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DOI: 10.1017/wet.2024.77

Short title: Weed resistance survey

Baseline survey reveals glyphosate and dicamba resistance in broadleaf weeds before sugar beet trait introduction

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Abstract

A pre-launch survey of broadleaf weeds was conducted to predict the weed management efficacy of a novel genetically engineered sugar beet with resistance traits for glyphosate, dicamba, and glufosinate. We targeted problematic broadleaf weed species prevalent in the sugar beet system, including kochia, common lambsquarters, Palmer amaranth, and redroot pigweed across sugar beet areas in Colorado, Nebraska, and Wyoming. The results revealed that a significant percentage of kochia populations in Colorado, Nebraska, and Wyoming exhibited resistance to glyphosate (94%, 98%, and 75%, respectively) and dicamba (30%, 42%, and 17%, respectively). Palmer amaranth populations had resistance frequencies for glyphosate and dicamba of 80% and 20% in Colorado and 20% and 3% in Nebraska, respectively. No resistance to the tested herbicides was identified in common lambsquarters or redroot pigweed. Glufosinate resistance was not identified for any species. Kochia and Palmer amaranth populations from Colorado and Nebraska exhibited glyphosate resistance primarily through 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*) gene amplification. However, one glyphosate-resistant kochia population from Wyoming lacked *EPSPS* gene amplification, indicating the presence of an alternative resistance mechanism. We identified the previously characterized *IAA16* G₇₃N substitution in a dicamba-resistant kochia population from Nebraska. However, dicamba-resistant kochia populations from Colorado did not possess this substitution, suggesting an alternative, yet-to-be-determined resistance mechanism. The widespread prevalence of glyphosate and dicamba resistance, coupled with the emergence of novel resistance mechanisms, poses a significant challenge to the long-term efficacy of this novel genetically engineered sugar beet technology. These findings underscore the urgent need for integrated weed management (IWM) strategies that diversify effective herbicide sites-of-action and incorporate alternative weed management practices within cropping systems.

Nomenclature: Glyphosate; dicamba; glufosinate-ammonium; kochia, *Bassia scoparia* (L.) A.J. Scott; common lambsquarters, *Chenopodium album* L; Palmer amaranth, *Amaranthus palmeri* S. Watson; redroot pigweed, *Amaranthus retroflexus* L. var. *salicifolius* I.M. Johnst.

Key words: Triple-stack event, kochia, Palmer amaranth, common lambsquarters, auxin herbicide

Introduction

The United States is a major global producer of *Beta vulgaris* (sugar beets), ranking fifth worldwide in 2022 with over 29 billion kg produced (USDA 2023). Effective weed control strategies are crucial for the success of this crop, as slow-growing sugar beet seedlings are poor competitors against weeds (Gerhards et al. 2017). Controlling weeds before and after sugar beet emergence is fundamental to maintaining yield and product quality (Bhadra et al. 2020). Before the introduction of genetically engineered traits, weed management in sugar beet production relied heavily on mechanical and cultural practices, as well as a limited number of herbicide options. This often led to challenges in weed management, potentially causing crop damage and yield loss (Lueck et al. 2017).

Since the introduction of Roundup Ready sugar beet in 2008, glyphosate has substantially facilitated weed management and reduced the impact of weeds on sugar beet farms (Morishita 2018). Glyphosate is extensively utilized in the current preemergence and postemergence weed management program in sugar beet systems. Dicamba, while not typically used directly in sugar beets, is often applied in rotational crops like wheat, barley, or corn and fallow (Bhadra et al. 2020; Cioni and Maines 2010). However, the efficacy of both glyphosate and dicamba has diminished in recent years, likely due to repeated use of glyphosate in sugar beet and both glyphosate and dicamba in rotational crops, which has accelerated the evolution of resistance in certain weed species (Jhala et al. 2020).

Kochia, Palmer amaranth, common lambsquarters, and redroot pigweed are among the most troublesome weeds in sugar beet systems, known for causing significant yield loss (Soltani et al. 2018; Van Wychen 2016). Kochia, a C₄ summer-annual broadleaf weed, is particularly notorious for its invasiveness, persistence, and prolific seed production (over 100,000 seeds m⁻²) (Kumar and Jha 2015). Moreover, kochia exhibits remarkable tolerance to abiotic stressors such as low soil temperature, drought, soil salinity, and heat (Kumar et al. 2019a). Its protogynous flowers promote outcrossing and gene flow, increasing genetic diversity and potentially accelerating the spread of herbicide resistance (Martin et al. 2020). Kochia populations have been reported with resistance to several modes of action, including acetolactate synthase (ALS) inhibitors, synthetic auxins, and EPSP synthase inhibitors (Heap 2024).

Common lambsquarters is an annual weed species that poses a significant challenge in sugar beet production (Bhadra et al. 2020). Capable of both self- and cross-pollination through

wind and insect vectors, this weed boasts high reproductive capacity, with single plant producing over 70,000 seeds. This prolific seed production contributes to its rapid spread and persistence in sugar beet fields. Resistance has been documented in common lambsquarters for photosystem II (PSII) and acetolactate (ALS)-inhibiting herbicides (Heap 2024). Reduced glyphosate translocation has been reported in common lambsquarters populations (Yerka et al. 2013). Glyphosate efficacy is affected by growth state in common lambsquarters, where plants above 7-cm exhibit greater tolerance than small plants (DeGreeff et al. 2018; Sivesind et al. 2011).

Palmer amaranth and redroot pigweed are problematic weed species in sugar beet production, both sharing prolonged emergence period that complicates management (Werle et al. 2014). Palmer amaranth, an annual, dioecious plant species, is a prolific seed producer (Ward et al. 2013). As an obligate outcrosser with high genetic diversity and pollen-mediated gene flow, it readily develops and spreads herbicide resistance (Jhala et al. 2020; Sosnoskie et al. 2012). Glyphosate and dicamba resistant Palmer amaranth populations have been reported in several U.S. states (Foster and Steckel 2022; Kumar et al. 2020; Vieira et al. 2018). Redroot pigweed possesses similar morphological characteristics to Palmer amaranth but is monoecious and more prevalent in Colorado. Redroot pigweed can be challenging to manage in sugar beet production (Jursík et al. 2008; Soltani et al. 2018).

Target-site resistance (TSR) to glyphosate, primarily through increased 5-enolpyruvylshikimate 3-phosphate (*EPSPS*) gene copy number, has been reported in kochia and Palmer amaranth (Gaines et al. 2019; Patterson et al. 2019). This gene amplification can lead to high resistance levels, depending on the number of gene copies replicated (Gaines et al. 2016; Giacomini et al. 2019; Godar et al. 2015). Target-site resistance to dicamba, involving mutations in the auxin receptor gene, has been reported in kochia populations, drastically reducing dicamba efficacy (LeClere et al. 2018; Wiersma et al. 2015). A thorough understanding of these resistance mechanisms is crucial for developing effective and sustainable weed management strategies, including the implementation diversified herbicide programs, crop rotation, and the integration of alternative weed control tactics (Brunharo et al. 2022).

