

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

## Research Article

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

### Keywords:

Triple-stack event; kochia; Palmer amaranth; common lambsquarters; auxin herbicide

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### Abstract

A prelaunch 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 sugar beet fields, including kochia, common lambsquarters, Palmer amaranth, and redroot pigweed 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* G<sub>73</sub>N 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 strategies that diversify effective herbicide sites-of-action and incorporate alternative weed management practices within cropping systems.

### Introduction

The United States is a major global producer of *Beta vulgaris* (sugar beets), ranking fifth worldwide in 2022 with more than 29 billion kg having been 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 in sugar beets, weed management in sugar beet production relied heavily on mechanical and cultural practices, and 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 used in current preemergence and postemergence weed management programs in sugar beet systems. Dicamba, while not used directly on sugar beets, is often applied with rotational crops such as 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 application of glyphosate on sugar beet fields and both glyphosate and dicamba on 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

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(Soltani *et al.* 2018; Van Wychen 2016). Kochia, a C<sub>4</sub> summer-annual broadleaf weed, is particularly notorious for its invasiveness, persistence, and prolific seed production (more than 100,000 seeds m<sup>-2</sup>) (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, thereby 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 5-enolpyruvylshikimate-3-phosphate synthase (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 exhibits a high reproductive capacity, with a single plant producing more than 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 herbicides that inhibit photosystem II (PS II) and ALS (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 taller than 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 a 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 herbicide resistance (Jhala *et al.* 2020; Sosnoskie *et al.* 2012). Glyphosate-resistant 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 *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). TSR to dicamba, which involves mutations in the auxin receptor gene, has been reported in kochia populations, which drastically reduces 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 postemergence 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-TSR (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 Palmer amaranth population from Anson County North Carolina was recently confirmed to be resistant to glufosinate when compared with susceptible lines of that weed from the same state (Jones *et al.* 2024).

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

To address the concerns of growers and to 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 2-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 autumn 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 37 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 8 km 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, 10 to 15 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 (Geo XH 2005 series; Trimble Boulder, Boulder, CO). Samples from Nebraska and Wyoming 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 10 to 15 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., Riviere-Ouelle, QC, Canada) in a plug tray (1.3 cm × 1.3 cm × 2.5 cm, TOP 200 Plug Tray 2.125 Deep Black; Griffin, Tewksbury, MA). Seedlings were grown to a height of 3 cm before being transplanted into larger pots (3.8 cm × 3.8 cm × 5.8 cm, Dillen CTS332PF Tray Black and 32 Pocket Square Carry Tray 03.00 Pot; Griffin). When plants were 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 ± 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<sup>-1</sup>; Bayer CropScience, St. Louis, MO) with ammonium sulfate at a concentration of 20 g L<sup>-1</sup>; dicamba (Engenia®, 280 g ae ha<sup>-1</sup>; BASF, Research Triangle Park, NC) with a nonionic surfactant (Induce®, BASF) at a concentration of 0.25% v/v, and glufosinate (Liberty®, 590 g ai ha<sup>-1</sup>; BASF) also 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; Hollandale, MN), calibrated to deliver 187 L ha<sup>-1</sup>. 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-wk 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 a previously established percentage scale (Owen et al. 2007) in which 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 via 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 with R statistical software (v. 4.1.2; R Core Team 2021) 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 the

glyphosate TSR mechanism was investigated for Palmer amaranth, because 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 d 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, Germantown MD). Genomic DNA extraction from each sample was conducted using a Zymo Quick DNA extraction kit (Zymo Research, Irvine, CA). 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, Waltham, MA). Relative *EPSPS* copy number was determined using quantitative polymerase chain reaction (qPCR) on the genomic DNA. The primers have been previously described (Gaines et al. 2021). The *ALS* gene was used as a single copy reference gene. Each qPCR reaction was 20 µL, including 10 µL of PerfeCTa *SYBR*<sup>®</sup> green Supermix (Quanta Biosciences, Beverly, MA), 1.2 µL of the forward and reverse primers (5 µM final concentration), 5 µL of gDNA (10 ng), and 2.6 µL of nuclease-free water. Reactions were performed in a CFX Connect Real-Time PCR machine (Bio-Rad Laboratories, Hercules, CA). The temperature for each of the reactions was used as follows: the denaturation step was held at 95 C for 3 min, followed by 30 cycles of denaturation at 95 C for 30 s, and an annealing/extension step at 72 C for 30 s. Fluorescence measurements were 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  $2^{\Delta\Delta C_t}$  ( $\Delta C_t = C_t^{ALS} - C_t^{EPSPS}$ ) method (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, CO, 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.

