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Inheritance of glyphosate resistance and cross-pollination rates under field conditions in kochia (*Bassia scoparia*)

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Research Article

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Abstract

Kochia [*Bassia scoparia* (L.) A.J. Scott] is an invasive species in the High Plains of the United States that poses formidable management challenges in agricultural systems, primarily due to its evolution of resistance to glyphosate. Resistance is due to a transposon-associated increase in 5-enolpyruvyl-3-shikimate phosphate synthase (*EPSPS*) gene copy number relative to the sensitive biotype. Factors behind the rapid spread of glyphosate-resistant biotypes are likely associated with certain aspects of *B. scoparia* biology, such as a protogynous flower morphology producing large amounts of pollen, that encourages outcrossing and favors high genetic diversity. Furthermore, its ability to tumble over long distances ensures a rapid spread of the resistance trait. Herein, we explore glyphosate resistance in *B. scoparia* in Colorado. There was no difference in *EPSPS* gene (Type I, Type II) and *FAR1* copy numbers between parent and progeny *B. scoparia* populations across multiple years (2018, 2020, and 2022), suggesting stable inheritance of glyphosate resistance. Further, the inheritance of glyphosate resistance was investigated using three specific microsatellites or simple sequence repeat (SSR) markers viz. 2656, 2896, and 1792. SSR marker analysis revealed an outcrossing rate of 78% and a selfing rate of 22% in *B. scoparia* progeny. By investigating the complex interplay between *B. scoparia*'s biology and genetics, this study investigates the inheritance of glyphosate resistance in *B. scoparia*, estimates the outcrossing rate under field conditions, and underscores the importance of developing effective management strategies to mitigate its impact on agricultural ecosystems.

Introduction

Kochia [*Bassia scoparia* (L.) A.J. Scott] is a resilient and adaptable plant species that has garnered attention for its ecological and agricultural impact (Geddes and Sharpe 2022; Kumar et al. 2019; Qadir et al. 2008). Native to Eurasia, *B. scoparia* has become widespread across North America, where it is a troublesome weed (Supplementary Figure 1). Historically, *B. scoparia* has been utilized for various purposes, including erosion control, forage production, and ornamental landscaping. However, its prolific seed production, rapid growth, and ability to thrive in diverse environmental conditions have contributed to its status as a major problematic weed in agricultural as well as non-crop settings.

A key adaptation for *B. scoparia*'s success is its C_4 photosynthetic pathway, coupled with a unique emergence and flowering strategy. As a C_4 plant, *B. scoparia* thrives in hot and dry environments due to efficient water use. This is particularly advantageous when it emerges early in spring, capitalizing on cool, moist conditions for initial growth while tolerating late spring frosts (Friesen et al. 2009). By delaying flowering until the hottest part of summer, *B. scoparia* leverages its limited use of moisture in arid conditions to attain its critical reproductive stage. This flexibility makes it a successful competitor in harsh conditions (Friesen et al. 2009). Genetic studies have revealed the existence of multiple genetic lineages within *B. scoparia* populations, reflecting both natural selection and human-mediated gene flow (Martin et al. 2020). This genetic diversity enables acclimation in response to environmental cues such as tolerance to high and low temperatures, low moisture, poor soils with high salt content, and nutrient availability (Kumar et al. 2019; Yadav et al. 2023; Figure 1).

Bassia scoparia has been a problematic weed in the High Plains of the United States for years, but its ability to evolve resistance to herbicides has complicated its management (Adeyemi et al. 2025). Various *B. scoparia* populations have evolved resistance, individually or in combinations, to synthetic auxins, photosystem II inhibitors, acetolactate synthase inhibitors, and glyphosate

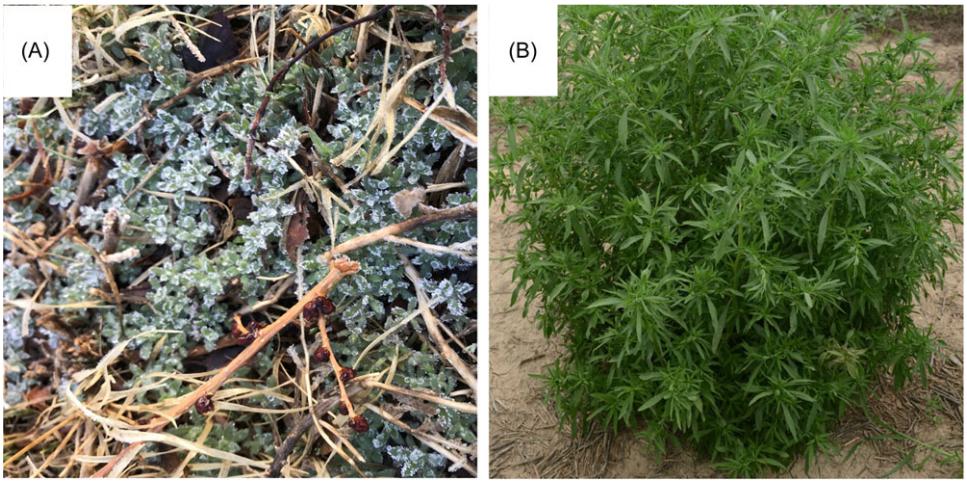

Figure 1. Examples of *Bassia scoparia* plants growing in Colorado, USA. (A) Seedlings can withstand exposure to freezing temperatures during spring cold snap and (B) resume growth without major injury. (Photos by PW).