The ongoing development of a sugar beet variety with a triple stack trait conferring resistance to glyphosate, dicamba, and glufosinate is anticipated to improve post-emergence weed management, particularly during the challenging early growth phase. While this stacked trait offers new possibilities for sugar beet weed management, the individual herbicides

(glyphosate, dicamba, and glufosinate) are not new to agriculture. Glufosinate is not currently used in sugar beet systems, but TSR and non-target site resistance (NTSR) to this herbicide has been reported in multiple weed species in different cropping systems. Carvalho-Moore et al. (2022) identified TSR to glufosinate in Palmer amaranth accessions from Arkansas due to increased chloroplastic glutamine synthetase gene copy number and overexpression. A resistant Italian ryegrass population from Oregon was able to metabolite glufosinate faster than susceptible populations (Brunharo et al. 2019). A North Carolina Palmer amaranth population from Anson County was recently confirmed to be resistant to glufosinate when compared to susceptible lines from the same state (Jones et al. 2024).

A previous survey (Westra et al. 2019) conducted from 2011 to 2014 in Colorado reported resistance in kochia to glyphosate and dicamba. However, there is limited available information regarding the current resistance to glyphosate, dicamba, and glufosinate in major weed species in sugar beet systems.

To address the concerns of growers and predict the efficacy of upcoming herbicide-resistant sugar beet traits, we conducted a resistance survey in 2020 and 2021 across sugar beet growing areas in Colorado, Nebraska, and Wyoming. The survey focused on four key weed species belonging to the Amaranthaceae family: kochia, Palmer amaranth, redroot pigweed, and common lambsquarters. Our objectives were two-fold: (1) to determine the geographical distribution of glyphosate, dicamba, and glufosinate resistance across these regions, and (2) to investigate whether resistance observed in kochia, and Palmer amaranth populations was due to previously documented TSR mechanisms.

Materials and Methods

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 in Colorado. Sample collection in Colorado included four species, kochia, Palmer amaranth, common lambsquarters, and redroot pigweed. In Nebraska, seeds of kochia, Palmer amaranth, and common lambsquarters were collected, while only kochia samples were collected in Wyoming. The collection was conducted by driving transects, ensuring a minimum distance of

eight kilometers between each cropping area. To minimize sampling bias, sugar beet fields along the border and on side roads were specifically targeted, regardless of reported herbicide efficacy. At each collection site, ten to fifteen plants of each target weed species were threshed, and the seeds were combined to form a population sample. The latitude and longitude coordinates of each area were recorded and georeferenced using a portable GPS device (Trimble Geo XH 2005 series, Trimble Boulder, Boulder, CO). Nebraska and Wyoming samples along with location data were sent to Colorado State University by mail from the Western Sugar Cooperative.

Greenhouse planting procedures

To obtain a representative sample from each collection site, seeds of kochia, common lambsquarters, redroot pigweed, and Palmer amaranth were threshed from ten to fifteen dried mature plants in the field and seeds were combined for each sampling location. Individual seeds from each population were then planted using pot soil LM-GPS germination, plugs and seedling (Lambert Peat Moss Inc, 106 chemin Lambert, Riviere-Ouelle, QC G0L 2C0, Canada) in a plug tray (1.3 cm by 1.3 cm by 2.5 cm, TOP 200 Plug Tray 2.125 Deep Black, Griffin, 1619 Main Street, Tewksbury, MA 01876, USA). Seedlings were grown to a height of 3-cm before transplanting into larger pots (3.8 cm by 3.8 cm by 5.8 cm) (Dillen CTS332PF Tray Black, 32 Pocket Square Carry Tray 03.00 Pot, Griffin, 1619 Main Street, Tewksbury, MA 01876, USA). At the 5 to 7-cm height, a total of 96 plants per population (32 individuals per herbicide) were screened for resistance to glyphosate, dicamba, and glufosinate alongside a known susceptible line, originally from western Nebraska (Preston et al. 2009). Plants were maintained in a greenhouse at $26/22 \pm 2$ C day/night, and a 14/10 h light/dark photoperiod. Additionally, they were irrigated daily to ensure they remained at field capacity of soil-less media.

Herbicide applications

Each collected population was individually screened for resistance to glyphosate, dicamba, and glufosinate. Plants 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 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®, BASF Corporation, 26 Davis Drive, Research Triangle Park) 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. Adjuvants were included based on herbicide-label recommendations for each herbicide. Herbicide applications were carried out using a single-nozzle spray chamber (DeVries Generation III Research Sprayer, MN, Hollandale), calibrated to deliver 187 L ha⁻¹. Phenotype was characterized by comparing each treated population to a known susceptible reference and an untreated control for each species. Individual plants that survived were visually assessed and categorized as resistant if they remained alive after a 4-week period, regardless of herbicide injury. 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 percentage scale (Owen et al. 2007), where populations with <1% survivors were categorized as susceptible, those with 1% to 19% survivors were classified as low resistance, and populations with >20% survivors were classified as resistant. Collection sites were georeferenced, and maps were created using QGIS software (version 3.28.3) from the QGIS Geographic Information System, Open-Source Geospatial Foundation Project (<http://qgis.org>). The WGS84 coordinate system (EPSG:4326) was used. The relationship between kochia glyphosate and dicamba resistance was examined by Fisher's Exact Test to investigate whether resistance to one herbicide was associated ($p < 0.05$) with resistance to the other. The general null hypothesis for this test is that categorical variables (phenotype classification) are independent (Nowacki 2017); in other words, glyphosate resistance has no influence on dicamba resistance and vice versa. Heatmaps were generated in R statistical software (version 4.1.2; R Core Team 2024) using the ggplot2 package (Wickham 2016). The matrix heatmaps were based on contingency tables displaying the proportions of populations classified as susceptible, low resistant, or resistant to both glyphosate and dicamba. Palmer amaranth associations were not analyzed due to the low sample size in this study.

Laboratory assays

Known TSR mechanisms were investigated for weed populations categorized as resistant (>20% survival). Glyphosate and dicamba TSR mechanisms were investigated for kochia, while only glyphosate TSR mechanism was investigated for Palmer amaranth, as there are no reports of dicamba TSR mechanisms for this species in the literature. Increased EPSPS copy number was

assessed for all collection sites where populations were categorized as resistant. Young tissue material (100 mg) was collected 28 days after glyphosate treatment from three randomly selected survivors and placed into a separated 2 mL Eppendorf tubes in liquid nitrogen and stored at -20 °C when not in use. Samples were homogenized using a TissueLyser II (QIAGEN Sciences, 19300 Germantown Rd Germantown MD 20874 USA). Genomic DNA extraction from each sample was conducted using a Zymo quick DNA extraction kit (Zymo Research, 17062 Murphy Avenue, Irvine, CA 92614, USA). 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, USA). 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, 100 Cummings Center Suite 407J Beverly, MA, USA), 1.2 µL of the forward and reverse primers [5 µM final concentration], 5µL gDNA (10 ng) and 2.6 µL of nuclease-free water. Reactions were performed in a BioRad CFX Connect Real-Time PCR machine (Bio-Rad Laboratories, Inc. 1000 Alfred Nobel Drive Hercules, CA 94547, USA). 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 C 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, thereby ensuring the reliability and accuracy of the PCR reaction. Relative *EPSPS* gene copy number was determined using the $2^{\Delta C_t}$ ($\Delta C_t = C_t^{ALS} - C_t^{EPSPS}$) (Schmittgen and Livak 2008). This method was applied to kochia and Palmer amaranth using three biological replicates, each from a different surviving plant, and two technical replicates per biological replicate. Mean and standard deviation of the mean of the relative *EPSPS* copy number was calculated for each population. To establish a reference for comparison and verify assay accuracy, a resistant kochia population from Akron, Colorado, with elevated *EPSPS* copy number (Gaines et al., 2016) and a known susceptible population were included as positive and negative controls, respectively. A non-template control with nuclease-

free water was included in each qPCR reaction to ensure accuracy and reliability of the method.