The dicamba TSR mechanism was investigated in populations of kochia classified as resistant in the previous greenhouse screening. The *AUX/IAA16* (GenBank accession number MF376149.1) gene was Sanger-sequenced to verify the presence of the previously reported G<sub>73</sub>N substitution in the degron region (LeClere et al. 2018). Young tissue material (100 mg) was collected 28 d 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 Quick RNA extraction kit following the manufacturer's recommendations (Zymo Research). Extracted RNA was checked for quality and quantity using a NanoDROP 1000 UV-Vis Spectrophotometer (Thermo Fisher), employing the same methodology used for glyphosate previously described. Subsequently, complementary DNA (cDNA) was synthesized

from the RNA product using a ProtoScript<sup>®</sup> II kit (Fisher Scientific) with random primers. PCR detection was performed using 1 µL of cDNA, 12.5 µL of EconoTaq<sup>®</sup> PLUS 2× Master Mix (Lucigen, Middleton, WI), 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<sup>®</sup> Nucleic Acid Gel Stain, 10,000X, 0.5 mL in dimethyl sulfoxide following the manufacturer's recommendations (Biotium Inc., Fremont, CA). The PCR products were then processed for Sanger sequencing by GENEWIZ (South Plainfield, NJ).

## Results and Discussion

### Glyphosate Resistance Status

A total of 37 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). Among these weed species, kochia was likely the most problematic in Colorado, Nebraska, and Wyoming sugar beet farms, because this weed species was present and widespread in most of the survey collection areas.

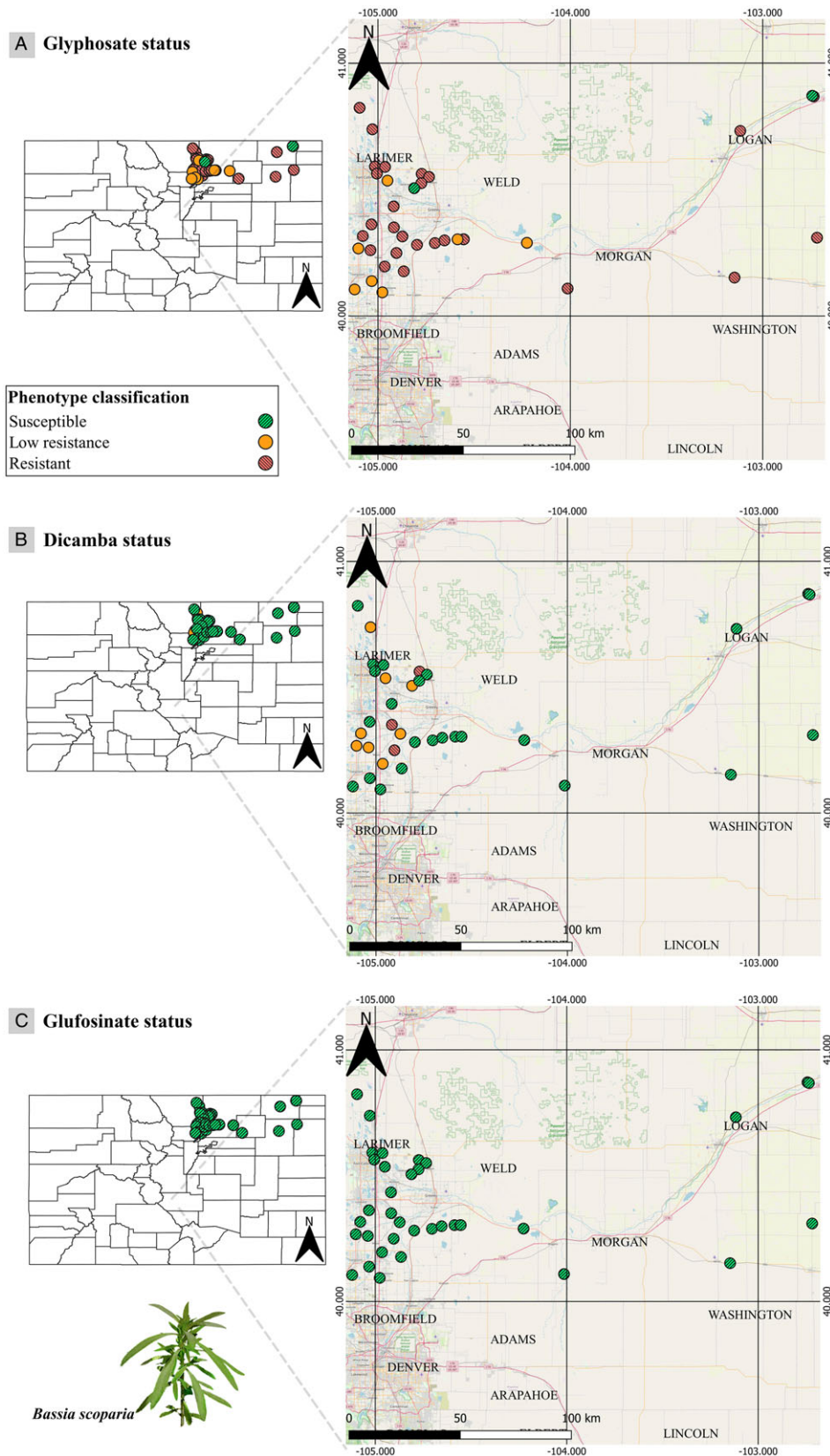
Screening of kochia accessions from Colorado revealed that around 75% of the collected samples were classified as resistant, 19% exhibited low resistance, and 6% were susceptible. In Nebraska (Figure 2A), 86% of populations were classified as resistant, 12% as low resistance, and only 2% as susceptible.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

