Brazil has led to the emergence of resistant weed species such as

Amaranthus palmeri, *Conyza sp*., *Chloris elata*, *Digilaria insularis*, and *Eleusine indica* (Correia and Durigan, 2010). Considering the current glyphosate resistance scenario, it is improbable that farmers will experience significant benefits from the GR technology included in the new triple-stack sugar beet event for managing kochia, especially in Nebraska where most populations were classified as resistant. Nonetheless, glyphosate may still have value in controlling other weed species in the region and can be combined with other modes of action to reduce the evolution of resistance.

For Palmer amaranth populations only a few numbers of accessions were identified and collected in Colorado (Supplementary Figure 1) and Nebraska (Supplementary Figure 4), but not in Wyoming. Based on our survey, four out of the five Palmer amaranth populations collected in Colorado were GR, representing 80% of the total population. For Nebraska, in the total of eight populations, one population was classified as resistant (13%), three as low resistant (38%) and four susceptible (48%) (Figure 1.2B). The relatively low number of Palmer amaranth populations on these areas could be attributed to the environmental conditions such as dry and cold weather that are distinct from the southwestern United States and northwestern Mexico where this species is indigenous (Ward et al., 2013). Despite the relatively low number of identified Palmer amaranth populations, it is alarming that the majority of these populations in Colorado have been classified as GR. Due to its dioecious nature, this species has a high potential for evolving and spreading resistance through gene flow via pollen similarly as kochia. The majority of the identified resistance mechanisms so far have been nuclear inherited, including gene amplification which contributes to the rapid herbicide evolution (Murphy and Tranel, 2019). In all surveyed populations classified as resistant, an increase in relative *EPSPS* gene copy number was observed compared to the negative control (Figure 1.7), which possessed approximately one copy of *EPSPS*. Resistance to glyphosate in Palmer amaranth accessions has been well-documented in various studies from different states in the United States. Gaines et al. (2010) reported that some populations of Palmer amaranth had 160-fold more copies of the *EPSPS* gene compared to a known susceptible population in a population from Georgia. While GR Palmer amaranth populations have been reported in twenty-six states (Heap, 2023), there have been no previous reports of GR Palmer amaranth in Colorado until now.

Common lambsquarters was surveyed in Colorado and Nebraska while redroot pigweed was identified in Colorado only. All the herbicides tested provided 100% control in common lambsquarters and redroot pigweed populations surveyed, and populations were classified as susceptible (Supplementary Figures 2, 3, 5). Populations of common lambsquarters have been identified as resistant to acetolactate synthase inhibitors and photosystem II inhibitors (Heap, 2023), but not to any of the three herbicides evaluated. Several studies have highlighted inconsistencies in glyphosate efficacy for controlling common lambsquarters. These inconsistencies could be attributed to species tolerance which may vary depending on the growth stage of the plant. Additionally, reduced efficacy of glyphosate could be influenced by environmental conditions such as rainfall occurring after herbicide applications. Schuster et al. (2007) observed a decrease in glyphosate efficacy going from 80% injury in plants with 2.5-cm to 55% in plants with 7.5 to 15-cm at 21 days after application. Sivesind et al. (2011) noticed a reduction in glyphosate efficacy associated with growth stage, where the ED_{50} (effective dose for 50% control) was three times higher in 20-cm plants compared to 10-cm plants. Enhanced glyphosate response in plants at the 5 to 7-cm growth stage was reported when compared to taller plants, particularly in cooler temperatures, when treated with glyphosate at a rate of 840 g ae ha^{-1} (DeGreeff et al., 2018). In our survey, common lambsquarters accessions were effectively controlled when treated at a height of 5 to 7 cm under controlled conditions in a greenhouse setting. These findings underscore the importance of timing and appropriate management strategies for this weed species.

There have been few reported cases of herbicide resistance in both common lambsquarters and redroot pigweed across different modes of action when compared to kochia and Palmer amaranth, with most cases being related to photosystem II inhibitors. It is well-documented that resistance to photosystem II inhibitors is primarily inherited maternally (Ghanizadeh et al., 2019). Unlike kochia and Palmer amaranth, these weed species have limited mechanisms for spreading resistance. Both species are predominantly autogamous, meaning that gene flow is predominantly restricted within their own seeds. A recent study by Moghadam et al. (2023) demonstrated that common lambsquarters and redroot pigweed exhibit low genetic diversity within populations but high diversity when compared to other populations.

This suggests that each population is distinct and requires an independent approach to weed management, with particular focus on controlling seed production and preventing seedbank replenishment. Here we highlight that the new sugar beet trait may facilitate the management of these two weed species since more option in post-emergence will be available for use, however it is important to consider that, although resistance cases are not present in our study, resistance to glyphosate and dicamba have been reported in redroot pigweed and common lambsquarters in other geographical locations (Heap, 2023; Rahman et al., 2014). It is crucial to avoid practices that could contribute to the gene flow of resistant seeds from one area that resistance is present to another that is not, for instance sharing machinery. Mitigating evolution of herbicide resistance in these species requires careful attention to seed dispersal and the implementation of effective management strategies.

3.2 Dicamba status

Kochia populations classified as resistant were identified in Colorado (Figure 1.1B) and Nebraska (Figure 1.2B) and low frequency in Wyoming (Figure 1.3B). In Colorado, 8% of the populations were categorized as resistant, 22% as low resistant, and 70% as susceptible. The survival frequency within the resistant populations in Colorado ranged from 56% to 88%. In Nebraska, 50 populations were surveyed, and one population was classified as DR, representing 2% of the total collection sites surveyed. Meanwhile, 40% of the populations showed low resistance, and 58% were susceptible. In Wyoming, 83% of the kochia populations surveyed were susceptible, 17% showed low resistance, and none were resistant. One Palmer amaranth population out of five collected in Colorado was classified as DR (Supplementary Figure 6) corresponding to 20% of survival frequency, and 80% were classified susceptible. For Nebraska out of 8 populations (Supplementary Figure 7), 0% was resistant, 38% as low resistance and 49% as susceptible (Figure 1.2B). Our data indicates a limited number of DR populations across these states when compared to glyphosate status, however a notable proportion of populations categorized as low resistant (with survival rates ranging from 1% to 20%) was identified. It is essential to reemphasize that this classification does not inherently imply that these populations are more sensitive to the herbicide compared to individual survivors within a resistant population. Rather, these populations may exhibit heterogeneity, justifying the heterogeneity and the resistant trait is likely segregating within each population. The frequency and uniformity of a resistant phenotype within a population will depend on the species' capacity to evolve and spread resistance, which is also strongly influenced by the management practices over the years (in-crop versus fallow applications) implemented on Sugar beet farms. In other words, a collection site that currently possess a low resistant population could potentially evolve to a resistant in subsequent years if the selection pression under dicamba is intensive, allowing survival to spread. It is worth noting that this topic remains controversial, as some authors may consider the resistance in development as a classification for population with low resistance frequency. Although there are no reports of dicamba-resistant (DR) kochia in sugar beet system until now, the issue of resistance to auxin-mimic herbicides is a growing problem in the United States, with reports of dicamba resistance emerging as early as the 1990s (Keith et al., 2011; Preston et al., 2009). Since then, several other cases have been reported in six states in the United States and in Canada (Beckie et al., 2019; Geddes et al., 2022; Heap, 2023; Kumar et al., 2019a; Westra et al., 2019). The rapid spread of GR kochia populations led to an increased use of dicamba as an alternative in several crop systems as well as the raised the number of resistance cases (Ou et al., 2018b). The majority of DR cases reported thus far have been identified in cereal crop systems where dicamba is extensively employed in-crop management, such as corn, sorghum, and wheat (Heap, 2023). A survey revealed a DR Palmer amaranth from Tennessee in DR soybean and cotton crop system, but the resistance mechanism remains unknown (Foster and Steckel, 2022). DR Palmer amaranth have not been reported in Colorado until now (Supplementary Figure 6), but further analysis is under processing to validate this phenotype.

All kochia and palmer populations from Colorado categorized as DR were also categorized as GR, whereas the population classified as DR from Nebraska was classified as low resistance for glyphosate. In our survey we observed that GR and DR kochia and Palmer amaranth is an emerging issue within the sugar beet areas in the Central Great Plains even before the trait is released. The combination of glyphosate and dicamba is a very common practice in the fallow season, however, studies have shown that this practice might not be the most optimal to manage weed resistance in some cases. Ou et al. (2018a) demonstrated that applying glyphosate and dicamba in combination led to reduced translocation of both herbicides, significantly compromising their performance leading to a poor control of kochia populations. Pesticide mixtures may mediate an evolutionary trade-off in weed resistance management where while using a combination of herbicides it may contributes with lower level of a TSR resistant mechanism, but increase chances of NTSR to evolve (Comont et al., 2020). Furthermore, Rigon et al. (2023) demonstrated that herbicide tank mixtures at sub-lethal doses may have led to a recurrent selection of barnyardgrass populations and decreased herbicide sensitivity during the years that may potentially be associated with selection of detoxifying genes and NTSR mechanisms. Currently, two known auxin TSR mechanisms have been identified in weeds, both occurring in the degron regions of Aux/IAA co-receptor genes. In kochia populations, an amino acid substitution in the degron region of the Aux/IAA co-receptor gene *IAA16* has been reported as the causative factor for the observed resistance phenotype. Similarly, in Indian hedge mustard a deletion has been identified in the degron region of the Aux/IAA co-receptor gene *IAA2*, resulting in resistance *(Figueiredo et al., 2022; LeClere et al., 2018)*.

The presence of the $G_{73}N$ amino acid substitution in the degron region of the *AUX/IAA16* was investigated as the TSR mechanism in three kochia populations from Colorado (A5, A22, and A32) and one from Nebraska (NBK 30) that were categorized as resistant in our previous screening. Upon sequencing the *AUX/IAA16* gene in dicamba-surviving individuals, populations from Colorado did not exhibit any amino acid substitution in this region. However, all surviving individuals from Nebraska population NBK30 had the $G_{73}N$ substitution (Figure 1.8). The $G_{73}N$ hinders the degradation of the AUX/IAA protein signaled through dicamba binding and TIR/AFB ubiquitination, preventing the release of auxin-responsive factors and leading to an auxin-mimic herbicide resistant phenotype (LeClere et al., 2018). These findings suggest that the Colorado population likely possesses a distinct and novel resistant mechanism that could either be a NTSR mechanism or a yet unknown TSR mechanism, while the DR phenotype observed in the Nebraska population is attributed to the known TSR mechanism, although it might contain additional mechanisms. While reports of auxin-mimic herbicides target-site resistance mechanisms are relatively limited, NTSR mechanisms to auxin-mimic herbicides have been documented in various studies and are often associated with cross-resistance to other modes of action (Dang et al., 2018; De Figueiredo et al., 2022; Souza et al., 2023).

While the new sugar beet trait may provide enhanced weed management capabilities, it is imperative to employ alternative herbicides or other weed management strategies during fallow periods rather than relying on dicamba alone. Research has shown that implementing diverse herbicide programs, particularly in conjunction with crop rotations, can be an effective strategy for controlling resistant populations of kochia (Sbatella et al., 2019). Therefore, adopting a comprehensive approach to weed management, tailored to the specific field conditions and considering the resistance history in the area and the weed species present, becomes fundamental.

3.3 Glufosinate status

Results in our survey revealed that all weed populations surveyed from Colorado, Nebraska, and Wyoming were controlled by glufosinate and consequently categorized as susceptible. This includes populations of Kochia and Palmer amaranth that exhibited resistance to glyphosate and dicamba (Figures 1.1C, 1.2C, 1.3C). While glyphosate and dicamba are extensively utilized in the current weed management system for sugar beets in the Central Great Plains, glufosinate is not currently included. One possible reason for this omission is the availability of more cost-effective options for eliminating weeds before planting sugar beets, which also provide a broader range of weed control. With the introduction of a new sugar beet trait resistant to glufosinate, its usage in the system is expected to increase in herbicide programs, particularly for postemergence applications.

To date, ten cases of herbicide resistance to glufosinate have been documented where most of them occurred in poaceous species and recently in *Amaranthus palmeri* (Heap, 2023). Increase in gene expression and gene amplification is the resistance mechanism in populations of Palmer amaranth from Arkansas (Carvalho-Moore et al., 2022). A novel point mutation $Ser_{59}Gly$ is in contact with important binding residues of glufosinate and was recently reported to confer resistance in *Eleusine indica* population from China (Zhang et al., 2022).

In our study glufosinate was effective in all species. We emphasize that the plants were treated in an early growth stage under controlled conditions in a greenhouse setting. Glufosinate is a contact herbicide that requires appropriate coverage, and the timing is crucial to achieve a proper performance. Weed growth state and species intrinsic characteristics (leaf type and angle cuticular waxes) should be accounted when using glufosinate (Takano and Dayan, 2020). Kumar et al. (2014) observed that the efficacy of glufosinate, applied at the same rate as in our study, was the lowest among the herbicide treatments for controlling 8-10 cm tall kochia populations, with control levels below 50%. Similarly, Duenk et al. (2023) noted that glufosinate application provided a poor control of common lambsquarters, velvetleaf, and redroot pigweed when they were above 5 cm, improving its performance to control common lambsquarters when added AMS, and they concluded that application timing is weed species specific.

Environmental conditions directly affect glufosinate performance, specifically, light intensity and low humidity can drastically decrease glufosinate efficacy on controlling weeds (Takano and Dayan, 2020). Colorado, Nebraska, and Wyoming possess a continental climate and generally experience a relatively low humidity climate with some fluctuations during the summer. These conditions have direct implications for glufosinate applications. Under dry conditions the absorption of glufosinate may be not optimal due to a rapid dryness of the droplets reducing its efficacy (Coetzer et al., 2001; Takano and Dayan, 2020).

Implementing an appropriate herbicide program is essential to prolong the effectiveness of glufosinate and ensure the sustainability of the HR sugar beet. Careful consideration should be given to employing a well-designed herbicide strategy, and especially considering the weed species in the area. For instance some weed species may respond differently when glufosinate is applied in a mixture or sequential, where in some cases, antagonistic likely occur when herbicide combinations are employed. Besançon et al. (2018) noted that when glufosinate and glyphosate were combined to control giant foxtail and velvetleaf there was a significant reduction of glyphosate translocation. The mixture of dicamba and glufosinate was antagonistic reflecting in poor control and percent mortality in Palmer amaranth populations (Priess et al., 2022b). In contrast, a synergistic interaction was observed using applications of glufosinate and dicamba to control sicklepod (Joseph et al., 2018). In giant ragweed glufosinate plus dicamba showed to have an addictive effect (Ganie and Jhala, 2017).