Dicamba TSR mechanism was investigated in populations of kochia classified as resistant in the previous greenhouse screening. The *AUX/IAA16* (GenBank: MF376149.1) gene was Sanger sequenced to verify the presence of the previously reported G₇₃N substitution in the degron region (LeClere et al., 2018). Young tissue material (100 mg) was collected 28 days after dicamba treatment from three randomly selected survivors and placed into separate 2 mL Eppendorf tubes in liquid nitrogen and stored at -80 C when not in use. RNA extraction was conducted using a Zymo Quick RNA extraction kit following the manufacturer's recommendations (Zymo Research, 17062 Murphy Avenue, Irvine, CA 92614, USA). Extracted RNA was checked for quality and quantity using a NanoDROP 1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), employing the same methodology used for glyphosate previously described. Subsequently, cDNA was synthesized from the RNA product using a ProtoScript® II kit (Fisher Scientific, 300 Industry Drive, Pittsburgh, PA 15275-1010, USA) with random primers. PCR detection was performed using 1 µL of cDNA, 12.5 µL of EconoTaq® PLUS 2× Master Mix (Lucigen, 2905 Parmenter Street, Middleton, WI 53562, USA), 2 µL of the forward primer, 2 µL of the reverse primer, and 7.5 µL of water, resulting in a total volume of 25 µL for each sample. PCR primers were described previously (Montgomery et al. 2024). PCR products were visualized on a 1.0% agarose gel stained with Biotium GelRed® Nucleic Acid Gel Stain, 10,000X, 0.5 mL in DMSO following manufacturer's recommendations (Biotium, Inc. 46117 Landing Parkway Fremont, CA 94538). The PCR products were then processed for Sanger sequencing by GENEWIZ (111 Corporate Boulevard, South Plainfield, NJ 07080).

Results and Discussion

Glyphosate resistance status

A total of 37 of sugar beet fields were surveyed across eastern Colorado to assess the presence of weeds at sugar beet harvest. In total, 97.30% of the surveyed fields were infested with kochia (Figure 1), 13.51% had Palmer amaranth (Supplementary Figure S1), 62.16% had common lambsquarters (Supplementary Figure S2), and 48.65% had redroot pigweed (Supplementary Figure S3). In Nebraska, 100% of the surveyed fields had kochia (Figure 2),

12% had Palmer amaranth (Supplementary Figure S4), and 22% had common lambsquarters (Supplementary Figure S5). In Wyoming, only kochia was targeted and was present in 100% of the surveyed fields (Figure 3) surveyed. Among these weed species, kochia was likely the most problematic in Colorado, Nebraska, and Wyoming sugar beet farms, as this weed species was present and widespread in most of the survey collection areas.

Screening of kochia accessions from Colorado revealed that around 75% of the collected samples were classified as resistant, 19% exhibited low resistance, and 6% were susceptible. In Nebraska (Figure 2A), 86% of populations were classified as resistant, 12% as low resistance, and only 2% as susceptible. In Wyoming (Figure 3A), 33% were categorized as resistant, 42% as low resistance, and 25% as susceptible.

With the commercialization of glyphosate-resistant sugar beet, Colorado, Wyoming, and Nebraska had 85 to 90% trait adoption within the first years (Khan 2010). After fifteen years of utilizing this technology, weed management strategies continue to heavily rely on glyphosate for 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 likely contributes to the evolution of resistance over time by selecting resistant populations. The evolution of glyphosate resistance in kochia populations is a significant issue in North America, recorded in multiple states across the United States and Canada (Heap 2024). For instance, a 2014 survey on kochia in 96 populations primarily from wheat-fallow systems in eastern Colorado showed that 23% of accessions were glyphosate resistant (Westra et al. 2019). In Canada, a 2018 survey in Manitoba identified a resistance rate of 59% in 315 sites, with the highest frequency of glyphosate resistant kochia in glyphosate resistant crops such as soybean and corn (Geddes et al. 2021). In southern Saskatchewan, researchers identified a high occurrence of glyphosate- and dicamba-resistant kochia populations, with 87% resistant to glyphosate and 45% to dicamba (Sharpe et al. 2023). Likewise, our study uncovered a significant proportion of glyphosate resistant populations (Figure 4), with frequencies of 94%, 98%, and 75% in Colorado, Nebraska, and Wyoming, respectively, considering both low-resistant and resistant populations.

In general, kochia exhibited minimal to no damage following glyphosate 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 5). This

explains the observed resistance phenotype, although additional underlying mechanisms could exist. For instance, one kochia population from Wyoming was classified as resistant, but individual survivors did not show an increased copy number (Figure 6). The resistance mechanism in this 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 increased *EPSPS* copies and reduced glyphosate efficacy, corresponding to increased resistance levels (Gaines et al. 2016; Godar et al. 2015). This resistance mechanism has been observed in various weed species, such as Palmer amaranth, weedy sunflower, and Russian thistle, none of which were 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 target enzyme amounts, reducing glyphosate effectiveness at the field rates (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 contributing 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, the 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 glyphosate resistant crop where they identified 78% and 70% of glyphosate-resistant 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 glyphosate-resistant 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 postemergence weed control in glyphosate-resistant soybean fields in Brazil has led to the emergence of resistant weed species such as horseweed [*Conyza sumatrensis* (Retz.) E.H. Walker], Italian ryegrass (*Lolium multiflorum* Lam.), sourgrass [*Digitaria insularis* (L.) Mez ex Ekman] (Adegas et al. 2022; Correia and Durigan 2010). Given the high prevalence of glyphosate resistance in kochia, especially in Nebraska, the glyphosate

resistance trait in the new triple-stack sugar beet may offer limited benefits for kochia management when used alone. Nonetheless, glyphosate remains an effective tool for managing other susceptible weed species and can be integrated into a broader IWM approach to prevent the evolution of herbicide resistance.