The glyphosate-resistance trait was adopted by 85% to 90% of sugar beet producers in the first year of commercialization in Colorado, Wyoming, and Nebraska (Khan 2010). After 15 yr of using 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 and provinces throughout the United States and Canada (Heap 2024). For instance, a 2014 survey of kochia in 96 populations primarily from wheat-fallow systems in eastern Colorado showed that 23% of accessions were resistant to glyphosate (Westra *et al.* 2019). In Canada, a 2018 survey in Manitoba identified a resistance rate of 59% at 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% found to be 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,

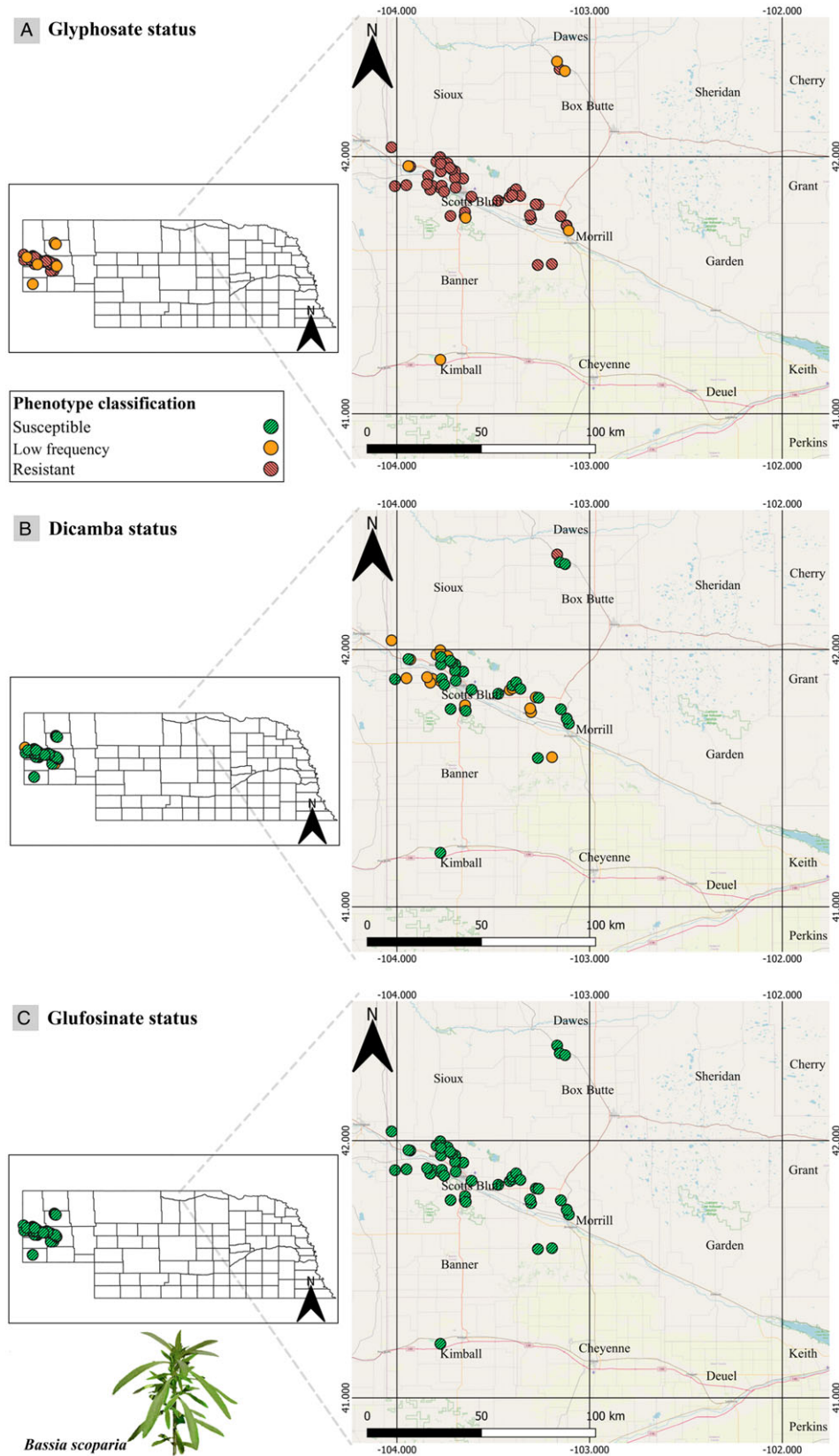
respectively, considering both low-resistant and resistant populations.

In general, kochia exhibited minimal to no damage following glyphosate treatment at the field-use rate. A 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 three) compared with a known susceptible single *EPSPS* gene copy reference (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; Yannicari *et al.* 2023). The presence of multiple copies of this gene results in more target enzymes, reducing glyphosate effectiveness at field-use 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 crops, where they identified 78% and 70% of glyphosate-resistant kochia populations in soybean and corn areas, respectively. The same authors observed an increase in glyphosate resistance ranging from 1% to 59% in just 5 yr. A survey conducted among stakeholders in Nebraska revealed that glyphosate was the primary postemergence 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.], and 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 integrated weed management (IWM) approach to prevent the evolution of herbicide resistance.