Considering these findings, our survey demonstrated that resistance to two of the three herbicides that the new sugar beet trait will be resistant to is already present in sugar beet areas of Colorado, Nebraska, and Wyoming, even prior to the release of the new trait. To ensure the long-term effectiveness of this new technology, it is crucial to implement proper stewardship practices for the next resistant trait, learning from the mistakes made in the current sugar beet crop system. Employing alternative herbicides for effective weed management, along with an integrated pest management approach, becomes critical to mitigate the evolution of resistance and preserve the utility of the new sugar beet trait.

FIGURES

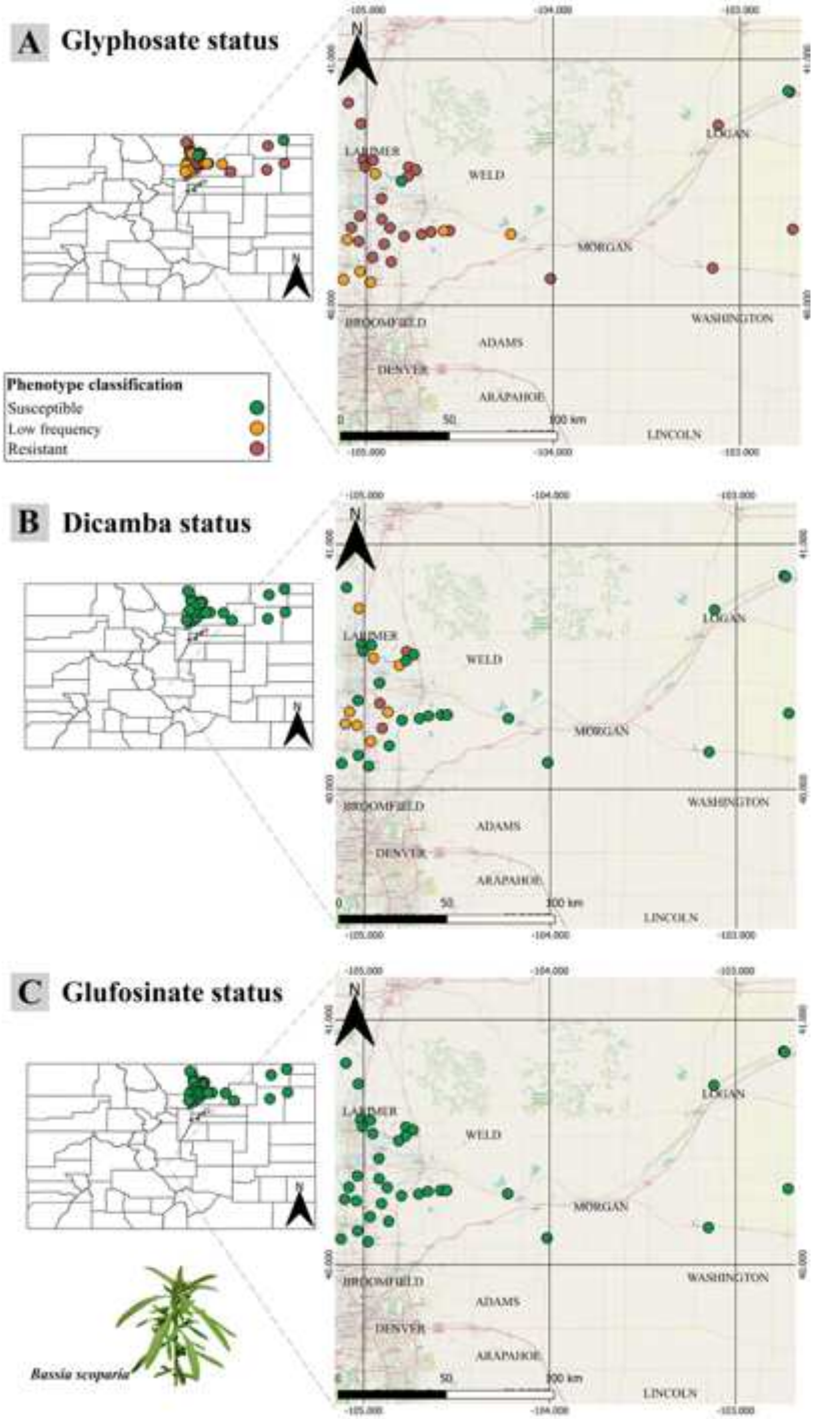

Figure 1.1: Geo-referenced map illustrating the kochia populations collected in Colorado during the fall of 2021. The dots on the map represent the locations of kochia populations, and their color signifies their response to glyphosate treatment (A), dicamba (B) and glufosinate (C). In the left corner, a separate map illustrates the distribution of the populations in a state overview. On the right side, a close-up map focuses on the main counties where the samples were collected. Populations classified as resistant (>20% survival)

are represented by red dots, while yellow dots indicate low frequency (1-19% survival), and green dots represent susceptible populations (0% survival).

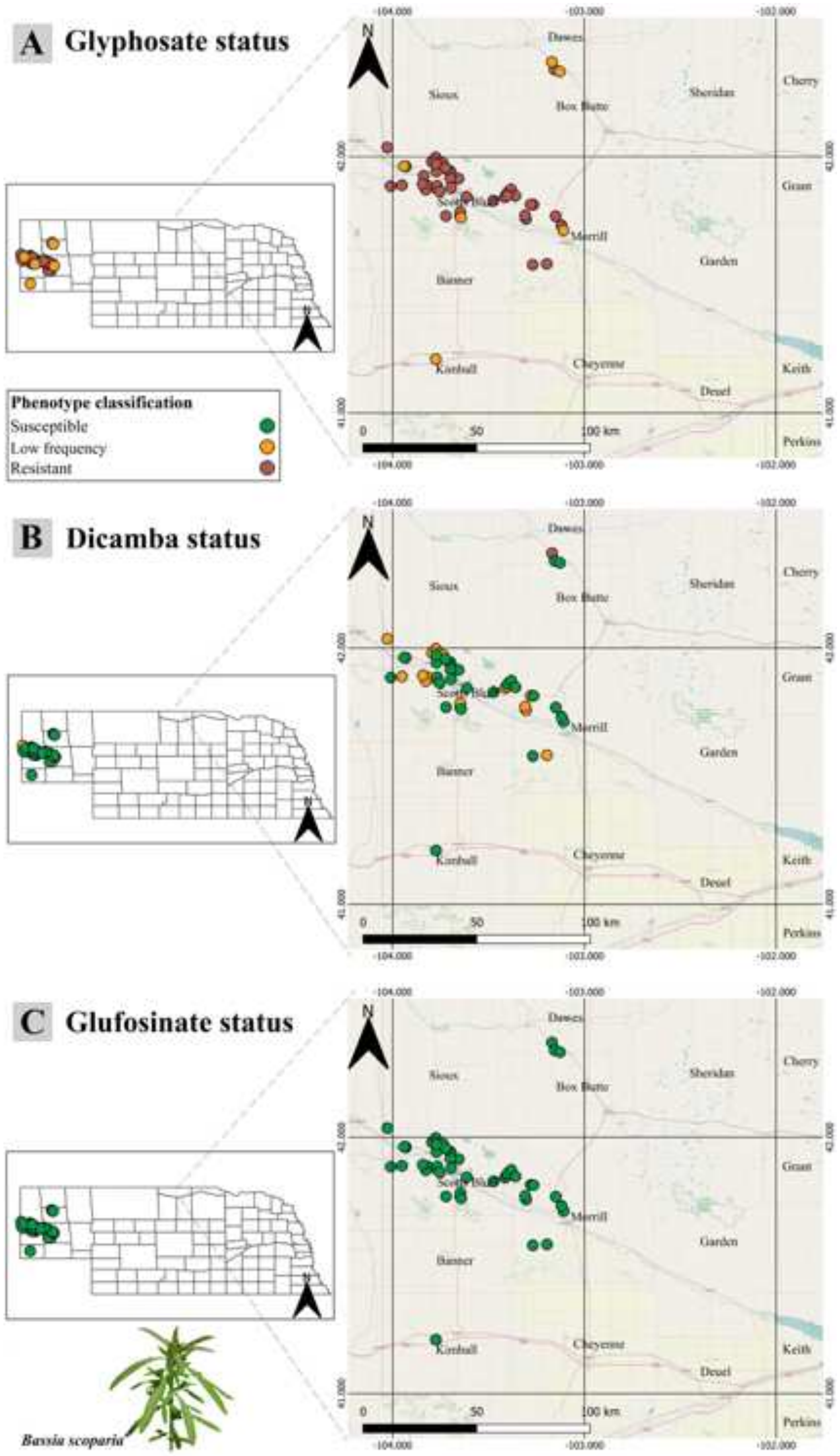

Figure 1.2: Geo-referenced map illustrating the kochia populations collected in Nebraska during the fall of 2020. The dots on the map represent the locations of kochia populations, and their color signifies their response to glyphosate treatment (A), dicamba (B) and glufosinate (C). In the left corner, a separate map illustrates the distribution of the populations in a state overview. On the right side, a close-up map focuses on the main counties where the samples were collected. Populations classified as resistant (>20% survival) are represented by red dots, while yellow dots indicate low frequency (1-19% survival), and green dots represent susceptible populations (0% survival).

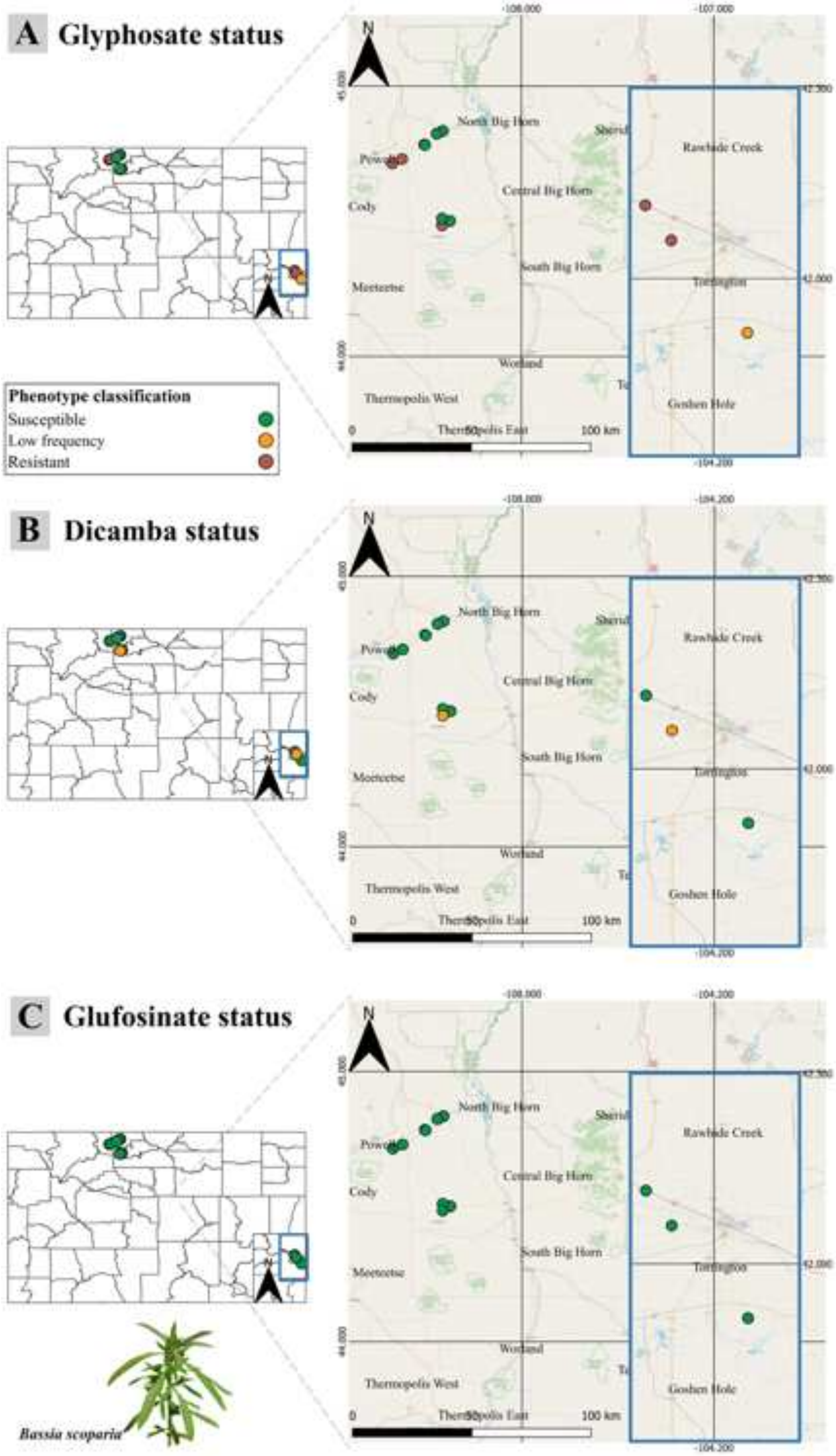

Figure 1.3: Geo-referenced map illustrating the kochia populations collected in Wyoming during the fall of 2020. The dots on the map represent the locations of kochia populations, and their color signifies their response to glyphosate treatment (A), dicamba (B) and glufosinate (C). In the left corner, a separate map illustrates the distribution of the populations in a state overview. On the right side, a close-up map focuses on the main counties where the samples were collected, including the highlighted blue square where a few samples were collected in the Southeast of Wyoming. Populations classified as resistant (>20% survival)

are represented by red dots, while yellow dots indicate low frequency (1-19% survival), and green dots represent susceptible populations (0% survival).