For Palmer amaranth populations only a few numbers of accessions were identified and collected in Colorado (Supplementary Figure S1) and Nebraska (Supplementary Figure S4), but not in Wyoming. Based on our survey, four out of the five Palmer amaranth populations collected in Colorado were glyphosate resistant, 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%) (Supplementary Figure S4). The relatively low number of Palmer amaranth populations in 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 glyphosate-resistant. Due to its dioecious nature, this species has a high potential for evolving and spreading resistance through gene flow via pollen similarly as kochia. Most of the identified resistance mechanisms so far have been nuclear inherited, including gene amplification, which contributes to the rapid evolution of herbicides (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 7), which possessed 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 glyphosate resistant Palmer amaranth populations have been reported in twenty-six states (Heap 2024), there have been no previous reports of glyphosate resistant 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 ALS-, PSII-inhibiting herbicides and very recently to auxin herbicide (Ghanizadeh et al. 2024; Huang et al. 2020; McKenzie-Gopsill et al. 2020). Several studies have highlighted inconsistencies in glyphosate efficacy for controlling common lambsquarters, likely due to species varying tolerance at different growth stages 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 2.5 cm plants to 55% in 7.5- to 15-cm plants at 21 days after application. Sivesind et al. (2011) noticed a reduction in glyphosate efficacy associated with growth stage, where the ED₅₀ (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 plants varying from 10 to 21-cm height, 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 TSR mechanisms 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. Common lambsquarters and redroot pigweed are predominantly autogamous, meaning that gene flow is predominantly by individual plants. 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 contribute to the management of these two species by providing additional post-emergence herbicide options. However, it is important to consider that resistance to glyphosate and dicamba has been reported in redroot pigweed and common lambsquarters in other regions (Heap 2024;

Rahman et al. 2014), highlighting the importance of integrating this technology into a diversified weed management programs to mitigate future resistance risks. Additionally, it is crucial to implement practices that prevent the spread of resistant seeds, such as thoroughly cleaning equipment between fields and using certified weed-free seed. Mitigating the evolution of herbicide resistance in these species requires careful attention to seed dispersal and the implementation of effective management strategies.

Dicamba resistance status

Kochia populations classified as resistant were identified in Colorado (Figure 1B) and Nebraska (Figure 2B) and at low frequencies in Wyoming (Figure 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 dicamba resistant, 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 categorized as resistant. One Palmer amaranth population out of five collected in Colorado was classified as dicamba resistant (Supplementary Figure S6) corresponding to 20% of survival frequency, and 80% were classified susceptible. For Nebraska out of 8 populations (Supplementary Figure S7), 0% were resistant, 38% as low resistance and 49% as susceptible. Our data indicates a limited number of dicamba resistant populations across these states when compared to glyphosate, however a notable proportion of populations categorized as low resistant (with survival rates ranging from 1% to 20%) were 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 frequency could potentially evolve to a population categorized as resistant in subsequent years if the

selection pressure for dicamba is intensive. 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.

There was no significant association between glyphosate and dicamba among resistant kochia populations from Colorado and Wyoming; however, in Nebraska, a relationship between the phenological classification was observed ($p < 0.05$) (Figure 8). In all three states, dicamba resistant kochia populations were always either resistant or low resistance to glyphosate. In contrast, there were populations glyphosate resistant that were not resistant to dicamba. This suggests that dicamba resistant populations are more likely to have glyphosate resistance, and fields with glyphosate resistance kochia may or may not contain dicamba resistance.

The lack of significance ($p > 0.05$) in Colorado and Wyoming does not necessarily indicate an absence of association between the two-way resistance. The contingency tables in this study (Figure 8) contained expected values lower than 5, which may compromise the statistical test. Low expected values and small sample sizes may result in reduced statistical power, increasing the likelihood of a Type II error, where a true effect is not detected (Freiman et al. 2019). The observed lack of association ($p > 0.05$) could also mean that the categorical variables are not linked, or in other words, resistance to both herbicides are independent, but this is unlikely the case as previously discussed.

Although there are no reports of dicamba-resistant kochia in sugar beet system, 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 2024; Kumar et al. 2019b; Westra et al. 2019). The rapid spread of glyphosate-resistant kochia populations led to increased use of dicamba as an alternative in several crop systems as well as raising the number of resistance cases (Ou et al. 2018a). Most dicamba-resistant 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 2024). A survey revealed a dicamba-resistant Palmer amaranth from Tennessee in dicamba-resistant soybean and cotton crop system, but the resistance mechanism remains unknown (Foster and Steckel 2022). Dicamba resistant Palmer amaranth has not been reported in Colorado until now (Supplementary Figure S6), and further analysis are under

processing to validate this phenotype.

All kochia and Palmer amaranth populations from Colorado, categorized as dicamba-resistant, were also categorized as glyphosate-resistant, whereas the population classified as dicamba-resistant from Nebraska was classified as low resistance to glyphosate. In this survey, we observed that glyphosate-resistant and dicamba-resistant kochia and Palmer amaranth are emerging issues 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. (2018b) 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 can have a dual evolutionary effect due to continuous selection. They may reduce TSR by combining herbicides from different chemical groups. but can increase the risk of NTSR developing through generalist mechanisms like enhanced metabolism (Comont et al. 2020). Rigon et al. (2023) demonstrated that herbicide 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, four known auxin TSR mechanisms have been identified in weeds, occurring in the degron region of Aux/IAA genes. In the presence of auxin, the degron region of Aux/IAA interacts with SCF^{TIR/AFB} complex, promoting the polyubiquitylation of the Aux/IAA repressor. This process leads to the transcription of genes that generate auxin responses through auxin responsive factors (de Figueiredo and Strader 2022). Mutations in the degron region can disrupt the interaction between auxin herbicides and the Aux/IAA-SCF^{TIR/AFB} complex, hindering polyubiquitylation of the repressor and thereby preventing auxin responses, which results in reduced herbicide efficacy. 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 (LeClere et al., 2018). A transposable element insertion in *IAA16* led to a disruption of a normal gene splicing, causing a substitution of a specific glycine in the degron region of Aux/IAA, which is associated with dicamba resistance in kochia (Montgomery et al. 2024). Very recently a new amino acid substitution was reported in the degron region of the *IAA16* gene in common lambsquarters associated with dicamba resistance (Ghanizadeh et al.

2024). In Indian hedge mustard a deletion has been identified in the degron tail region of the Aux/IAA co-receptor gene *IAA2*, resulting in 2,4-D resistance (Figueiredo et al. 2022b). Enhanced metabolic detoxification of fluroxypyr and reduced translocation of 2,4-D have been reported in kochia and Sumatran fleabane, respectively (Leal et al. 2022; Todd et al. 2024).

The presence of the G₇₃N 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 (NEK 30) that were categorized as resistant in our survey 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 the Nebraska population NEK30 had the G₇₃N substitution (Figure 9). The G₇₃N 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 populations likely possess a distinct and novel resistance mechanism, which could be a non-target site resistance (NTSR) mechanism or a yet unknown TSR mechanism. In contrast, the dicamba resistant phenotype observed in the Nebraska kochia population is attributed to the known TSR mechanism, though it may also involve 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; Figueiredo et al. 2022a; 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.

Glufosinate resistance status

Our survey found no glufosinate resistance in any of the weed populations sampled from Colorado, Nebraska, and Wyoming. This includes populations of Kochia and Palmer amaranth that exhibited resistance to glyphosate and dicamba (Figures 1C, 2C, 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. The current lack of glufosinate use in sugar beet systems may be due to the availability of more cost-effective pre-plant herbicides with a broader weed control spectrum. 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 post-emergence applications.