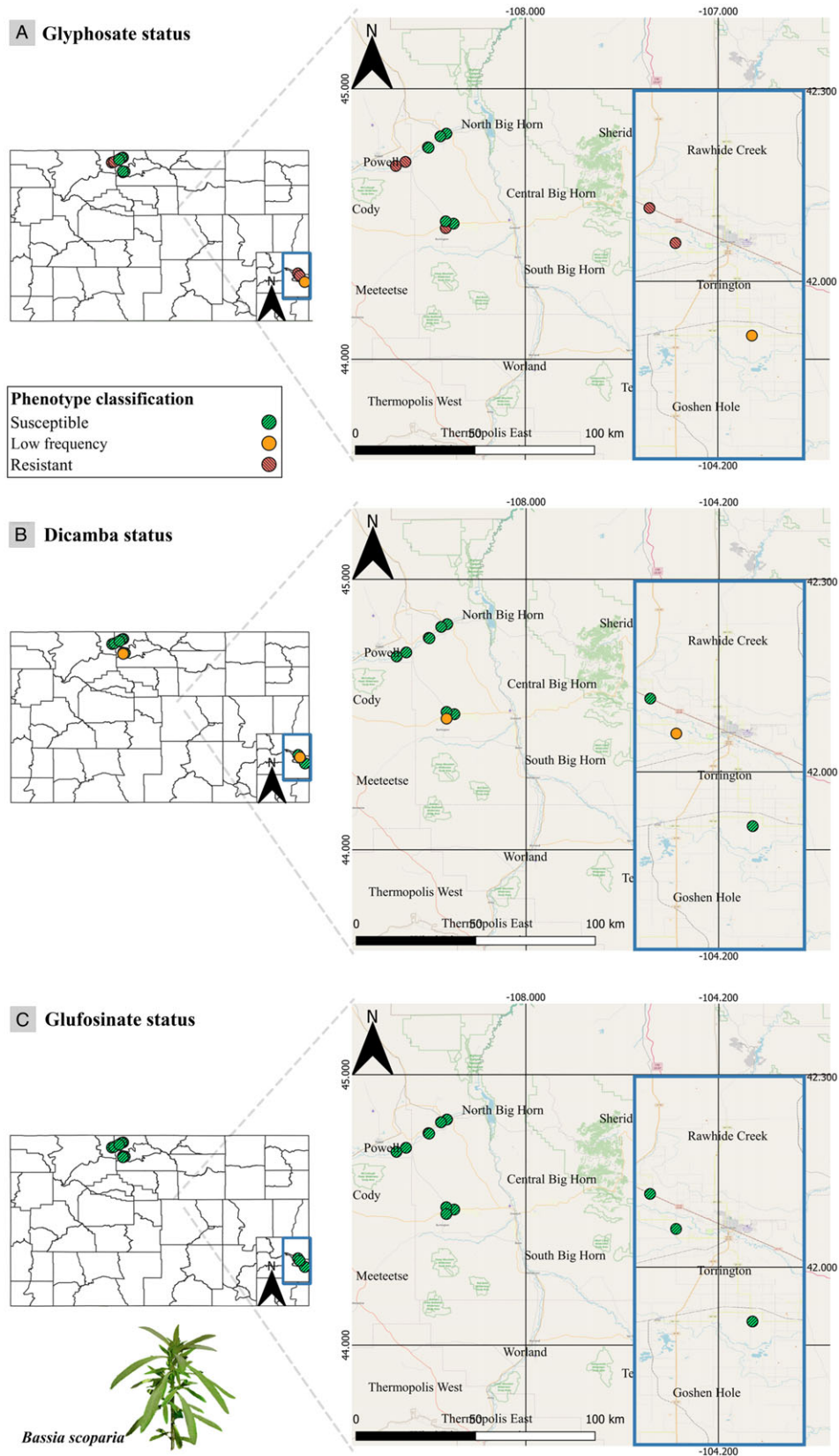
Only a few accessions of Palmer amaranth were identified and collected in Colorado (Supplementary Figure S1) and Nebraska (Supplementary Figure S4), and none were collected in Wyoming. Based on our survey, four out of the five Palmer amaranth