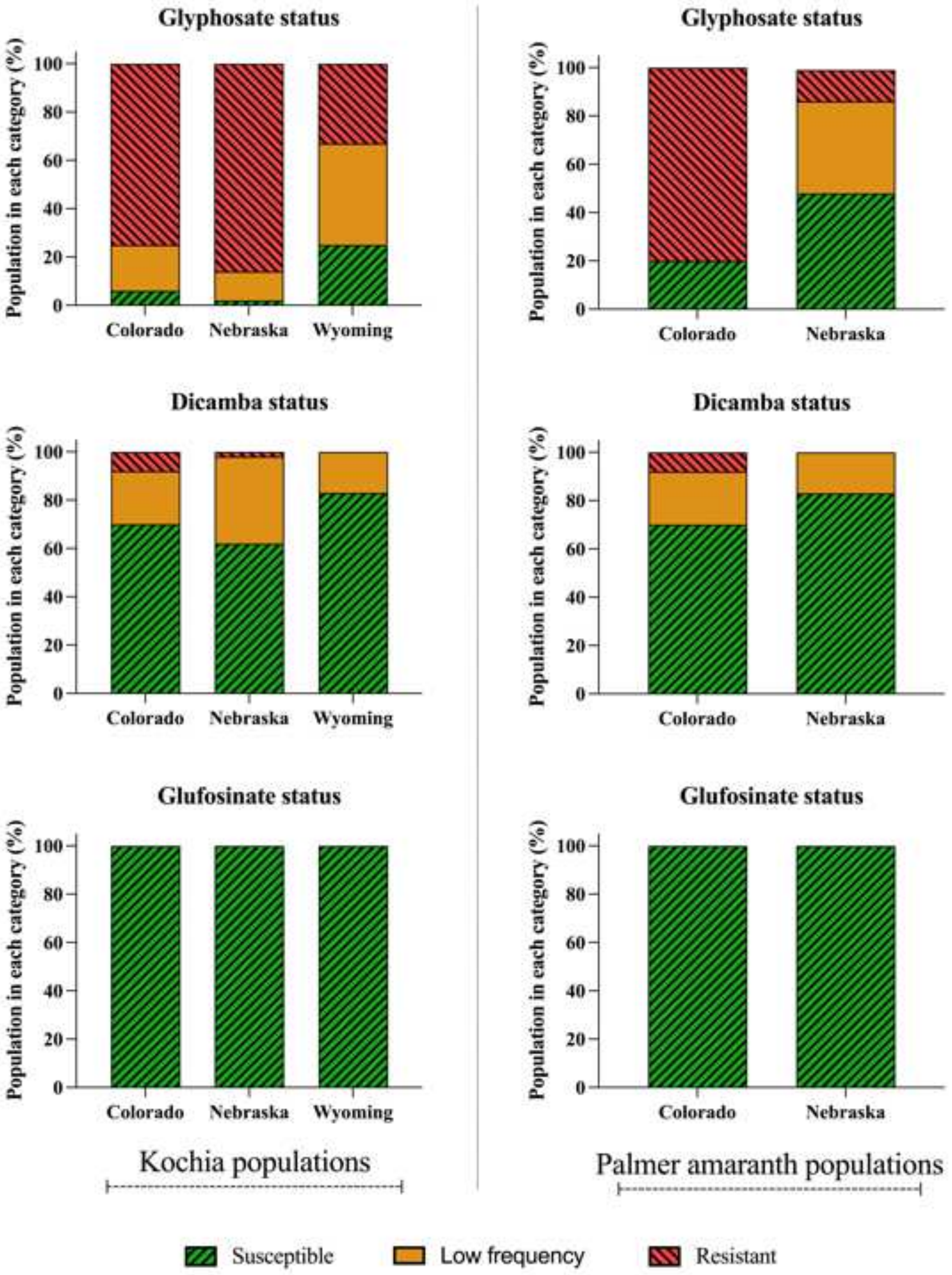

Figure 1.4. Frequency of observed phenotypes of kochia (left) and Palmer amaranth (right) populations collected from Colorado, Nebraska and Wyoming during the fall of 2020 and 2021, following treatment in a greenhouse setting with glyphosate, dicamba, and glufosinate. Bar colors represent the phenotype characterization where: green (dashed to the right) are susceptible (0% survival), yellow represents low resistance (1% to 19% survival), and red (dashed to the left) are populations classified as resistant (>20% survival).

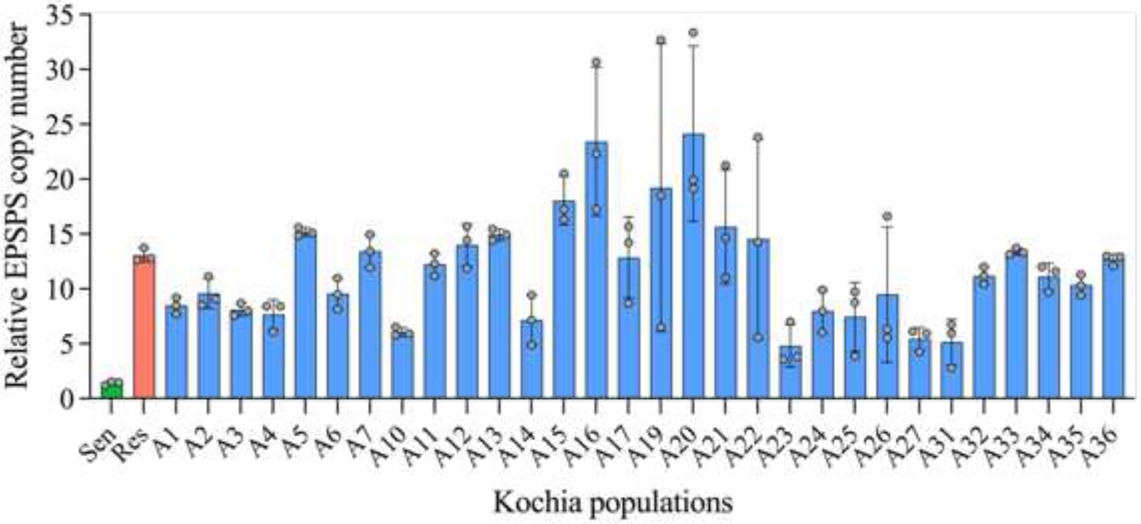

Figure 1.5. Relative *EPSPS* gene copy number in kochia populations collected from Colorado. The green and red bars represent the sensitive and resistant references (Sen and Res), respectively. The blue bars labeled as A represent resistant populations (>20% survival) surveyed from Colorado. Each bar represents the mean and standard deviation of the relative *EPSPS* copy number from three biological replicates (shown as grey circles) within each population.

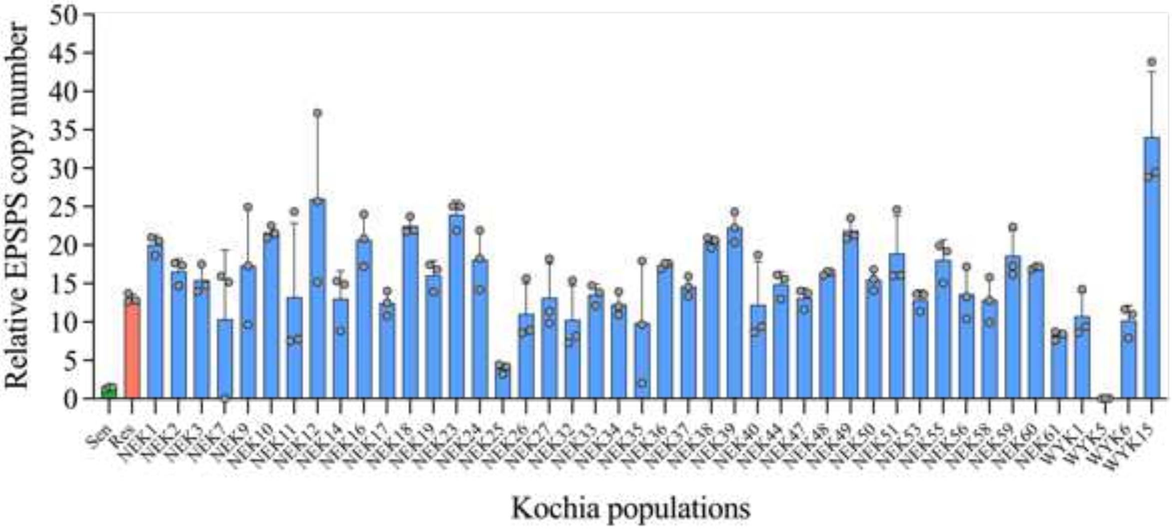

Figure 1.6. Relative *EPSPS gene* copy number in kochia populations collected from Nebraska and Wyoming. The green and red bars represent the sensitive and resistant references (Sen and Res), respectively. The blue bars labeled as NEK represents Nebraska kochia populations and WYK represents Wyoming kochia populations. Each bar represents the mean and standard deviation of the Relative EPSPS copy number from three biological replicates (shown as grey circles) within each population.

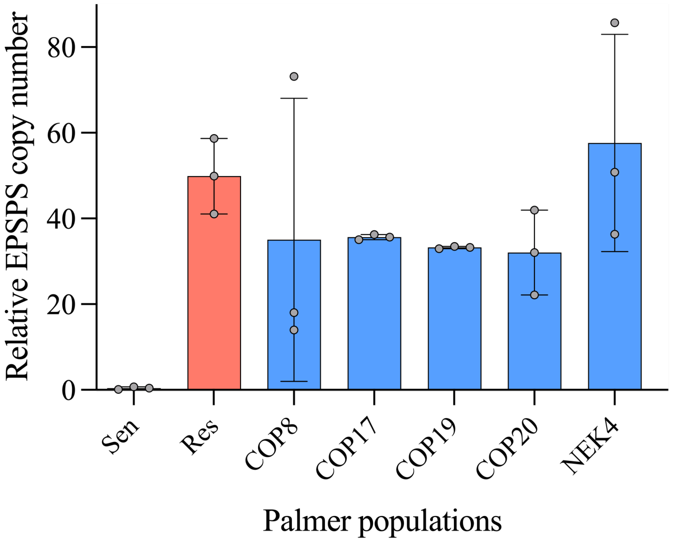

Figure 1.7. Relative *EPSPS gene* copy number in Palmer amaranth populations collected from Colorado and Nebraska. Known sensitive (Sen) and resistant (Res) Palmer amaranth populations were used as positive and negative controls. The blue bars labeled as COP represent Colorado palmer populations classified as resistant (>20% survival), while the blue bar labeled as NBP represents a Nebraska Palmer amaranth population. Each bar represents the mean and standard deviation of the Relative EPSPS copy number from three biological replicates (shown as grey circles) within each population.

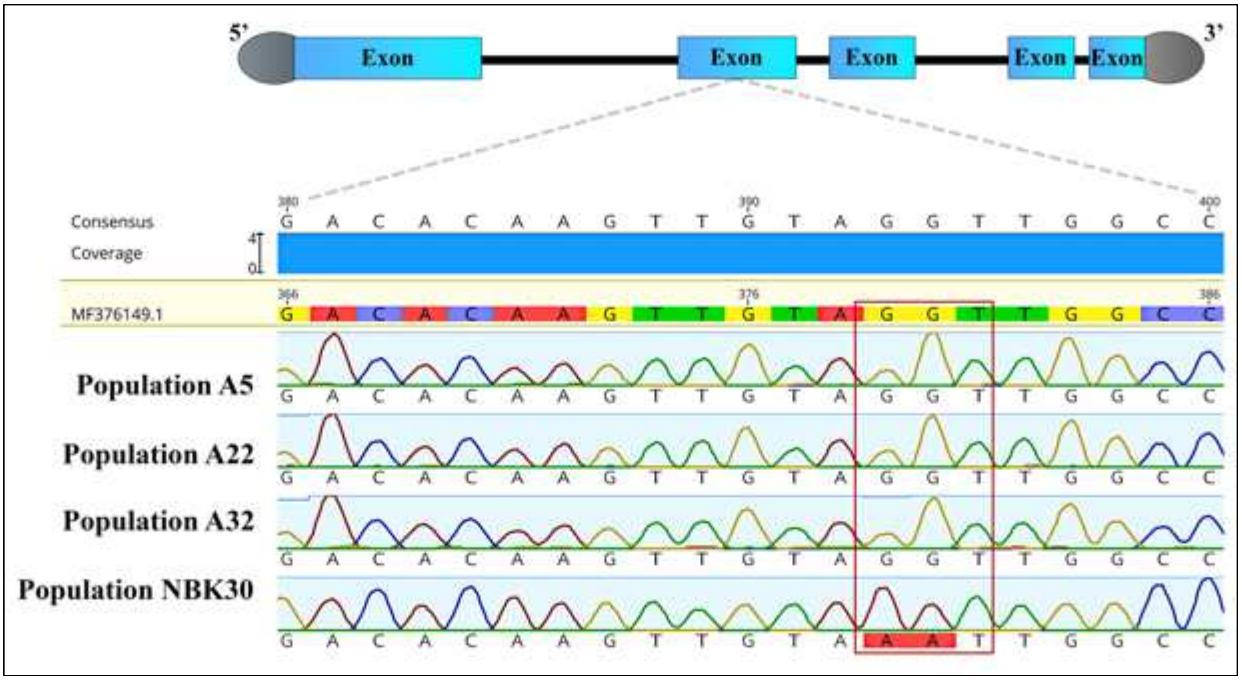

Figure 1.8. The top illustration shows the gene structure of kochia *IAA16* gene. The 5’ and 3’ untranslated regions (UTRs) are represented by grey circles, while the exons are shown as blue boxes. The introns are indicated by black lines. The bottom section displays sanger sequencing chromatograms representing kochia population classified as dicamba resistant from Colorado (A5, A22, and A32) and Nebraska (NBK 30). The region highlighted within the red rectangle is associated with the dicamba resistant phenotype ($G_{73}N$), where sequence GGT is the wild type allele encoding G and AAT is the mutant allele encoding N. MF376149.1 was used as the GenBank reference for IAA16 susceptible allele.

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CHAPTER 2: CHAFF LINING IMPACTS ON WEED EMERGENCE AND SEED VIABILITY IN DRYLAND WHEAT, CORN, AND GRAIN SORGHUM

INTRODUCTION

The emergence of herbicide-resistant (HR) weed populations has become a significant problem in agronomic crop systems in the United States and worldwide, posing a serious threat to yields and leading to increasing economic costs (Peterson et al., 2018). Misuse of herbicide programs and excessive reliance on chemical weed management practices are closely associated with the evolution of resistance (Hicks et al., 2018). Resistant weed populations have been identified in various dryland crop systems, including cereal crops like wheat and corn in the United States (Soni et al., 2022; Westra et al., 2019). Given the continuous evolution of herbicide resistance and the lengthy process of registering and introducing new herbicide modes of action to the market (He et al., 2022), it is imperative to adopt integrated weed management approaches that incorporate alternative tactics to ensure sustainable weed control and extend the lifespan of available chemical options.