To date, ten cases of herbicide resistance to glufosinate have been documented where most of them occurred in poaceous species and recently in Palmer amaranth (Brunharo et al. 2019; Carvalho-Moore et al. 2022; He et al. 2023; Priess et al. 2022b). An 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 S₅₉G is in contact with important binding residues of glufosinate and was recently reported to confer resistance in a goosegrass (*Eleusine indica* L.) 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 (5- to 7-cm height) under controlled conditions in a greenhouse setting. Glufosinate is a contact herbicide that requires appropriate coverage, and the timing is crucial to achieve an effective control. Plant sensitivity to glufosinate varies considerably by species and likely depends on the amount that reaches the target enzyme, glutamine synthetase (GS). For instance, when the same rate of glufosinate was applied to the leaves of grasses (johnsongrass and ryegrass) and broadleaf species (kochia and Palmer amaranth), lower herbicide concentrations were found in grasses. This reduced GS inhibition and less visual injury (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 least effective among the herbicide treatments for controlling 8 to 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 height, but glufosinate performance increased with the addition of the adjuvant ammonium sulfate.

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 not be 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, antagonism 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. 2022a). 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 additive effect (Ganie and Jhala 2017).

Our survey reveals that resistance to two of the three herbicides (glyphosate and dicamba) to which the new sugar beet trait will confer resistance is already prevalent in sugar beet production areas of Colorado, Nebraska, and Wyoming, even prior to the new traits release. This underscores the critical need for proactive stewardship and IWM strategies to preserve the long-term effectiveness of this new technology. Lessons learned from the current sugar beet cropping systems, where overreliance on glyphosate has led to widespread resistance, should guide the development and implementation of diversified weed management programs. Employing alternative herbicide sites-of-action, along with an IWM approach, becomes critical to mitigate the evolution of resistance and preserve the utility of the new sugar beet trait.

Practical Implications

Surveys play a crucial role in the early detection of resistance, enabling the implementation of effective management strategies. With the impending release of a genetically engineered sugar beet trait resistant to glyphosate, dicamba, and glufosinate, significant changes are expected in weed management practices, particularly in in-crop weed control. Growers associated with the Western Sugar Cooperative, who funded this study, have expressed concerns about the potential widespread resistance to these active ingredients. Although glyphosate resistance in kochia and Palmer amaranth is widespread across the United States, research specifically within sugar beet systems has been limited. This study provides valuable insights into the resistance status and frequency of problematic weed species in current sugar beet systems across Colorado, Nebraska, and Wyoming to the three active ingredients. Additionally, our findings reveal the first occurrence of glyphosate and dicamba-resistant Palmer amaranth populations in Colorado and dicamba-resistant kochia populations within a sugar beet system in Colorado and Nebraska. 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. This study also provides the first assessment of glufosinate resistance in sugar beet weeds in this region. The widespread occurrence of glyphosate and dicamba kochia and palmer resistance in certain areas has direct implications in how those must be managed once the new trait is released. To minimize resistance evolution and safeguard the long-term efficacy of this new technology, it is crucial to implement proactive stewardship practices. Growers should adopt IWM strategies that include crop rotation, using cover crops, employing mechanical weed control, diversifying herbicide sites-of-action, and avoiding repeated use of the same herbicide. Building upon lessons learned from the current sugar beet crop system will be essential to ensure the sustainable success of this new technology.

Acknowledgments

The authors acknowledge the Western Sugar Cooperative for their assistance in identifying field sites and collecting weed seeds.

Funding

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.

Competing interests: The authors declare none.

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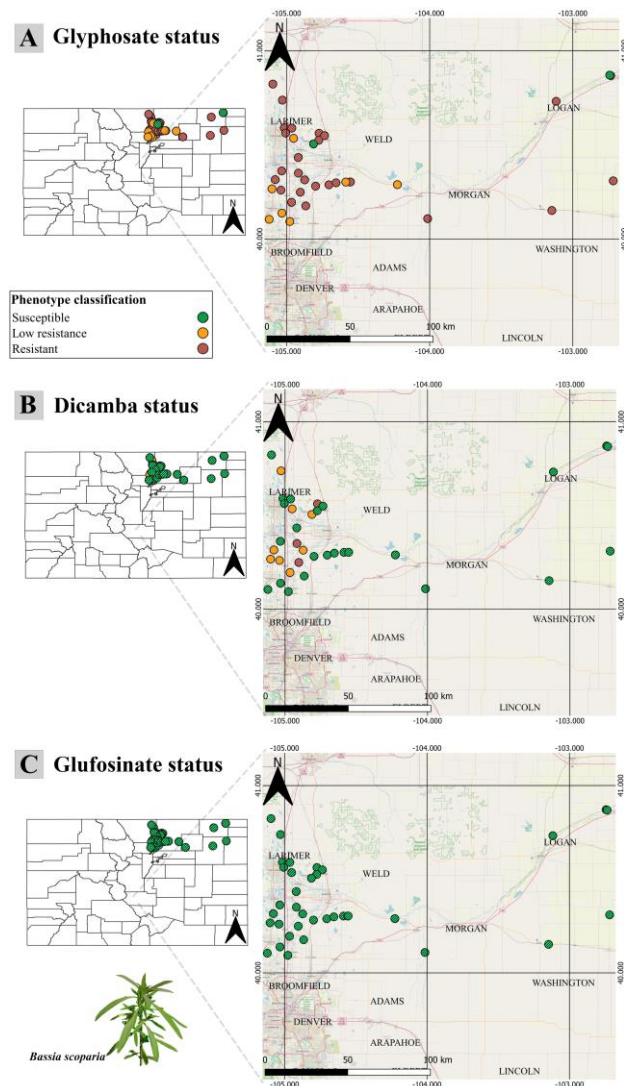


Figure 1: Geo-referenced map illustrating the *Bassia scoparia* (kochia) populations collected in Colorado during the fall of 2021. The dots on the map represent the locations of kochia populations, and their color signifies their response to glyphosate treatment (A), dicamba (B) and glufosinate (C). 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).