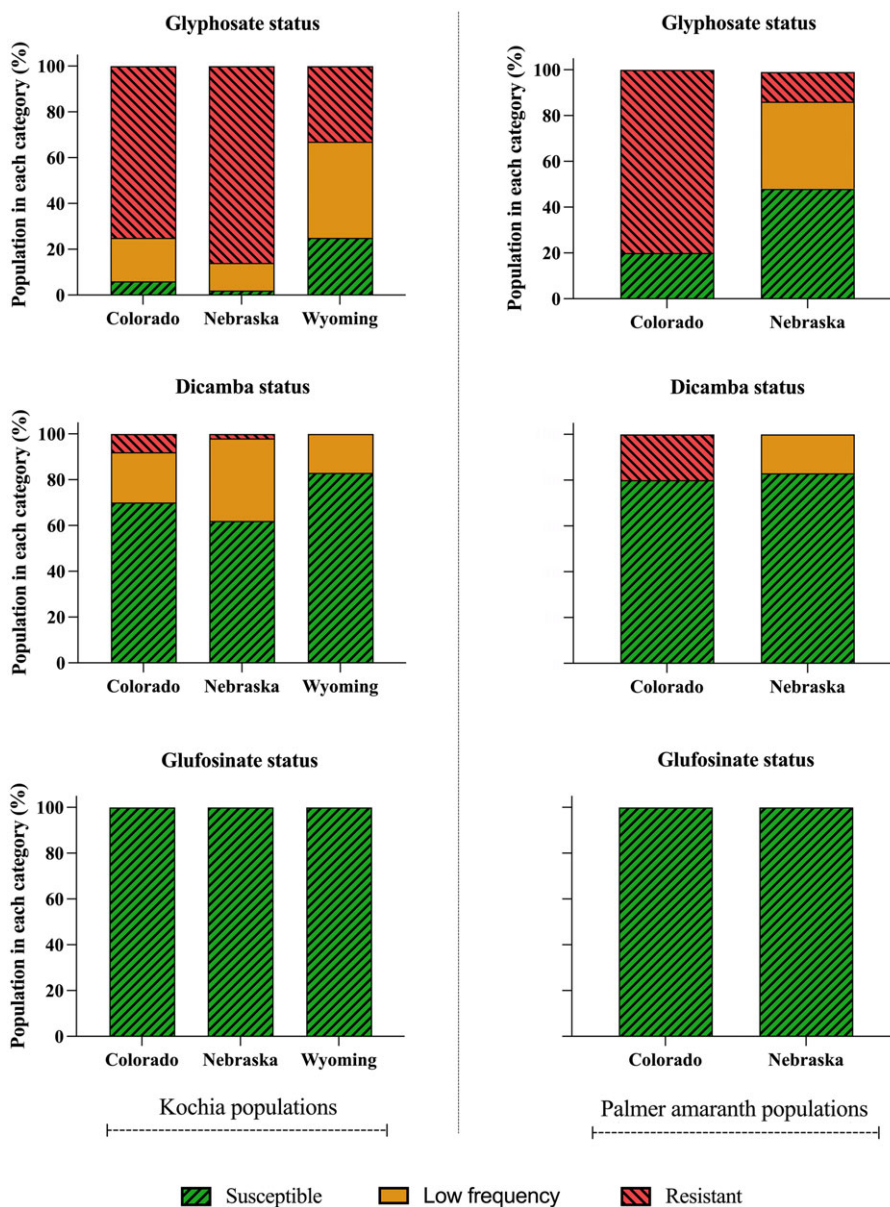
**Figure 1.** Geo-referenced map illustrating the *Bassia scoparia* (kochia) populations collected in Colorado during fall 2021. The dots on the map represent the locations of kochia populations, and their color signifies their response to glyphosate treatment (A), dicamba (B), and glufosinate (C). On the left a map illustrates the distribution of the populations in a state overview. On the right, 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, yellow dots indicate low frequency (1% to 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 fall 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). On the left, a map illustrates the distribution of the populations in a state overview. On the right, 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, yellow dots indicate low frequency (1% to 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 fall 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). On the left, a map illustrates the distribution of the populations in a state overview. On the right, a close-up map focuses on the main counties where the samples were collected, including the highlighted blue squares where a few samples were collected in southeastern Wyoming. Populations classified as resistant (>20% survival) are represented by red dots, yellow dots indicate low frequency (1% to 19% survival), and green dots represent susceptible populations (0% survival).

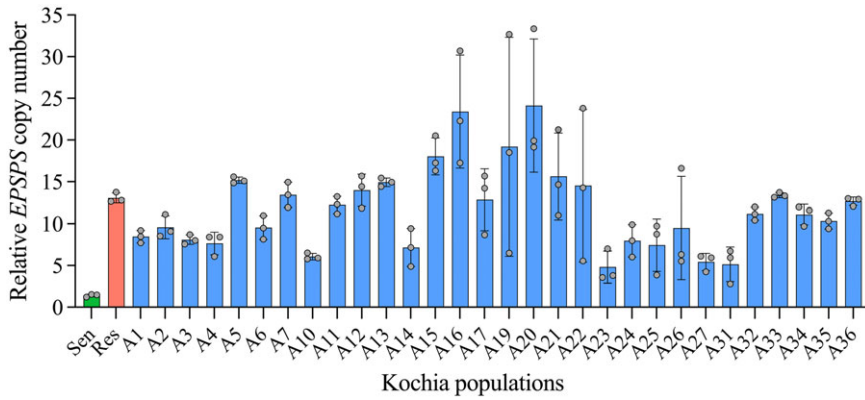


**Figure 4.** Frequency of observed phenotypes of kochia (left) and Palmer amaranth (right) populations collected from Colorado, Nebraska and Wyoming during fall 2020 and 2021, following treatment in a greenhouse setting with glyphosate, dicamba, and glufosinate. Bar colors represent the phenotype characterization: green (dashed to the right) represent susceptible populations (0% survival), yellow represents low resistance (1% to 19% survival), and red (dashed to the left) represent populations classified as resistant (>20% survival).