Harvest weed seed control (HWSC) was developed in Australia as an alternative weed management strategy in cereal farming to reduce herbicide usage and mitigate the spread of HR populations, particularly in ryegrass (*Lolium rigidum Gaud.*) (Walsh et al., 2022). The implementation of HWSC may present various challenges depending on the specific location, highlighting the importance of understanding each situation individually (Akhter et al., 2023). For instance, this approach has shown enhanced mechanical effectiveness in dry and hot environmental conditions when synchronized with the crop harvest period (Schwartz-Lazaro et al., 2017). HWSC aims to suppress weed seed emergence by employing methods such as destruction, removal, or confinement of weed seeds in the field. In previous studies, HWSC approaches have showed promising potential for implementation in the United States (Beam et al., 2019; Shergill et al., 2020a; Soni et al., 2020). Several HWSC systems have been developed, including narrow window systems, bale direct systems, impact mills, chaff carts, chaff tramlining, and chaff lining.

Chaff lining is a cost-effective technique used in HWSC to trap weed seeds within a concentrated band of chaff mulch residue collected during crop harvesting. Compared to other HWSC techniques, chaff lining is very easy to implement. It involves confining weed seeds in a narrow layer of chaff mulch, which is then strategically placed in residue lines behind the harvester and left on the soil surface (Shergill et al., 2020b). The efficacy of this approach depends on the quantity of weed seeds present during harvesting, focusing on those seeds that have not dispersed prior to the harvest (Shergill et al., 2020b). Promising results have been observed using this strategy to reduce the emergence of both broadleaf and grass weed seeds (Broster et al., 2018; Ruttledge et al., 2018). However, variations in effectiveness have been reported, likely due to site-specific factors where this method was implemented (Ruttledge et al., 2018).

While the efficacy of other HWSC methods have been reported in the United States (Norsworthy et al., 2020; Norsworthy et al., 2016; Schwartz-Lazaro et al., 2017; Shergill et al., 2020a), limited information is available regarding the efficacy of chaff lining. Additionally, it remains unclear whether the presence or absence of chaff covering buried weed seeds has an impact, particularly under suboptimal environmental conditions. Therefore, the objectives of our study are threefold: (1) to evaluate the effectiveness of different types of chaff in reducing weed seed emergence under field conditions in Colorado, Oklahoma and Kansas, (2) to assess whether the presence of chaff covering weed seeds in the field influences seed decay, and (3) to investigate the potential effects of temperature and humidity conditions on weed seed emergence in relation to the presence of chaff.

MATERIALS AND METHODS

2.1 Chaff influence on weed seed emergence

A field trial was conducted across three states, involving two locations (replicates) within each state: Colorado (Fort Collins and Akron), Oklahoma (Lahoma and Perkins), and Kansas (Hays and Tribune). The objective of the trial was to investigate the impact of different chaff treatments (high chaff, low chaff, and no chaff) on seed decay and its subsequent influence on weed seed emergence when planted. Ten weed species were selected for the experiment based on their relevance to the local crop systems (Table

2.1). Five hundred weed seeds were enclosed within organza bags measuring 25 x 38 cm and then situated on the soil surface within the field. Chaff lining was employed as the method of choice, and the bags were covered with different levels of chaff, including low chaff, high chaff, or no chaff. These various chaff treatments were consistently maintained for a period of 3 months in the field. The experimental design adhered to a factorial, strip-split-block design, featuring four replications. The factors under consideration were chaff densities (high, low, and no chaff), weed species and emergence. After the specified field period, the bags containing the weed seeds were collected and transferred to the greenhouse. In the greenhouse, each experimental unit was comprised of planting 100 intact seeds from each of the organza bags that had been previously kept in the field. These seeds were planted in individual containers measuring 1.3 cm by 1.3 cm by 2.5 cm. The greenhouse environment was carefully controlled, with daily irrigation to maintain field capacity. The temperature was maintained within a range of 22 to 26 degrees Celsius, and a photoperiod regimen of 10-14 hours of light per day was provided. After a 30-day period, the emerged weed seeds were counted to assess the impact of the chaff treatments applied in the field on subsequent weed seed emergence. Emergence counts were compared across various chaff levels, including low chaff and high chaff, as well as the control group with no chaff treatment. Statistical analysis was conducted using a two-way ANOVA, considering the factors of chaff densities and weed species. Significance was determined using Dunnett's test, with a predefined significance level of $p < 0.05$. This analysis aimed to assess the impact of chaff treatments in comparison to the no chaff treatment on weed seed emergence.

2.2 Temperature and humidity effects on germination

A controlled condition experiment was conducted in different growth chambers to investigate the impact of temperature and humidity (Table 2.2) on weed seed emergence in the presence or absence of chaff. Seven weed species were selected based on their prevalence as problematic weeds in the central Great Plains region. To assess seed viability a germination test was performed prior the experiment. The controlled condition experiment was investigated in weed species described in Colorado only (Table 2). Three chaff types (wheat, corn, and sorghum) were utilized, and the species tested were based on their relevance in each of those dryland crop systems. For each weed species, 500 seeds were placed in organza bags measuring approximately 10.16 x 15.24 cm and arranged in trays with three technical replicates. The trays were divided into chaff treatments (covered with chaff) and no chaff treatments (without chaff). The chaff density applied was determined based on the field experiment previously discussed and adjusted for the tray area, while in the growth chamber experiment, only the high chaff density was used (3,000 kg ha^{-1}). The trays containing the weed species were kept in growth chambers with constant temperature and humidity ranges (Table 2.2) for a duration of 90 days. After this period, 100 seeds from each replicate were collected randomly from the trays to investigate the combined effect of temperature, humidity, and chaff on seed germination. Seeds were then sown in soil-less plug flats, and seedlings were grown in cells measuring 1.3 cm by 1.3 cm by 2.5 cm (American Clay Works, Denver CO) under greenhouse conditions. The greenhouse conditions, including environmental parameters and irrigation, were consistent with the previous description. One month after planting, the emergence of weed seeds was quantified. The collected data was analyzed using R v.4.0.3, and the "emmeans" (Lenth, 2018) package was employed to calculate estimated marginal means and perform post hoc comparisons for each temperature/humidity treatment within each weed species. To investigate the potential impact of chaff and no chaff conditions on the mean weed seed emergence under different temperature and humidity conditions, the Tukey test with a significance level of $p < 0.05$ was applied to evaluate the differences in weed seed emergence among all varying temperature and humidity levels across the weed species investigated in our study.

RESULTS AND DISCUSSION

2.3 Chaff influence on weed seed emergence

Overall, seed viability was not significantly affected by chaff density (Figure 2.1). Most weed seeds subjected to the low and high chaff treatments exhibited similar germination rates in the greenhouse, with no significant differences ($p < 0.05$) compared to seeds without chaff interference. However, certain weed species showed significantly higher emergence when exposed to the chaff treatments. Specifically, feral rye and barnyard grass seeds in the Akron and Fort Collins locations displayed greater emergence under the chaff treatments compared to the absence of chaff (Figure 2.1). Conversely, no significant differences in emergence between chaff treatments were observed for jointed goatgrass or kochia in both field trials conducted in Colorado. In the Fort Collins field trial, downy brome seeds exhibited a significant decrease ($p < 0.05$) in emergence under both low chaff (90% decrease) and high chaff (96% decrease) treatments. Notably, this decrease was not consistently observed in Akron, where emergence actually increased under both low and high chaff conditions. In the field trials conducted in Oklahoma and Kansas, there were no differences between chaff levels for any of the weed species tested (Supplementary Figure 8, Supplementary Figure 9). It is important to note that seed viability was generally low across all treatments in locations in Oklahoma and Kansas, but the reasons behind this are not clear. These findings highlight the unpredictable and complex nature of chaff lining impacts on weed seed emergence, with different weed species exhibiting varying responses to the factors present in their respective environments. While chaff may act as a physical barrier to suppress seed germination, it may also provide short-term protection to seeds in certain agroecosystems. Similar to our study, Walsh et al. (2021) conducted field trials to assess the impact of chaff on weed seed survival and observed that ryegrass germination increased when exposed to high chaff levels (> 8,000 kg ha^{-1}) compared to lower chaff levels. However, in a different experimental location, ryegrass germination was lower with higher chaff amounts than with lower chaff densities. The authors also emphasized that chaff density does not appear to influence the viability of weed seeds in the field.

Location-specific environmental factors, including climate, seed predators, allelopathic compounds, crop residue, and soil microbiome, may influence seed decay (Sarabi, 2019; Sias et al., 2021). The presence of chaff may generate unfavorable conditions that will impact on seed decay. However, chaff alone is unlikely to have a significant short-term impact on seed decay. Instead, as an organic matter it may perhaps contribute as a substrate to microoganisms activity in association with other environmental factors over a longer period in a field setting. Weed seeds can remain viable in the field regardless of chaff density, emphasizing the importance of practices that minimize disturbance of the mulch layer to prevent seed dispersion, which would likely enable better conditions for germination. Increasing organic matter can promote seed decay by providing a substrate for native seed pathogens (Müller-Stöver et al., 2016). Soil with high organic matter content has been associated with greater microbial activity, resulting in increased enzymatic activity that can suppress seedling growth in species such as green foxtail (*Setaria viridis* [L.] Beauv.) and inhibit bindweed (*Convolvulus arvensis* L.) (Kremer and Li, 2003).

Chaff lining, like other weed management practices, should not be solely relied upon as a standalone strategy to mitigate weed adaptation and maintain long-term efficacy and sustainability. While chaff lining has shown promise in weed control, it is essential to consider the broader implications and potential limitations of this method. The implementation of HWSC methods, including chaff lining, may result in the loss of nutrients due to the removal of chaff and straw, which can potentially increase fertilizer costs (Spoth et al., 2022). Furthermore, chaff lining alone is unlikely to provide effective weed control due to the influence of various factors previously discussed, including intrinsic weed species characteristics. For example, targeted weeds should have a similar life cycle to the crop, allowing their capture during crop harvest (Shergill et al., 2020b). In situations where weed species, such as kochia, are immature and green during the harvest in certain cereal crop systems, it is advisable to consider alternative management strategies, such as herbicide treatment, to augment the efficiency of Herbicide-Resistant Weed Seed Control (HWSC) methods, including chaff lining. Moreover, it's essential to recognize that weed seeds already existing in the seed bank may persist and remain unaffected, thus requiring the implementation of supplementary weed management measures. Furthermore, the dormancy level and the duration of these mechanisms in weed seeds can also significantly impact the long-term effectiveness of chaff lining as a weed control strategy. Therefore, a comprehensive weed management approach that considers multiple factors, such as seed bank dynamics and seed dormancy, along with the integration of diverse strategies, is essential to ensure effective weed control and maximize the utility of chaff lining.

2.4 Temperature and humidity effects on germination

The presence of chaff, exposed to varying temperature and humidity intervals, did not have a significant impact ($P < 0.05$) on reducing weed seed germination compared to seeds that were not incubated in chaff conditions. Temperature and relative humidity did not affect ($P < 0.05$) seedling emergence of barnyardgrass, jointed goatgrass, Palmer amaranth, or yellow foxtail, particularly when compared to the conditions of the storage room (RH20 and T4). However, for downy brome and feral rye, increased temperature and humidity result in negative impacts on seed emergence (Figure 2.2). Seeds that were incubated at T30 had a significant decrease in emergence compared to T20 at the same humidity level (RH80). As feral rye is known to have low to no dormancy (Burger and Ellstrand, 2014; Stump and Westra, 2000), these results imply that temperature and humidity together may be negatively influencing seed decay and leading to poor emergence in feral rye.

For kochia seeds, germination rate was 0% when seeds were incubated at RH80 and T30 (Figure 2.2). However, when seeds were incubated at the same humidity but lower temperature (T20), an increase in germination was observed, although it was not significantly different from the RH80 and T30 treatment. Kochia seeds exposed to the two high humidity treatments were different ($P < 0.05$) from the rest of the treatments, including the stored room condition. Germination increased when kochia seeds were incubated in dry treatments (RH20) compared to humid treatments (RH80), regardless of the temperature. This suggests that humidity was the main factor reducing germination (RH80 and T20) and inhibiting germination (RH80 and T30) in kochia seeds, although it appears that temperature, in conjunction with high humidity, may accelerate the process of seed decay. Unlike many other weed species, kochia seeds are known to have low to no dormancy and short persistence in the field (Beckie et al., 2018; Dille et al., 2017; Everitt et al., 1983; Thompson et al., 1994). These results suggests that high humidity affected kochia seed viability, resulting in poor or complete suppression of emergence observed in a greenhouse assay.

Mickelson and Grey (2006) observed that greater soil water content led to increased wild oat seed mortality, resulting in a cumulative decline in seed bank and they hypothesized that the relationship between moisture and seed decay is associated with increased activity of microorganisms. Relative humidity has a close association with specific microorganisms, while some microorganisms develop in dry conditions, some require moisture to develop (Gupta et al., 2017). Seed-borne microorganisms can be present on the seed surface and/or internally and may play a role in promoting seed decay depending on favorable conditions for pathogenic microbes to infect. Seeds with a prolonged viability period may possess complex defense mechanisms to protect against pathogens (Baskin and Baskin, 1985; Pollard, 2018). Among weed seeds, it appears that some have more physical and/or chemical defense when compared to others, which may lead to a different scenarios for seed decay, as noted in our study, when the tested species are exposed to similar environmental conditions. For instance, Davis et al. (2008) reported that kochia was the weed species with least physical protection in the thickness of the seed coat compared to other weed species tested. Seeds exposed to moisture may release exudates and trigger fungi spore germination that could potentially use nutrients from seeds (Wagner and Mitschunas, 2022). This explanation may explain why downy brome, feral rye, and kochia exhibited significant reductions in germination rates in our study nuder high relative humidity conditions. Other weed species tested may possess diverse chemical and physical attributes that were sufficient to protect them during the three-month period of exposure in our study. Korres et al. (2018) observed that the viability of Palmer amaranth and waterhemp seeds was greatly affected by a combination of factors such as burial conditions, time, and specific soil conditions at the site studied. These findings highlight the complex interplay of biotic and abiotic factors in weed seed suppression. In the context of our study, while chaff did not demonstrate a consistent relationship with temperature and humidity variations in impacting seed decay, it is important to recognize that interactions among chaff, soil type, microbes, insects, adverse environmental conditions, and seed longevity collectively may contribute to weed seed suppression in different circumstances within a natural environmental setting.