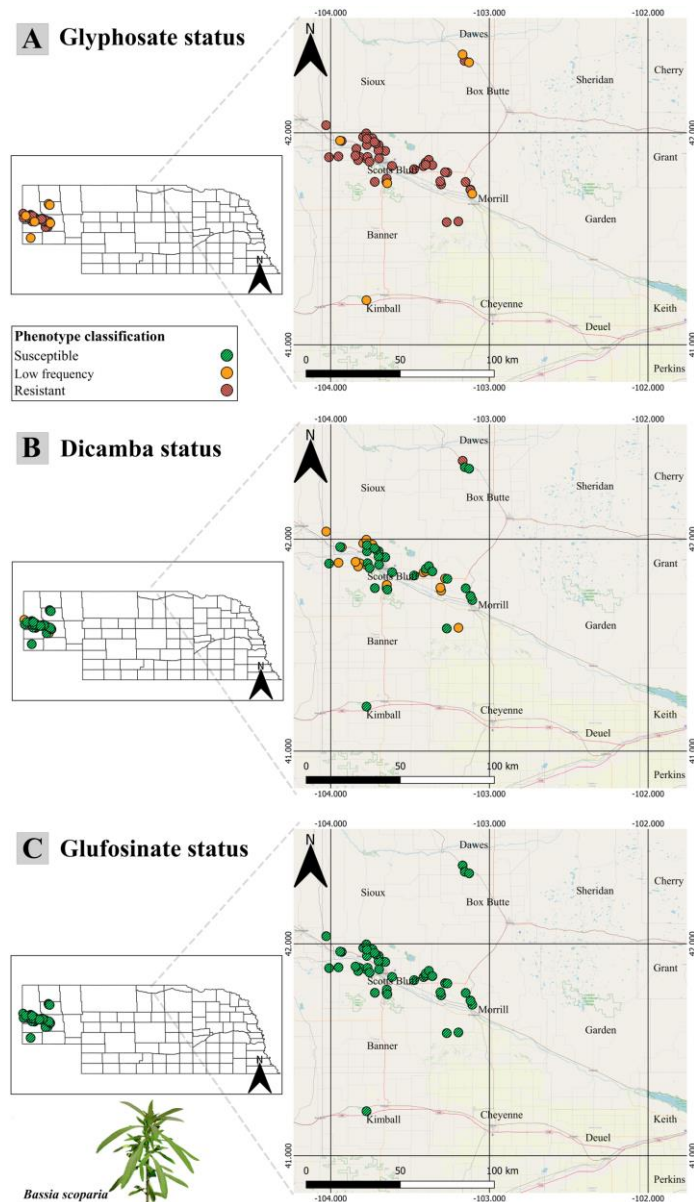


Figure 2: Geo-referenced map illustrating the *Bassia scoparia* (kochia) populations collected in Nebraska during the fall of 2020. The dots on the map represent the locations of kochia populations, and their color signifies their response to glyphosate treatment (A), dicamba (B) and glufosinate (C). 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).

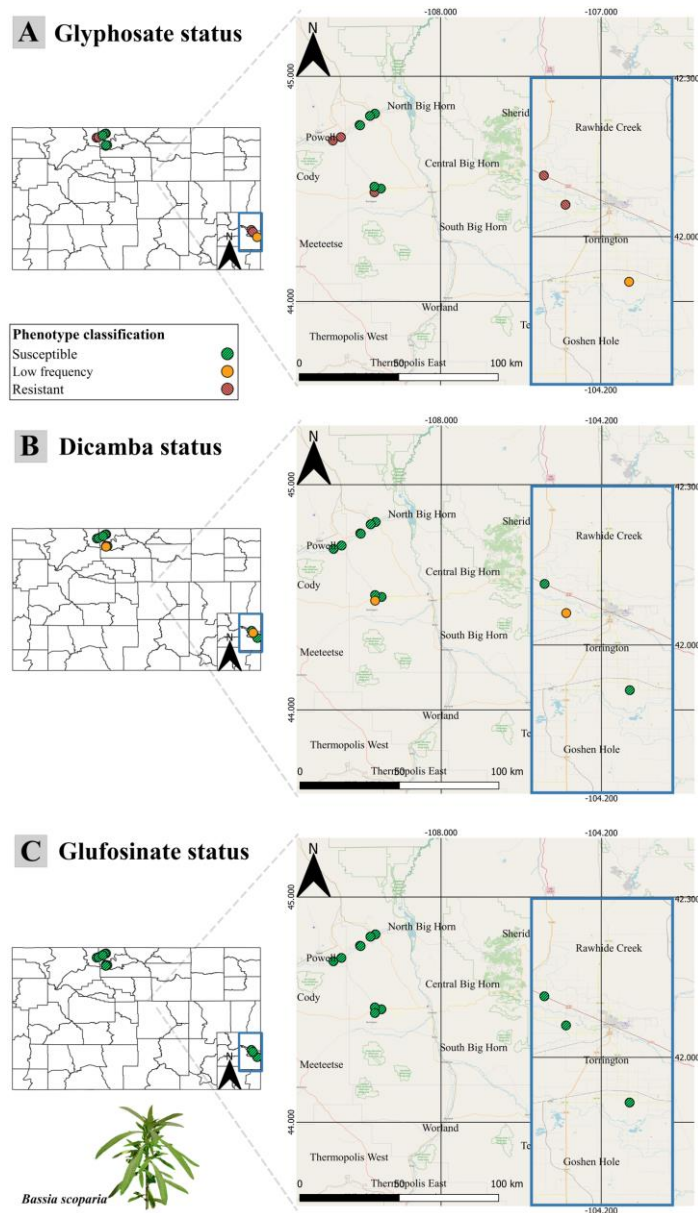


Figure 3: Geo-referenced map illustrating the *Bassia scoparia* (kochia) populations collected in Wyoming during the fall of 2020. The dots on the map represent the locations of kochia populations, and their color signifies their response to glyphosate treatment (A), dicamba (B) and glufosinate (C). 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)