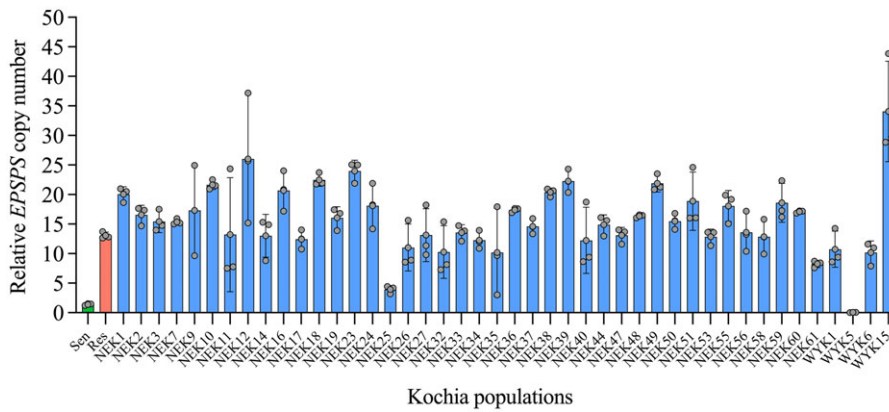
populations collected in Colorado were resistant to glyphosate, representing 80% of the total population. For Nebraska, of the eight populations collected, one was classified as resistant (13%), three as low resistant (38%) and four as susceptible (48%) (Supplementary Figure S4). The relatively low number of Palmer amaranth populations in these areas could be attributed to dry and cold weather, which is distinct from the southwestern United States and northwestern Mexico, where this species is indigenous (Ward *et al.* 2013). Despite the relatively low number of Palmer amaranth populations that were identified, it is alarming that the majority of these populations in Colorado have been classified as being resistant to glyphosate. 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 rapid herbicide resistance evolution (Murphy and Tranel 2019). In all surveyed populations classified as resistant, an increase in relative *EPSPS* gene copy number was observed compared with 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 parts of the United States. Gaines *et al.* (2010) reported that some populations of Palmer amaranth had 160-fold more copies of the *EPSPS* gene than a known susceptible population from Georgia. While glyphosate-resistant Palmer amaranth populations have been

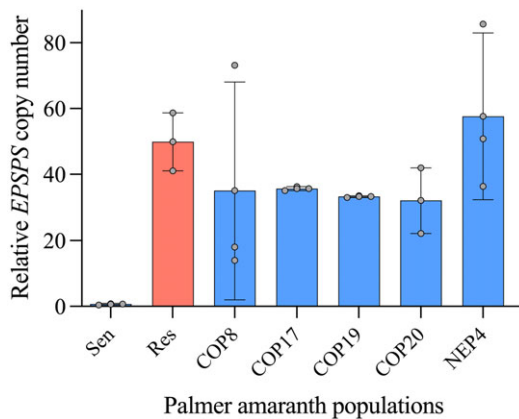




**Figure 5.** Relative *EPSPS* gene copy number in kochia populations collected from Colorado. The green and red bars represent the sensitive and resistant references (Sen and Res), respectively. The blue bars labeled as A represent resistant populations (>20% survival) surveyed from Colorado. Each bar 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.

reported in 26 states (Heap 2024) including recent confirmation of high *EPSPS* gene copy number in glyphosate-resistant Palmer amaranth in New York (Butler-Jones et al. 2024), there have been no previous reports of glyphosate-resistant Palmer amaranth in Colorado until now.

Common lambsquarters was surveyed in Colorado and Nebraska, whereas redroot pigweed was identified in Colorado only. All the herbicides tested provided 100% control of common lambsquarters and redroot pigweed populations surveyed, and populations were classified as susceptible (Supplementary Figures 2, 3, and 5). Populations of common lambsquarters have been identified as resistant to ALS- and PS II-inhibiting herbicides, and very recently, to auxin herbicides (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 the species' varying tolerance to the herbicide at different growth stages. 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 from 80% injury in 2.5-cm plants to 55% in 7.5- to 15-cm plants at 21 d after application. Sivesind et al. (2011)

noticed a reduction in glyphosate efficacy associated with growth stage, where the ED<sub>50</sub> (the effective dose for 50% control) was three times higher in 20-cm plants compared with 10-cm plants. Enhanced glyphosate response in plants at the 5- to 7-cm growth stage was reported compared with plants that varied from 10 to 21 cm in height, particularly in cooler temperatures, when treated with glyphosate at a rate of 840 g ae ha<sup>-1</sup> (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 PS II inhibitors. It has been well documented that resistance to PS II inhibitors is primarily maternally inherited (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 occurs 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 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 postemergence 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 of the United States (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