Effectiveness of chaff as part of harvest weed seed control (HWSC) programs in the field may be attributed to its role as a physical barrier as suggested by our results that may consistently suppress seed germination over time. However, further studies are necessary to fully understand the impact of chaff in conjunction with other biotic and abiotic factors, as well as to assess the long-term utility and applicability of this approach. The comprehensive exploration of these factors will provide valuable insights for the development of effective and sustainable weed management strategies.

TABLES

Table 2.1: Weed species and crop chaff utilized for investigating chaff influence on weed seed emergence in the field settings and seed decay in the greenhouse settings.

State	Crop chaff	Weed species			
Colorado	Wheat	Downy brome	Feral rye	Jointed goatgrass	
	Corn	Kochia	Palmer amaranth		
	Sorghum	Barnyardgrass	Yellow foxtail		
Oklahoma	Wheat	Downy brome	Wild mustard	Wild oat	
	Corn	Barnyardgrass	Kochia	Palmer amaranth	Yellow foxtail
	Sorghum	Barnyardgrass	Kochia	Palmer amaranth	Yellow foxtail
Kansas	Wheat	Barnyardgrass	Tansy mustard	Wild mustard	Yellow foxtail
	Corn	Kochia	Palmer amaranth	Tansy mustard	Yellow foxtail
	Sorghum	Kochia	Palmer amaranth	Tansy mustard	Yellow foxtail

Table 2.2: Controlled Condition Experiment: Temperature and Relative Humidity Measurements

Temperature (°C)	Relative humidity (%)
4	20
10	20
20	20
20	80
30	20
30	80

FIGURES

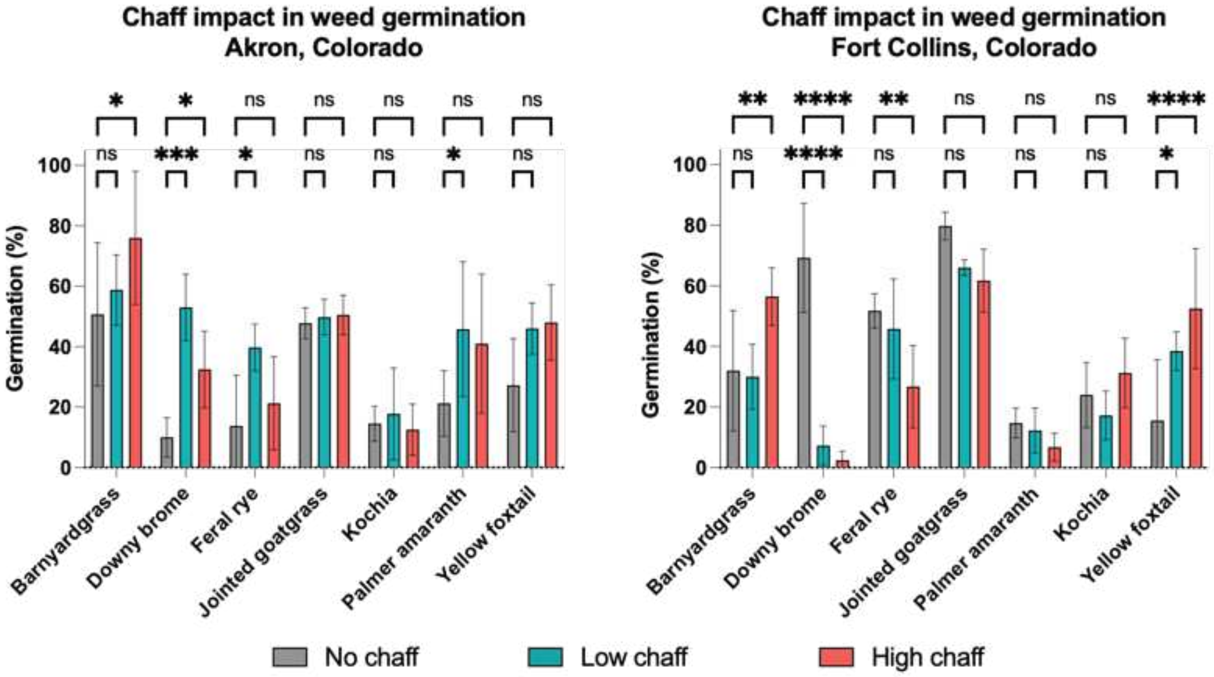

Figure 2.1. Germination percentage of weed species under different levels of chaff in Colorado. Bar graph showing the mean germination percentages (%) of various weed species across three levels of chaff: no chaff (grey bars), low chaff (green bars), and high chaff (red bars). Error bars represent the standard deviation of germination from four technical replicates. Statistically significant differences were determined using a two-way ANOVA multiple comparison test ($p < 0.05$, Dunnett's test). Asterisks (*) indicate significant differences compared to the no chaff treatment

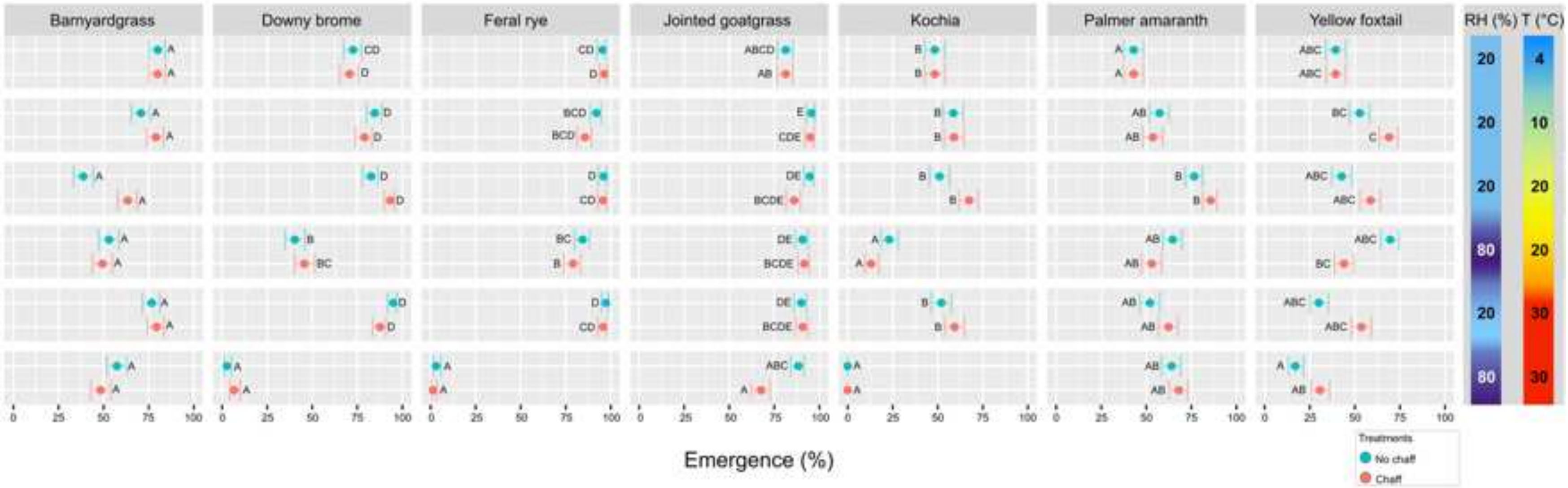

Figure 2.2. Influence of temperature, humidity, and chaff on weed seed germination. Wheat chaff was utilized for jointed goatgrass, feral rye, and downy brome, while corn chaff was employed for palmer amaranth and kochia. Sorghum chaff was chosen for barnyardgrass and yellow foxtail. The figure illustrates the impact of temperature, humidity, and the presence or absence of chaff on weed seed germination. The mean values with standard error are depicted by green dots (no chaff) and red dots (chaff), based on three technical replicates containing 100 weed seeds each. The color gradients in the right column represent the corresponding relative humidity (RH %) and temperature (T°C). Statistical differences between treatments are illustrated by different letters, indicating significant variations determined by the Tukey test ($p < 0.05$).

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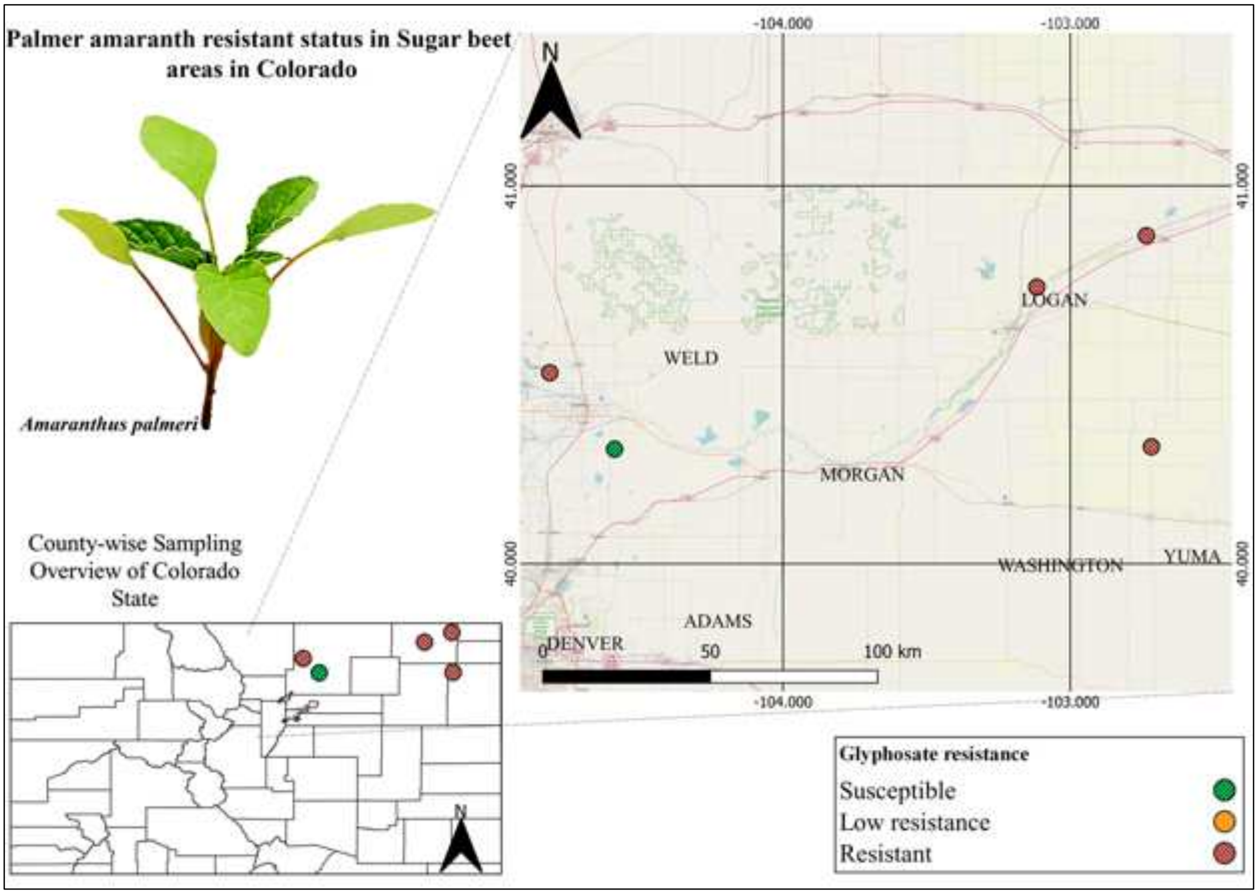
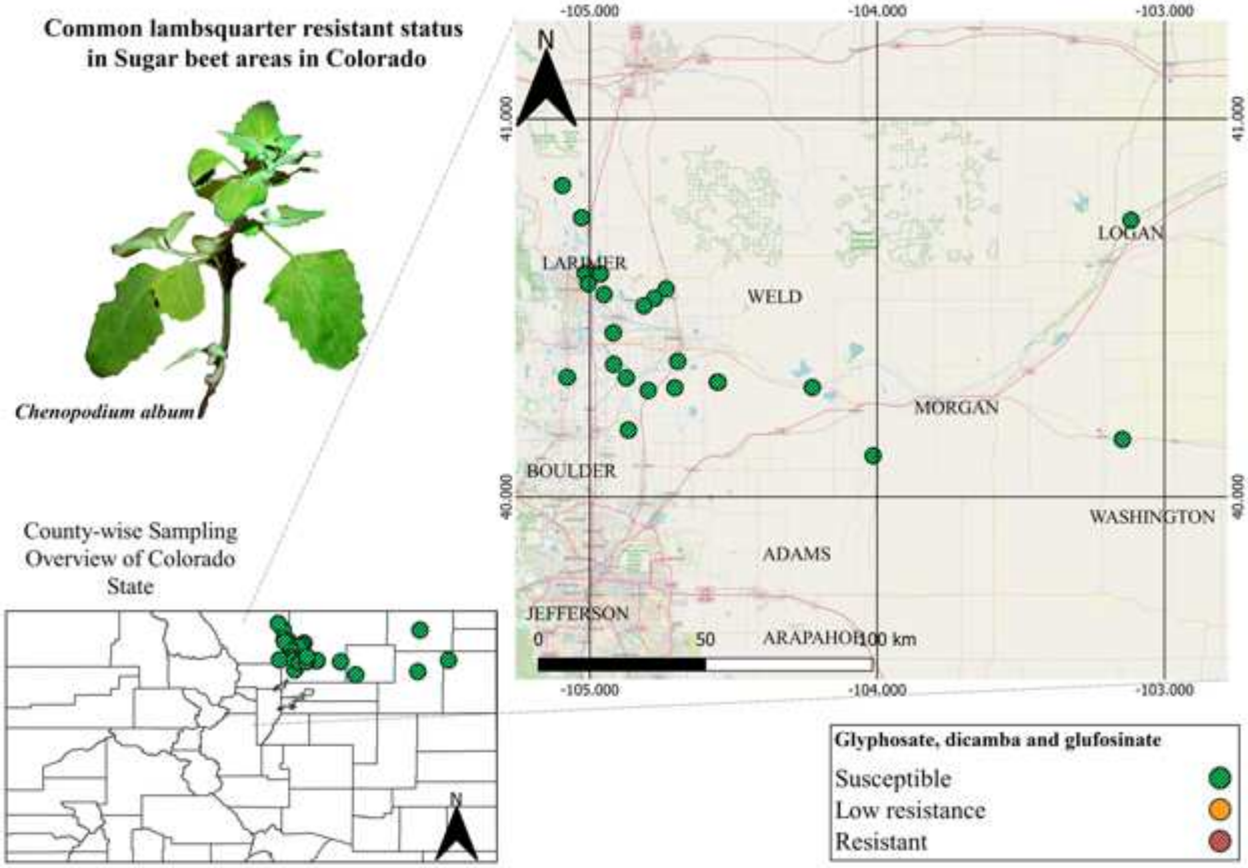
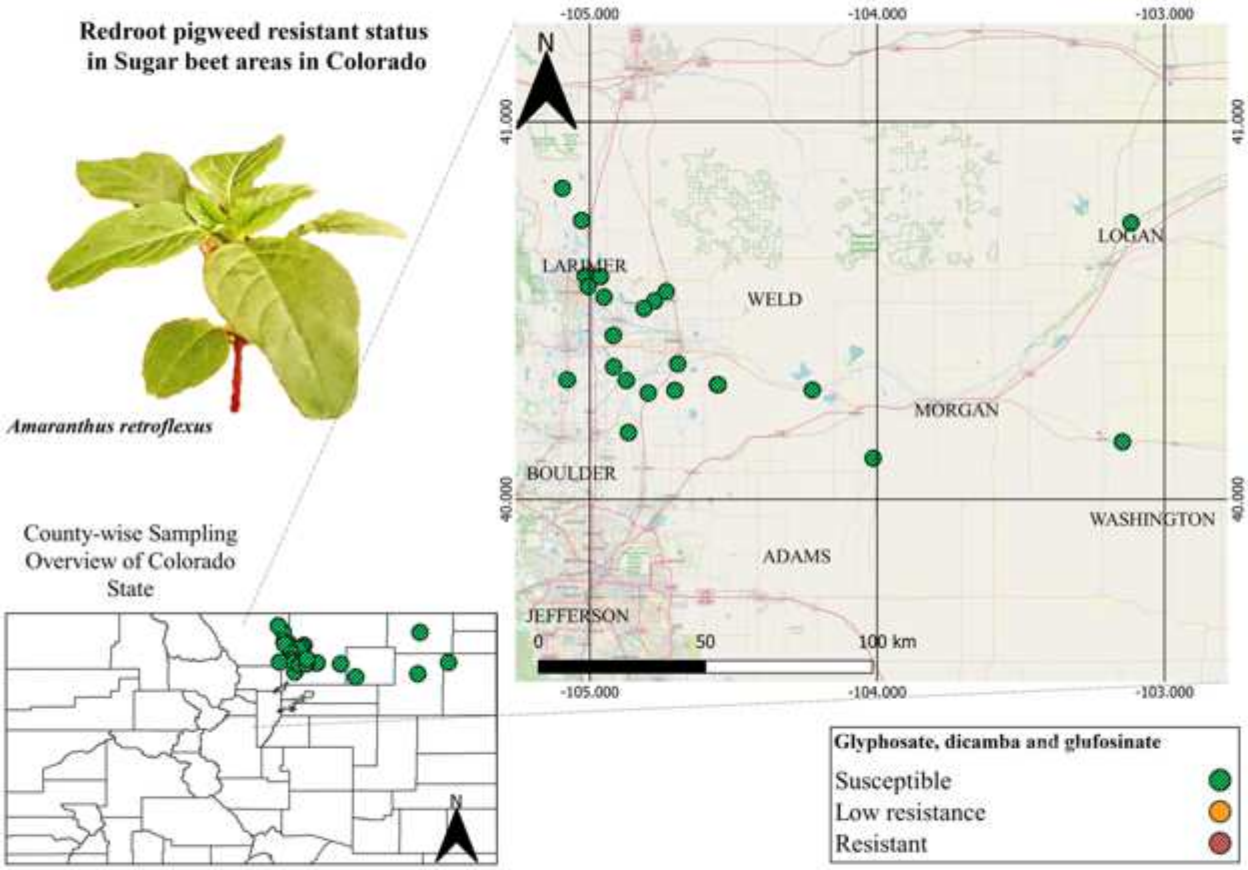
APPENDICIES