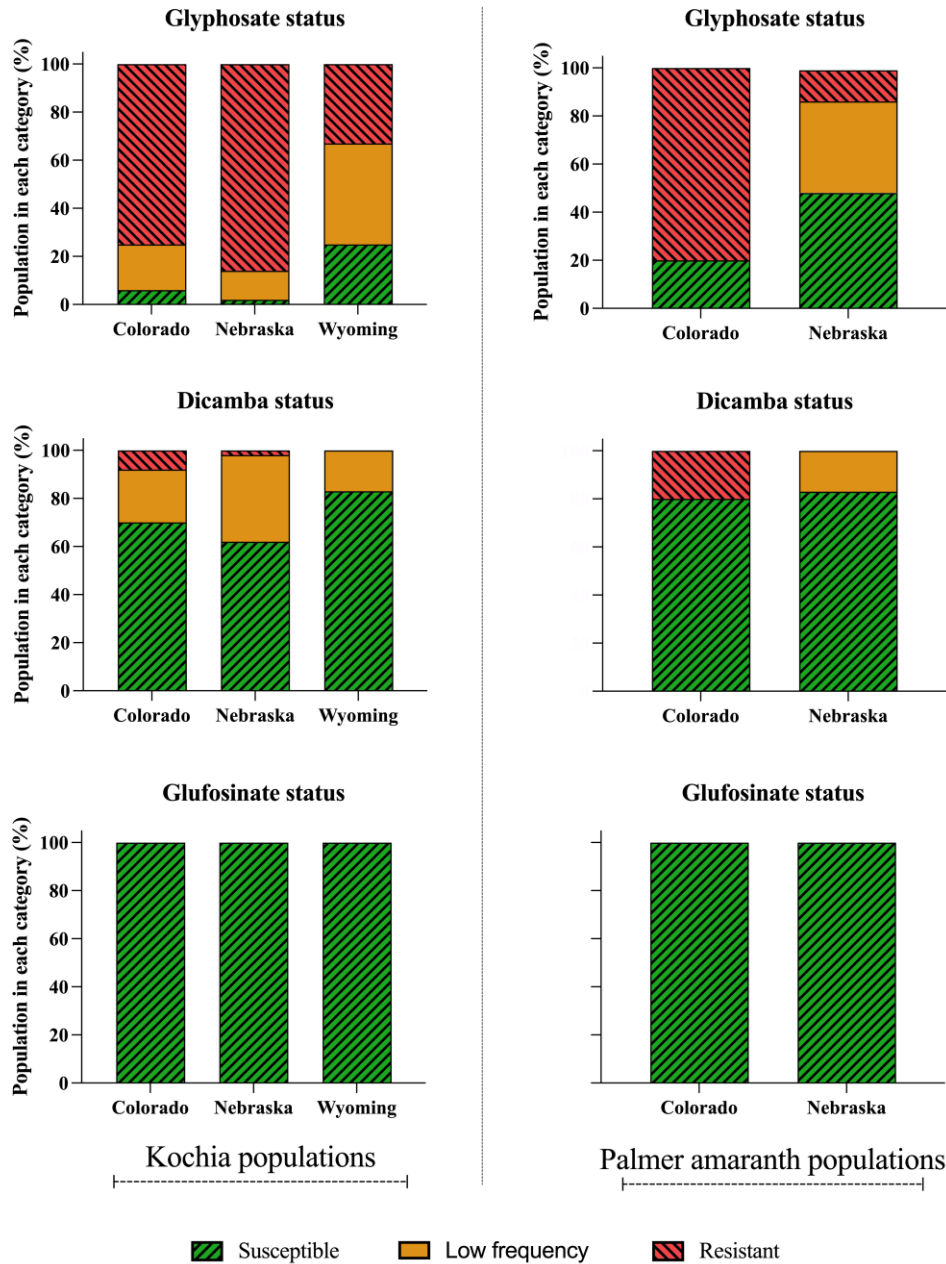


Figure 4. Frequency of observed phenotypes of kochia (left) and Palmer amaranth (right) populations collected from Colorado, Nebraska and Wyoming during the fall of 2020 and 2021, following treatment in a greenhouse setting with glyphosate, dicamba, and glufosinate. Bar colors represent the phenotype 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).

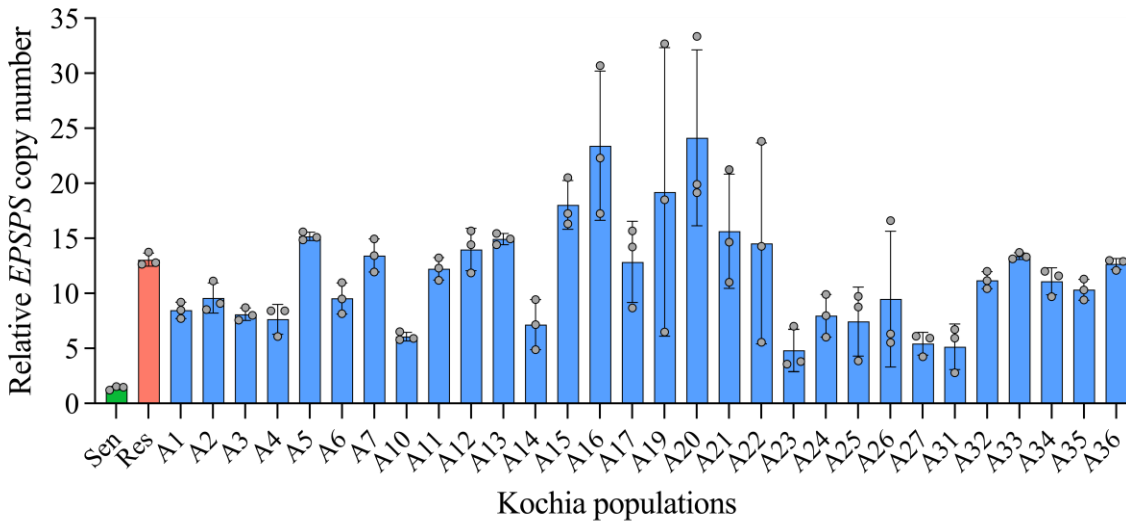


Figure 5. Relative EPSPS gene copy number in kochia populations collected from Colorado. The green and red bars represent the sensitive and resistant references (Sen and Res), respectively. The blue bars labeled as A represent resistant populations (>20% survival) surveyed from Colorado. Each bar represents the mean of the relative *EPSPS* copy number from three biological replicates (shown as grey circles) within each population, with error bars indicating the standard deviation.

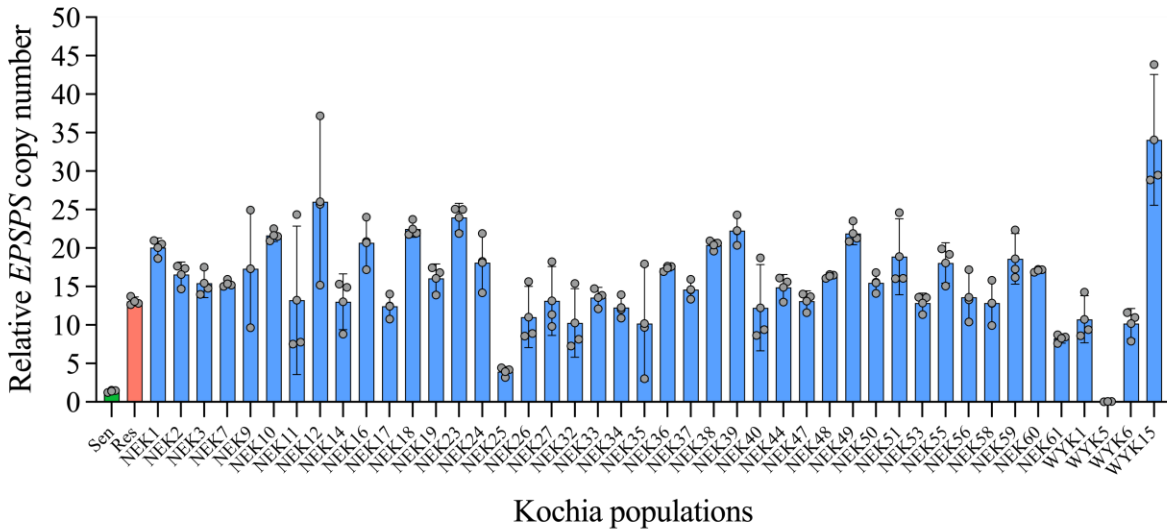


Figure 6. Relative EPSPS gene copy number in kochia populations collected from Nebraska and Wyoming. The green and red bars represent the sensitive and resistant references (Sen and Res), respectively. The blue bars labeled as NEK represent Nebraska kochia populations, and WYK represents Wyoming kochia populations. Each bar represents the mean of the relative *EPSPS* copy number from three biological replicates (shown as grey circles) within each population, with error bars indicating the standard deviation.

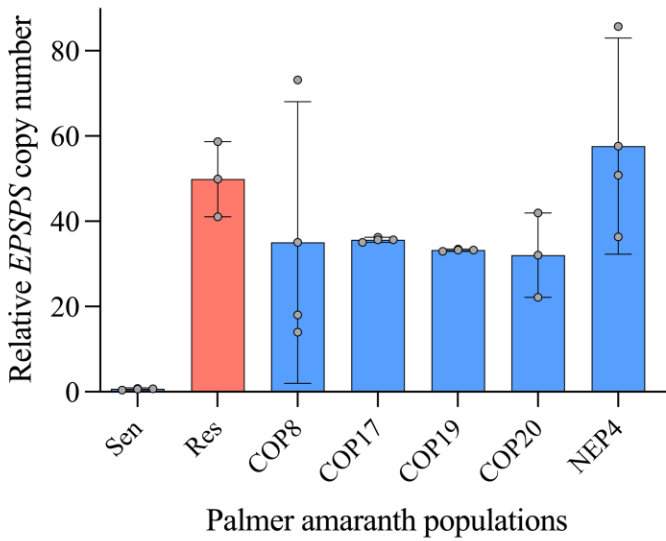


Figure 7. Relative *EPSPS* gene copy number in Palmer amaranth populations collected from Colorado and Nebraska. Known sensitive (Sen) and resistant (Res) Palmer amaranth populations were used as positive and negative controls. The blue bars labeled as COP represent Colorado Palmer amaranth populations classified as resistant (>20% survival), while the blue bar labeled as NEP 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.