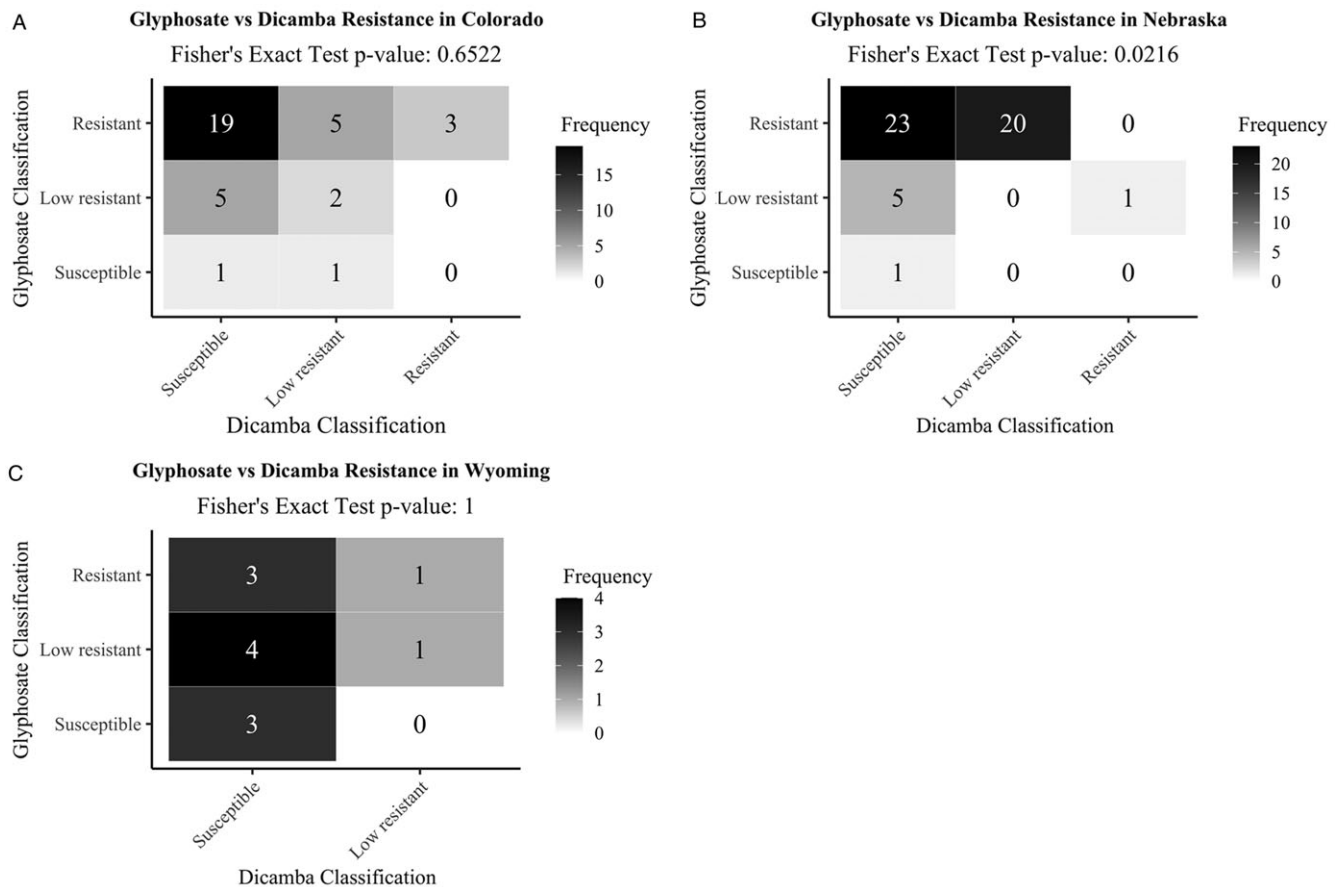
Kochia populations classified as resistant were identified in Colorado (Figure 1B) and Nebraska (Figure 2B) and at low frequencies in Wyoming (Figure 3B). In Colorado, 8% of the populations were categorized as resistant, 22% as low resistant, and 70% as susceptible. The survival frequency within the resistant populations in Colorado ranged from 56% to 88%. In Nebraska, 50 populations were surveyed, and one 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 were 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 eight populations (Supplementary Figure S7), 0% were resistant, 38% demonstrated low resistance and 49% were susceptible. Our

data indicate a limited number of dicamba-resistant populations exist in these states compared to glyphosate resistance; 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 with individual survivors within a resistant population. Rather, these populations may exhibit heterogeneity, and 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 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, and 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 demonstrated low resistance to glyphosate. In contrast, there were glyphosate-resistant populations that were not resistant to dicamba. This suggests that dicamba-resistant populations are more likely to have glyphosate resistance, and fields with glyphosate-resistant 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 five, which may compromise the statistical test. Low expected values and small sample sizes may result in reduced statistical power, thus increasing the likelihood of a Type II error, in which 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 is independent, but this is unlikely the case, as was previously discussed.