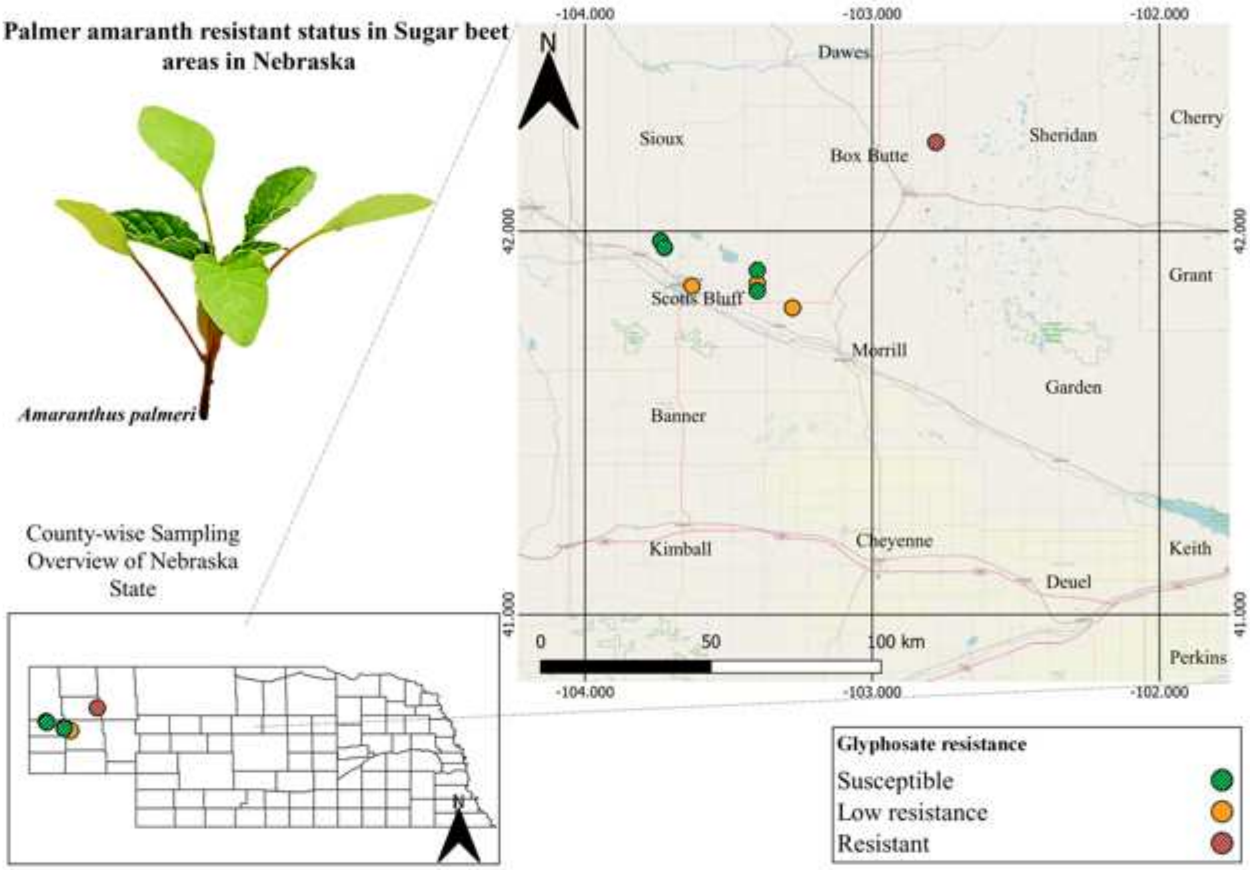
Supplementary Figure 1. Geo-referenced map illustrating the Palmer amaranth populations collected in Colorado during the fall of 2021. The dots on the map represent the locations of Palmer amaranth populations, and their color signifies their response to glyphosate treatment. In the bottom left corner, a separate map illustrates the distribution of the collected populations. On the right side, a close-up map focuses on the main counties where the samples were collected. Populations classified as resistant are represented by red dots, while yellow dots indicate low resistance, and green dots represent susceptible populations.



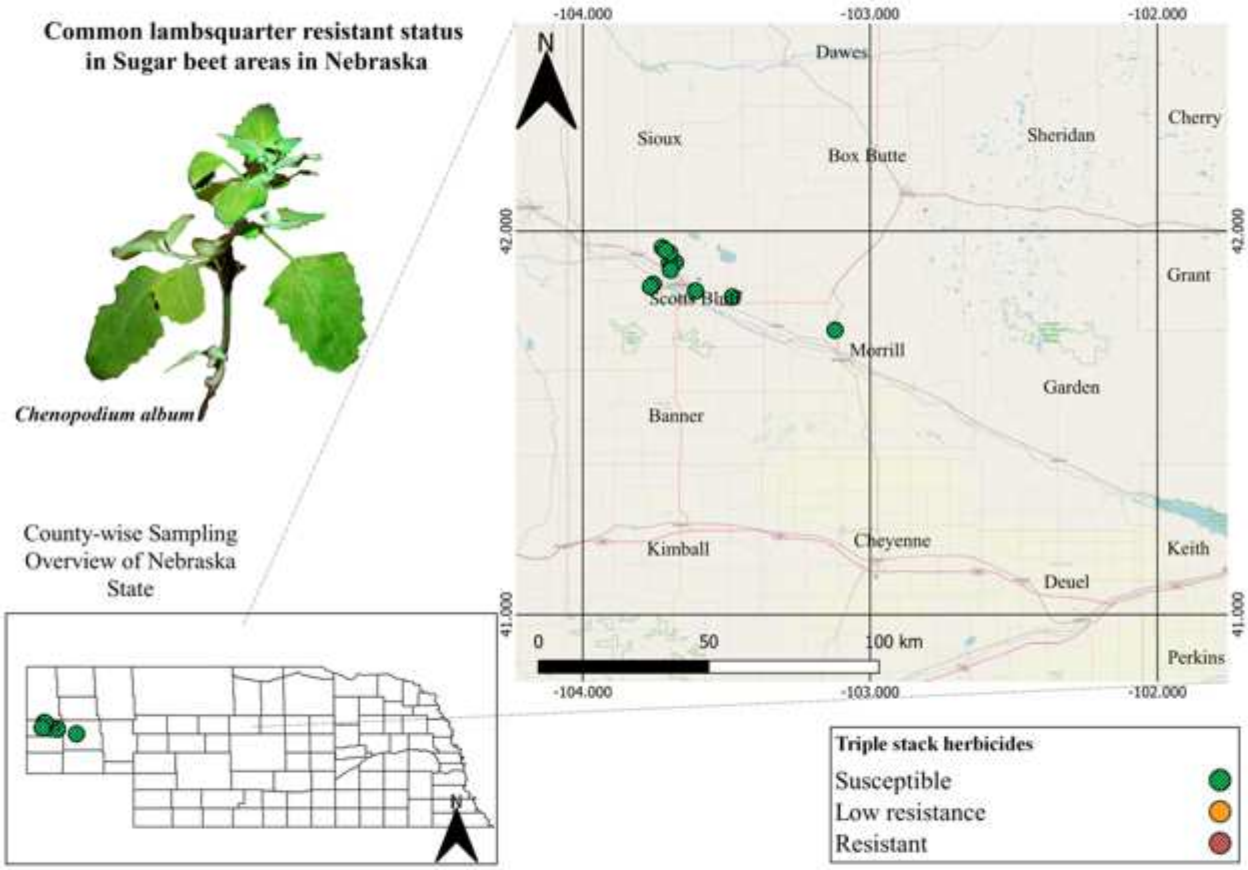
Supplementary Figure 2. Geo-referenced map illustrating the common lambsquarters populations collected in Colorado during the fall of 2021. The dots on the map represent the locations of common lambsquarters populations, and their color signifies their response to glyphosate, dicamba and glufosinate treatments. In the bottom left corner, a separate map illustrates the distribution of the collected populations. On the right side, a close-up map focuses on the main counties where the samples were collected. Populations classified as resistant are represented by red dots, while yellow dots indicate low resistance, and green dots represent susceptible populations.



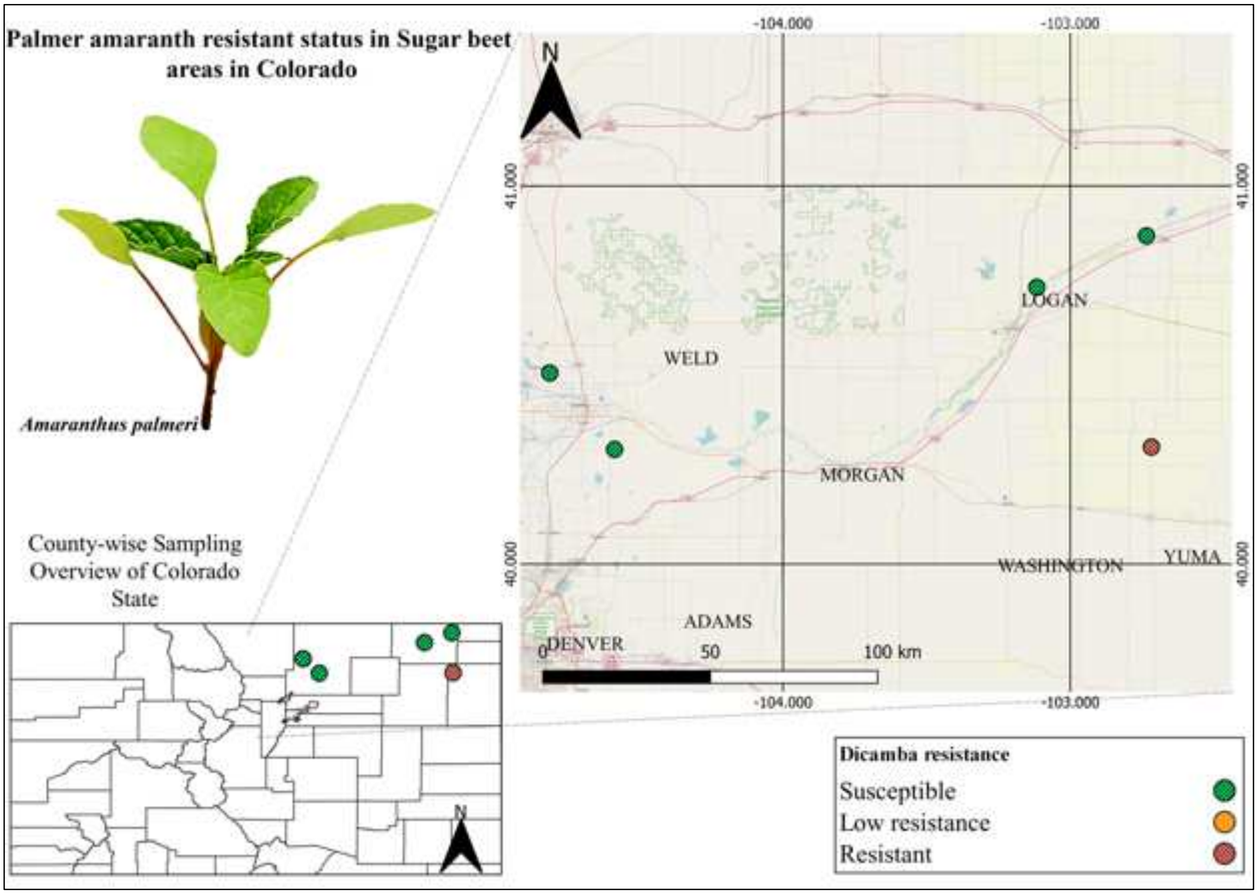
Supplementary Figure 3. Geo-referenced map illustrating the redroot pigweed populations collected in Colorado during the fall of 2021. The dots on the map represent the locations of redroot pigweed populations, and their color signifies their response to glyphosate treatment. In the bottom left corner, a separate map illustrates the distribution of the collected populations. On the right side, a close-up map focuses on the main counties where the samples were collected. Populations classified as resistant are represented by red dots, while yellow dots indicate low resistance, and green dots represent susceptible populations.