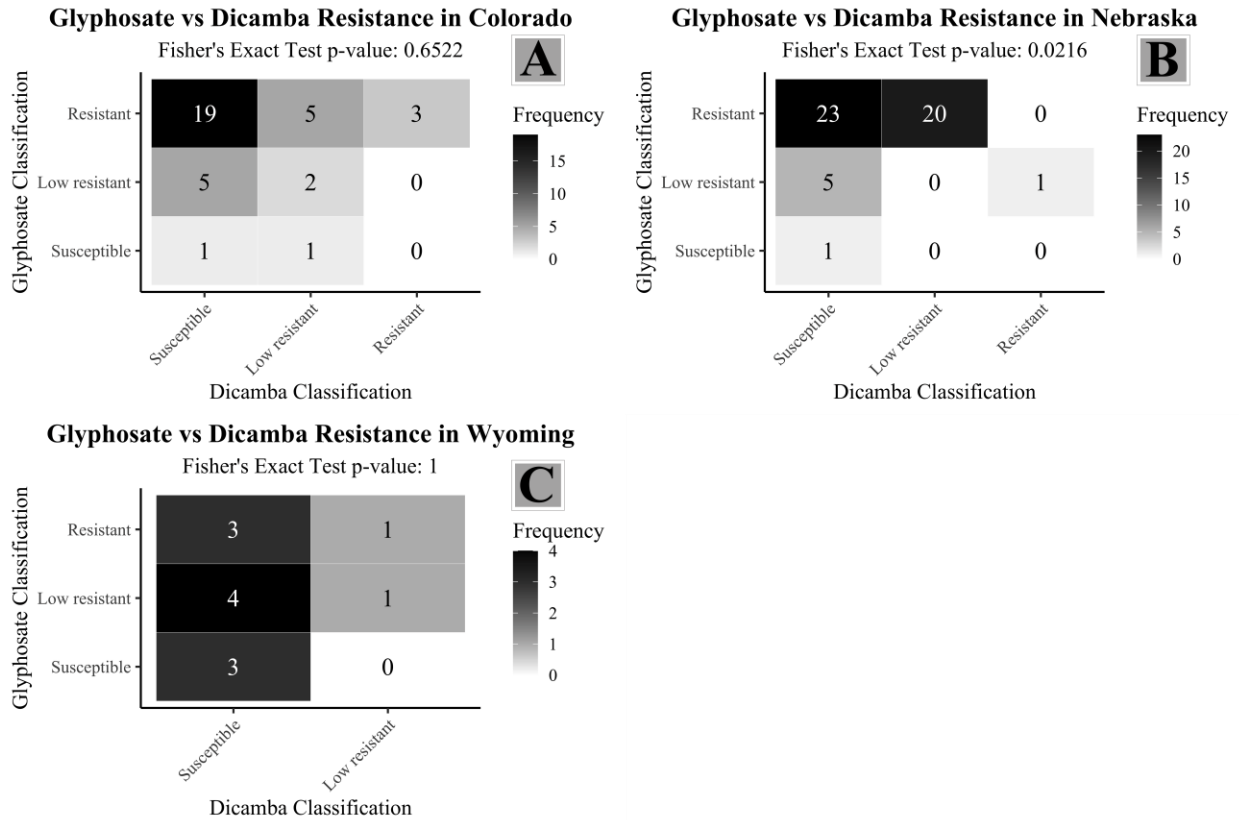


Figure 8: Matrix heatmaps of glyphosate and dicamba resistance in kochia populations across Colorado (A), Nebraska (B), and Wyoming (C). Heatmaps show the frequency of kochia populations categorized by phenotypic classifications (susceptible, low resistant, and resistant) to glyphosate and dicamba in Colorado, Nebraska, and Wyoming. The colors represent the number of observations in each category, with darker shades indicating higher frequencies. Fisher's Exact Test was performed to assess the statistical significance of associations between glyphosate and dicamba resistance. The test statistics and p-values are displayed within each heatmap. Associations are considered significant if the p-value is less than 0.05.

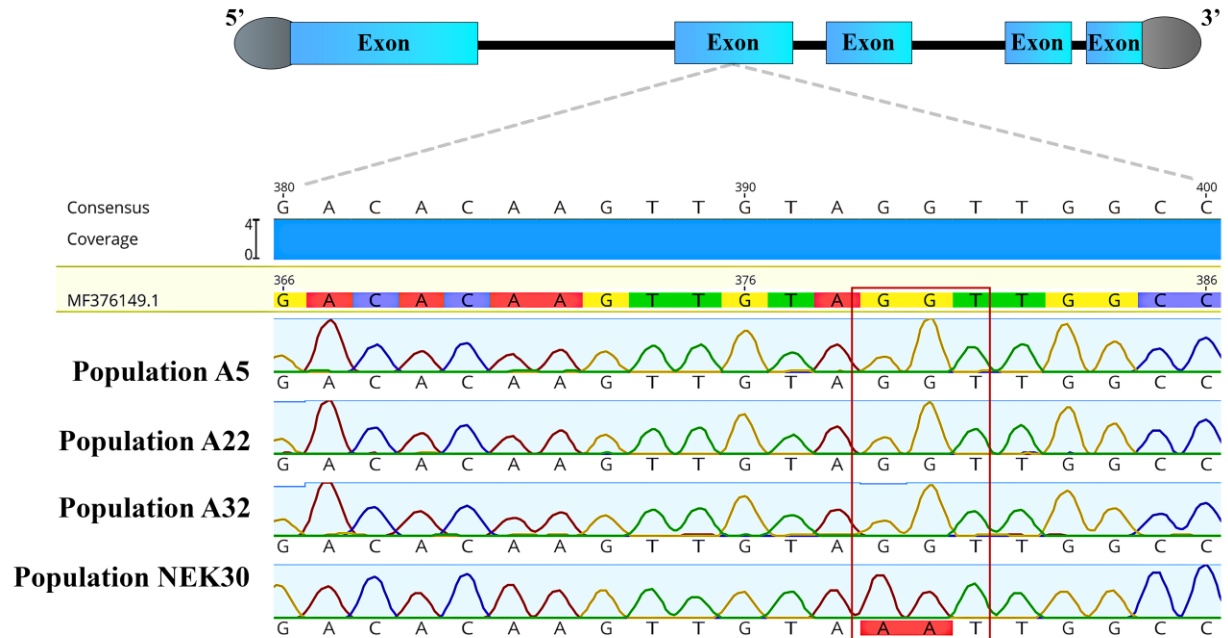


Figure 9. The top illustration shows the gene structure of kochia *IAA16* gene. The 5' and 3' untranslated regions (UTRs) are represented by grey circles, while the exons are shown as blue boxes. The introns are indicated by black lines. The bottom section displays sanger sequencing chromatograms representing kochia population classified as dicamba resistant from Colorado (A5, A22, and A32) and Nebraska (NEK 30). The region highlighted within the red rectangle is associated with the dicamba resistant phenotype (G₇₃N), 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.