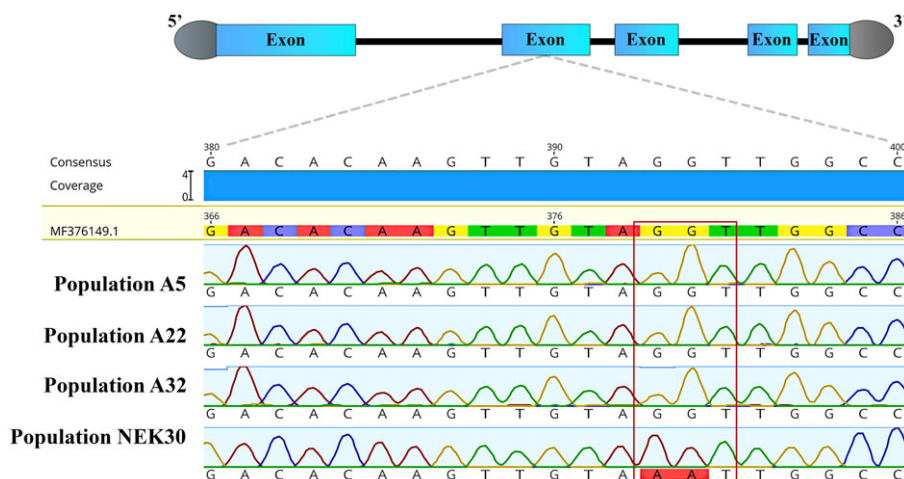
Although there are no reports of dicamba-resistant kochia in sugar beet systems, the issue of resistance to auxin-mimic herbicides is a growing problem in the United States, with reports of dicamba resistance emerging as early as the 1990s (Keith *et al.* 2011; Preston *et al.* 2009). Since then, several other cases have been reported in six states and provinces 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 has led to an 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 such as corn, sorghum, and wheat, where dicamba is extensively employed in crop management (Heap 2024). A 2021 survey revealed a dicamba-resistant Palmer amaranth from Tennessee in dicamba-resistant soybean and cotton crop systems, 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 analyses are underway to validate this phenotype.



**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. A 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  $< 0.05$ .

All kochia and Palmer amaranth populations from Colorado categorized as dicamba-resistant were also categorized as glyphosate-resistant, whereas the population from Nebraska that was classified as dicamba-resistant was classified as having 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-growing areas in the Central Great Plains even before the trait is released. The use of a 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 for managing 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 and 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 they can also increase the risk of NTSR developing through generalist mechanisms such as enhanced metabolism (Comont et al. 2020). Rigon et al. (2023) demonstrated that herbicide mixtures at sublethal doses may have led to a recurrent selection of barnyardgrass [*Echinochloa crus-galli* (L.) P.Beauv.] 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 proteins. In the presence of auxin, the degron region of Aux/IAA interacts with the SCF<sup>TIR/AFB</sup> 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<sup>TIR/AFB</sup> 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 that is 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 (de Figueiredo et al. 2022b). Enhanced metabolic detoxification of



**Figure 9.** The top illustration shows the gene structure of the kochia *IAA16* gene. The 5' and 3' untranslated regions 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 three kochia populations from Colorado classified as dicamba resistant (A5, A22, and A32) and one from Nebraska (NEK 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.

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  $G_{73}N$  amino acid substitution in the degron region of the *AUX/IAA16* gene complex 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  $G_{73}N$  substitution (Figure 9). 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 populations likely possess a distinct and novel resistance mechanism, which could be an 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; de 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, or Wyoming. This includes populations of kochia and Palmer amaranth that exhibited resistance to glyphosate and dicamba (Figures 1C, 2C, and 3C). Although glyphosate and dicamba are used extensively in current weed management systems for sugar beets in the Central Great Plains, glufosinate is not currently included. The current lack of glufosinate use on sugar beet may be due to the availability of more cost-effective preplant herbicides with a broader weed control spectrum. With the introduction of a new sugar beet trait that confers resistance to glufosinate, its use on the crop is expected to increase, particularly with postemergence applications.

To date, 10 cases of herbicide resistance to glufosinate have been documented. Most of them occurred in poaceae 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,  $S_{59}G$ , is in contact with important binding residues of glufosinate and was recently reported to confer resistance in a population of goosegrass (*Eleusine indica* L.) in China (Zhang *et al.* 2022).

In our study, glufosinate was effective on all species. We emphasize that the plants were treated at 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 of its application 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. 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 resulted in reduced glutamine synthetase 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 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 an application of glufosinate provided poor control of common lambsquarters, velvetleaf, and redroot pigweed when they were taller than 5 cm in 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 weeds (Takano and Dayan 2020). Colorado, Nebraska, and Wyoming have a continental climate and generally experience relatively low humidity 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, thereby 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 herbicide-resistant 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 sequentially, where in some cases, antagonism will 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, as reflected in poor control and percent mortality in Palmer amaranth populations (Priess et al. 2022a). In contrast, a synergistic interaction was observed when glufosinate and dicamba were applied to control sicklepod [*Senna obtusifolia* (L.) Irwin & Barneby] (Joseph et al. 2018). Glufosinate plus dicamba were shown to have an additive effect on giant ragweed (*Ambrosia trifida* L.) (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 trait's 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 herbicide resistance, enabling the implementation of effective management strategies. With the impending release of a genetically engineered sugar beet trait that confers resistance 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 sugar beet systems in Colorado and Nebraska. Furthermore, we report that all dicamba-resistant kochia populations tested in Colorado lack a known TSR 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 kochia and palmer exhibiting resistance to glyphosate and dicamba 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.

**Supplementary material.** To view supplementary material for this article, please visit <https://doi.org/10.1017/wet.2024.77>

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**Competing interests.** The authors declare none.

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