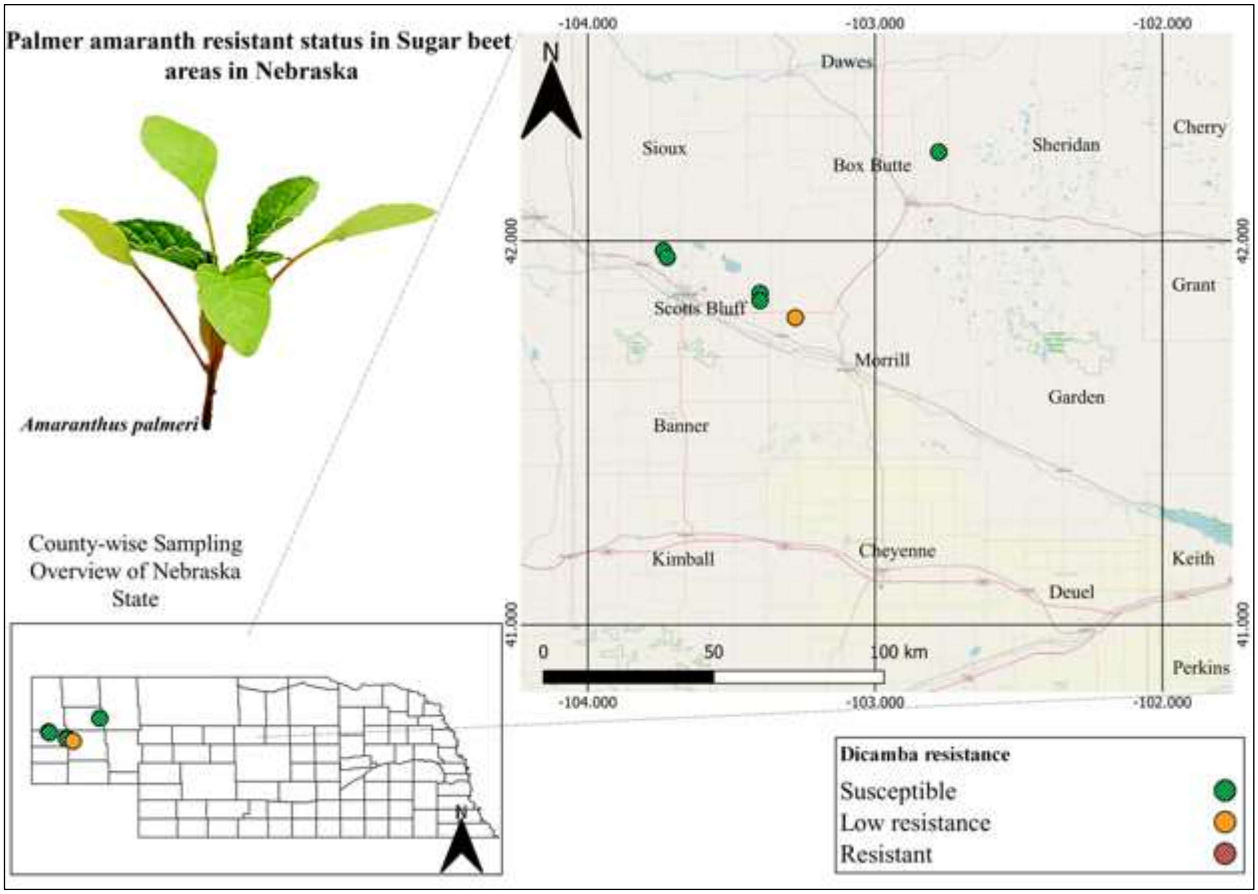
Supplementary Figure 4. Geo-referenced map illustrating the Palmer amaranth populations collected in Nebraska during the fall of 2020. The dots on the map represent the locations of Palmer amaranth populations, and their color signifies their response to glyphosate treatment. In the bottom left corner, a separate map illustrates the distribution of the collected populations. On the right side, a close-up map focuses on the main counties where the samples were collected. Populations classified as resistant are represented by red dots, while yellow dots indicate low resistance, and green dots represent susceptible populations.



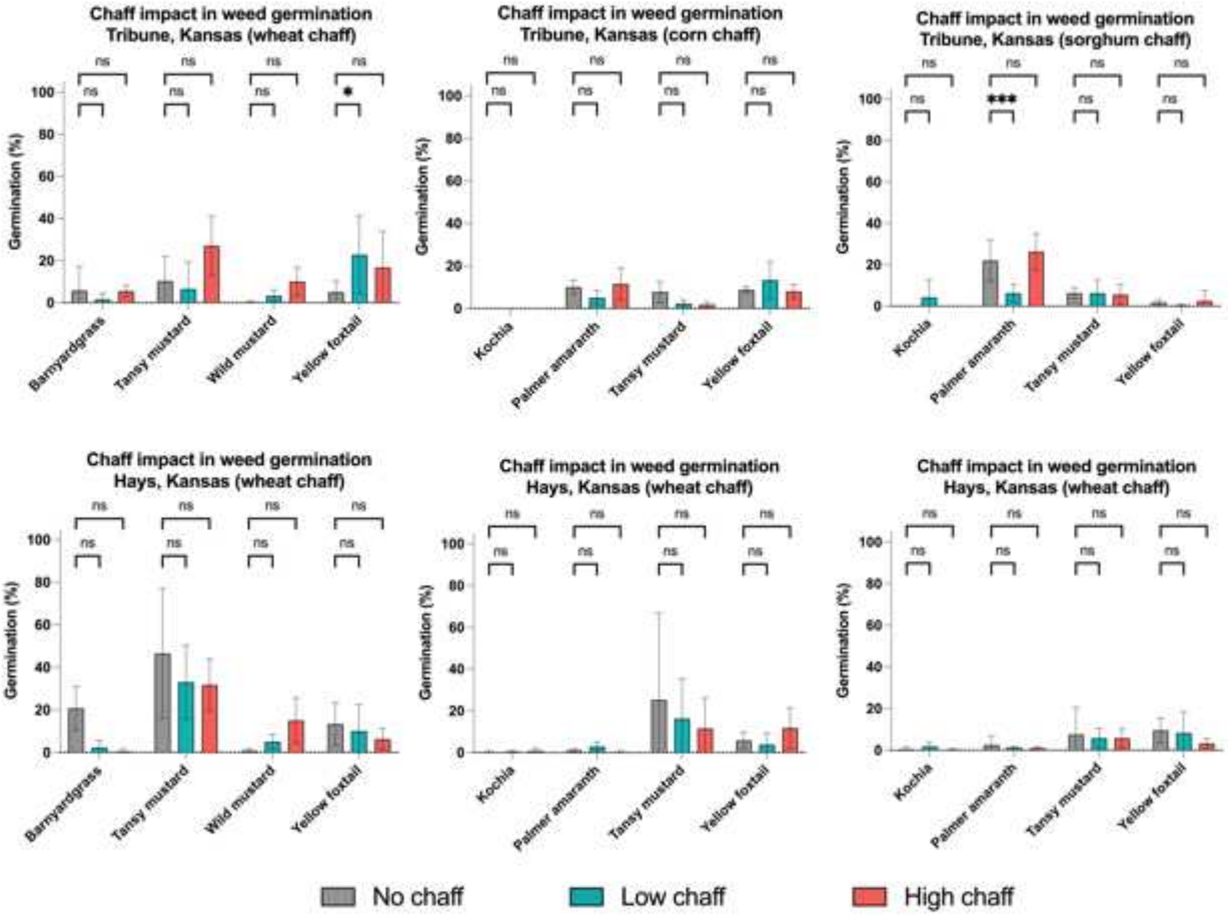
Supplementary Figure 5. Geo-referenced map illustrating the common common lambsquarters populations collected in Nebraska during the fall of 2020. The dots on the map represent the locations of common common lambsquarters populations, and their color signifies their response to glyphosate treatment. In the bottom left corner, a separate map illustrates the distribution of the collected populations. On the right side, a close-up map focuses on the main counties where the samples were collected. Populations classified as resistant are represented by red dots, while yellow dots indicate low resistance, and green dots represent susceptible populations.



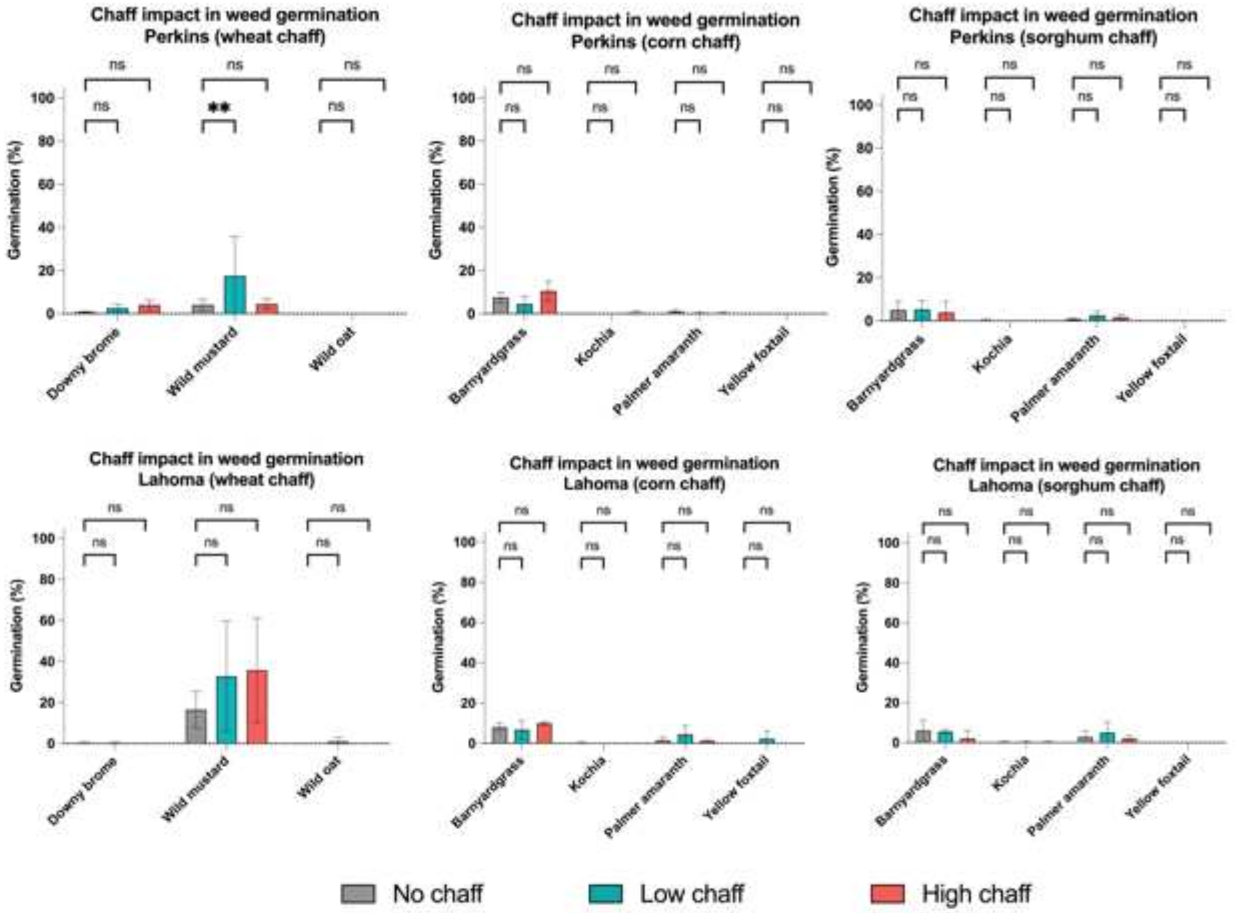
Supplementary Figure 6: Geo-referenced map illustrating the Palmer amaranth populations collected in Colorado during the fall of 2021. The dots on the map represent the locations of kochia populations, and their color signifies their response to dicamba treatment. In the bottom left corner, a separate map illustrates the distribution of the collected populations. On the right side, a close-up map focuses on the main counties where the samples were collected. Populations classified as resistant are represented by red dots, while yellow dots indicate low resistance, and green dots represent susceptible populations.



Supplementary Figure 7. Geo-referenced map illustrating the Palmer amaranth populations collected in Nebraska during the fall of 2020. The dots on the map represent the locations of Palmer amaranth populations, and their color signifies their response to dicamba treatment. In the bottom left corner, a separate map illustrates the distribution of the collected populations. On the right side, a close-up map focuses on the main counties where the samples were collected. Populations classified as resistant are represented by red dots, while yellow dots indicate low resistance, and green dots represent susceptible populations.



Supplementary Figure 8. Germination percentage of weed species under different levels of chaff in Kansas. Bar graph showing the mean germination percentages (%) of various weed species across three levels of chaff: no chaff (grey bars), low chaff (green bars), and high chaff (red bars). Error bars represent the standard deviation of germination from four technical replicates. Statistically significant differences were determined using a two-way ANOVA multiple comparison test ($p < 0.05$, Dunnett's test). Asterisks (*) indicate significant differences compared to the no chaff treatment.



Supplementary Figure 9. Germination percentage of weed species under different levels of chaff in Oklahoma. Bar graph showing the mean germination percentages (%) of various weed species across three levels of chaff: no chaff (grey bars), low chaff (green bars), and high chaff (red bars). Error bars represent the standard deviation of germination from four technical replicates. Statistically significant differences were determined using a two-way ANOVA multiple comparison test ($p < 0.05$, Dunnett's test). Asterisks (*) indicate significant differences compared to the no chaff treatment.

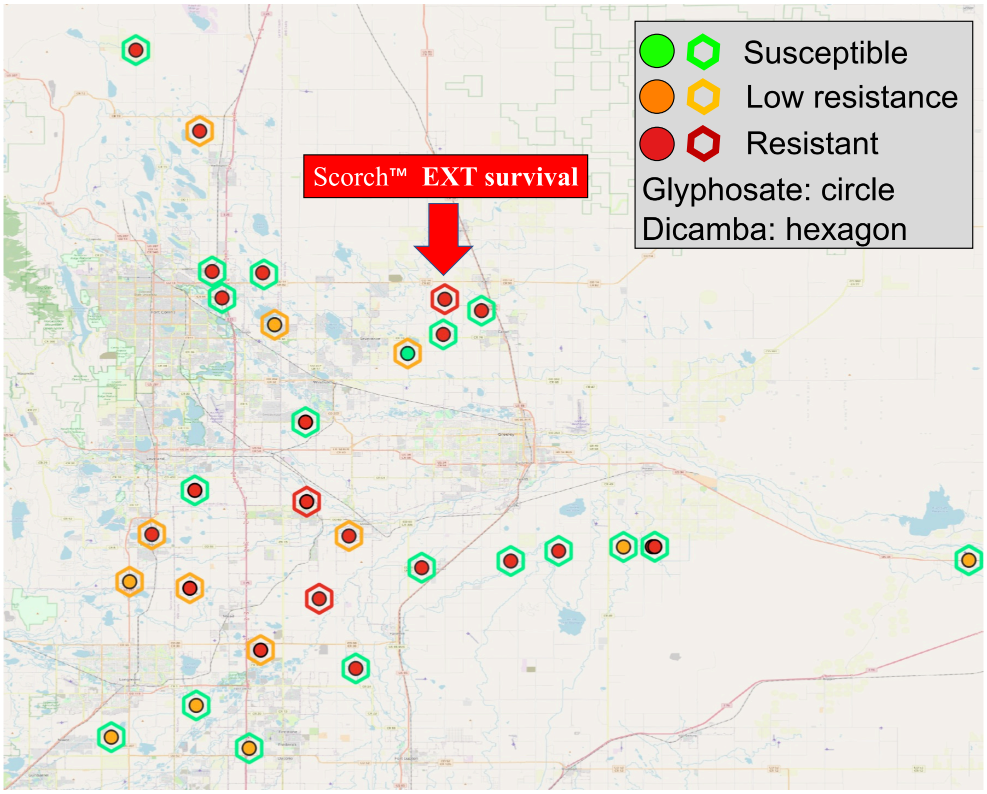
INVESTIGATION OF CROSS RESISTANCE TO AUXIN-MIMIC HERBICIDES IN A DICAMBA RESISTANT KOCHIA POPULATION

Based on the previous survey of sugar beet farms, populations classified as resistant to dicamba were further investigated for resistance to an herbicide mixture called Scorch™ EXT, which contains dicamba, 2,4-D, and dichloprop as active ingredients. Among the four populations classified as resistant in Colorado, Nebraska, and Wyoming survey, survivors were identified in population A5 (21%), population A22 (3%), and population A32 (3%). These populations were located in Larimer County, Colorado (Supplementary Figure 10). The survivors from these populations were self-pollinated for one generation to produce seeds for further analysis.

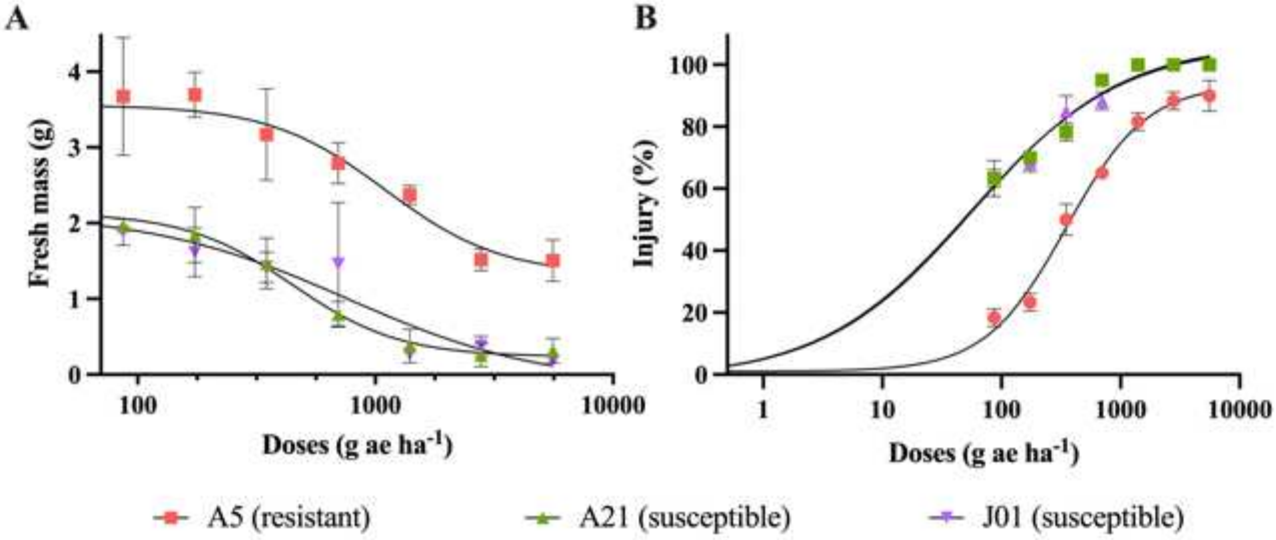
Based on the observed survival frequency of the herbicide mixture treatment, only population A5 was classified as resistant (>20%). To determine the resistance factor, a dose-response curve was performed, including two known susceptible populations (J01 and A9). Scorch EXT was applied at rates of 0, 87, 174, 349, 698, 1395, 2790, and 5580 g ae ha^{-1}. The herbicide applications were conducted using a laboratory chamber single-nozzle sprayer (DeVries Generation III Research Sprayer, MN, Hollandale), calibrated to deliver 187 L ha^{-1}.

The resistance factor (RF) of population A5 was low. The ED_{50} of J01 and A5 was 688.99 and 2720.68, respectively, generating a RF of 3.94. Population A21 was slightly more sensitive than J01 with a ED_{50} of 511.20, which generates A5 RF of 5.32. It is evident that A5 required a slightly higher dose of the herbicide mixture to achieve control (Supplementary Figures 11, 12) compared to the two known susceptible populations. Resistance to this herbicide mixture has not been reported previously.

There is no mutation detected in the degron region, as discussed earlier. The selection pressure from Scorch EXT is likely low since this herbicide mixture was recently released, and there are other usual options for desiccating the area prior to planting sugar beet. As the resistance was not identified in the other dicamba-resistant populations, it is unlikely that the lower susceptibility of population A5 to dicamba explains this phenotype. This suggests that other resistant mechanisms may be evolving in this population, reducing the efficacy of the other two active ingredients in the mixture. Although the resistance factor in population A5 is not high, the resistance mechanism in this population may be non-target site, possibly involving resistance to other herbicides from different chemical groups. However, this possibility has not been tested.



Supplementary Figure 10. Geo-referenced kochia glyphosate and dicamba resistant populations in Colorado. Where survival score: <2% (● or ⬡), between 2 and 20% (● or ⬡), and >20% (● or ⬡).



Supplementary Figure 11. Dose-response curve (A) and visual injury assessment (B) of the mixture dicamba, 2,4-D, and dichlorprop in kochia survival population (A5) and known susceptible (A9, J01) at 21 days after treatment. Each data point signifies the mean and standard deviation error of three biological replicates.

Supplementary Figure 11. Symptomology response of kochia resistant (A5) and susceptible (J01, A9) populations to the herbicide mixture containing dicamba, 2,4-D, and dichlorprop at 21 days after treatment.

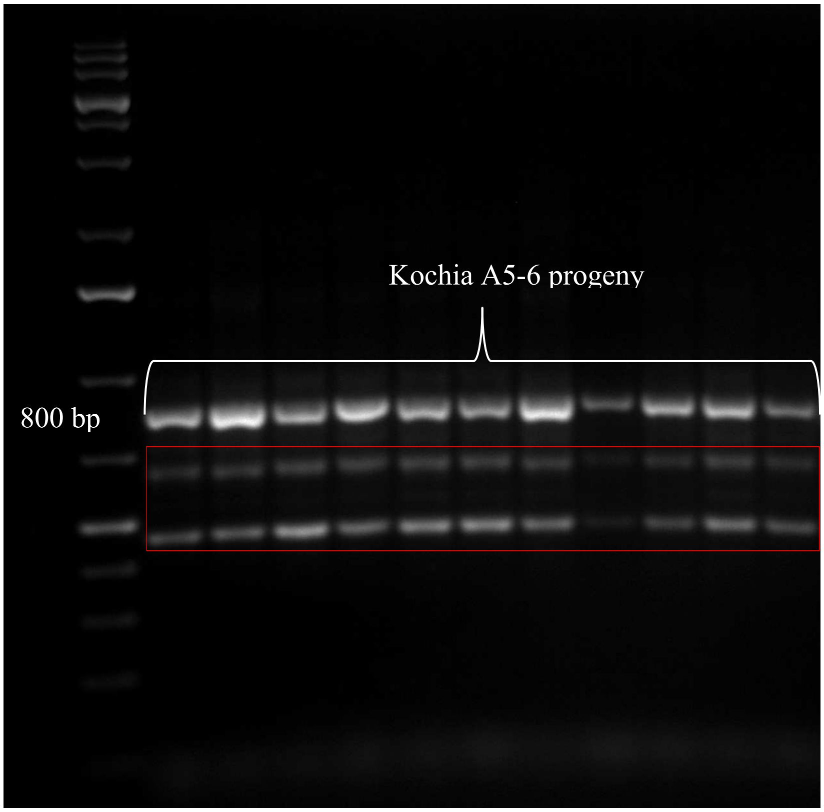
A DELETION IN THE IAA16 DEGRON REGION IDENTIFIED IN A KOCHIA DICAMBA RESISTANT SURVIVOR

The sugar beet survey identified DR populations, which were later genotyped to confirm the presence of an amino acid substitution discussed in Chapter 1. Among the individuals from population A5, four were genotyped. Three of them lacked any amino acid substitution, while one individual exhibited multiple mutations and a deletion spanning over 300 base pairs. PCR was conducted using the same set of primers used for all DR populations, ruling out issues with primer specificity or dimers.

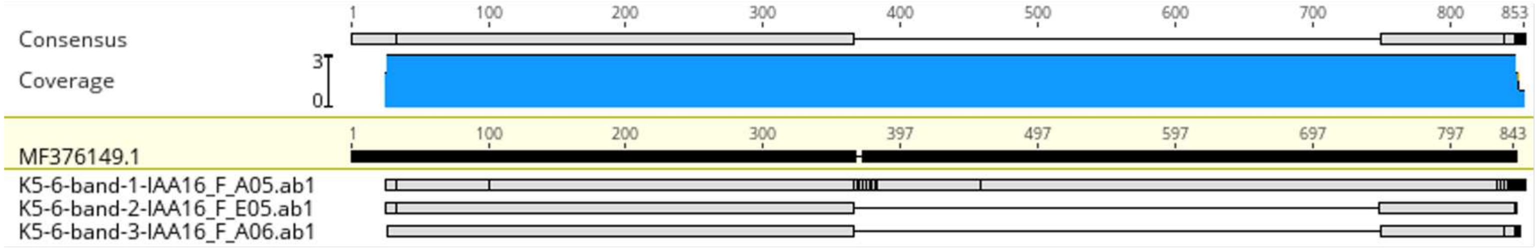
Subsequently, the genetically distinct individual with mutations and deletion was self-pollinated, and eleven progeny individuals were subjected to a PCR. Gel electrophoresis analysis of these progeny individuals revealed three bands in all cases (Supplementary Figure 12). These bands were purified, Sanger sequenced, and the results are presented in Supplementary Figure 13.

The role of these mutations and deletion in contributing to the resistant phenotype is not yet clear, and ongoing investigations are underway for these populations. An individual possessing solely the deletion without the associated mutations was not identified based on the electrophoresis gel analysis. It was previously discussed in Chapter 1 that the G73N mutation is the only known cause of dicamba resistance in kochia. A deletion in the degron IAA2 has been reported to cause 2,4-D resistance in wild mustard.

This study highlights the significant genetic diversity that can exist within a weed population, with individuals showing different genotypes from others within the population. Cross-resistance was not observed in individuals with mutation, while survival to dicamba, 2,4-D, and dichlorprop in a mixture was noted in individuals without the mutation, as previously discussed.



Supplementary Figure 12. Gel electrophoresis showing the PCR amplification products of individuals from the progeny derived from the plant harboring mutations and a deletion. Each lane represents an individual, and the gel shows the presence of three distinct bands in all individuals. Highlighted in red demonstrates the bands with deletion.



Supplementary Figure 13. Sanger sequencing results for each of the bands observed in the electrophoresis assay. The figure displays the complete IAA16 sequence from the three bands visualized in the gel electrophoresis, including exons only. The first band possesses single nucleotide polymorphisms while the second and third have a deletion.