(Kumar et al. 2019; Varanasi et al. 2015). With respect to glyphosate resistance, most plant species, including *B. scoparia*, normally have a single copy of 5-enolpyruvyl-3-shikimate phosphate synthase (*EPSPS*). Cases of resistance involving *EPSPS* copy number variation (CNV) have arisen by different means (Johnson et al. 2025). For example, in Palmer amaranth (*Amaranthus palmeri* S. Watson), a 297-kb replicon containing multiple additional open reading frames for additional genes and repetitive DNA elements is amplified as extrachromosomal circular DNA with plants having more than 100 copies of *EPSPS* (Koo et al. 2018). In *B. scoparia*, this process is driven by the presence of an *FHY3*/*FAR1* transposable elements (Jugulam et al. 2014; Patterson et al. 2019; Wiersma et al. 2015), with some plants having as many as 20 *EPSPS* copies (Godar et al. 2015).

As with other weeds, effective management of glyphosate-resistant (GR) *B. scoparia* requires integrated weed management strategies that incorporate diverse approaches, such as herbicide rotation, use of alternative herbicides with different modes of action, adoption of cultural practices, and development of nonchemical control methods (Kumar et al. 2019). Proactive monitoring of herbicide resistance and early detection of resistant populations are essential for implementing timely and targeted management interventions to mitigate the spread of resistance and preserve the efficacy of herbicides for weed control in agricultural systems (Torbiak et al. 2024).

Herein, we report a survey for (1) frequency of glyphosate resistance in field-collected *B. scoparia* populations; (2) the inheritance pattern of gene copy number for total *EPSPS*, the two types of *EPSPS* CNV, and the associated transposon in a field study; and (3) the frequency of outcrossing in a field study.

Materials and Methods

Pollen Scanning Electron Microscopy

Naturally dried pollen was separated from mature flowers and prepared for scanning electron microscope observation according to Lynch and Webster (1975). The grains were sprinkled evenly on double-sided conductive adhesive on gold–palladium metallic stubs and coated with a gold sputter coater (Vacuum Desk II Gold Sputter Coater, Denton North America, Moorestown, NJ USA). The pollen grains were observed using a field emission scanning electron microscope (JSM-6500, JEOL USA, Peabody, MA USA) at the Center for Imaging and Surface Science of Colorado State University (Fort Collins, CO).

Geographic Localization of GR Bassia scoparia and Wind Analysis

The locations and dates of reports of GR *B. scoparia* were obtained from the International Herbicide-Resistant Weed Database (Heap 2024; accessed: July 2024). Twenty-year (January 2001 to December 2020) average monthly wind speed and direction were obtained from the National Aeronautics and Space Administration (NASA) Langley Research Center Prediction of Worldwide Energy Resource (POWER) Project funded through the NASA Earth Science/Applied Science Program (https://power.larc.nasa.gov/).

Plant Material for Field Survey and Treatment

A field survey was conducted in 2015 to assess glyphosate resistance in *B. scoparia* across Colorado. *Bassia scoparia* seeds were collected in autumn (October to November) from field and roadside locations along transects, with a minimum distance of 16 km between sites (Supplementary Table 2). Within crop fields, seeds were harvested from individual *B. scoparia* plants that had survived the entire growing season. To create composite samples for each location, seeds from 5 to 20 plants were pooled. Seeds were then transferred to the Colorado State University greenhouse for screening Seedlings were treated with glyphosate (RoundUp WeatherMax®, Bayer, St. Louis, MO USA) at a rate of 870 g ai ha^{-1} with 20 g L^{-1} ammonium sulfate using a moving overhead single-nozzle sprayer (DeVries, Hollandale, MN) calibrated to deliver 187 L ha^{-1}.

Bassia scoparia accessions were classified as susceptible (≤ 20% survival) or resistant (>20% survival) based on their response to the discriminating glyphosate rate corresponding to 870 g ae ha^{-1}. Georeferenced collection sites were mapped using Arc Catalogue and ArcMap (v. 10.2.1) to visualize spatial patterns of glyphosate resistance across Colorado and facilitate comparisons over time (Khater et al. 2022).

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Table 1. Forward and reverse primer sequences for *ALS* (control), *EPSPS*, Type I, Type II repeats, and mobile genetic element (*FAR1*).

Repeats	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
ALS	CCAGAAAAGGCTGCGATG	CTGACTCGCTCTGATTCCA
EPSPS	CGCTATATGTTGGATGCTCTAAG	CACTCCTATTCTCTTTACCAGC
Type I	GACGGAAATACCCTCAATATAGACA	ACGCCCAAGATGTACATTGATA
Type II	GACGGAAATACCCTCAATATAGACA	CATGCCTTTGATGTCCAAGTTT
FAR1	GAAGATAGCGAGACGTTTGAG	CGGCTTGATCGGTTAAGATAC

Plant Material for Inheritance Study

Survivor (parent) plant samples were collected during autumn from different Roundup Ready® sugar beet (*Beta vulgaris* L. subsp. *vulgaris*) fields in each year (2018, 2020, 2022). For 2018, parental tissues were randomly collected from a field at coordinates 40.617°N, 105.017°W in northern Colorado, and seeds were brought to Colorado State University. Collection for 2020 was performed within the same field at 40.6°N, 105.017°W, but using a more spatially targeted approach. Mature plant samples 1 to 11 were collected from the west side of the field, samples 12 to 22 were clusters of survivors on the east side of the same field, samples 23 to 31 were isolated from the central part of the field, and samples 32 to 44 were gathered from the northwest part of the field from August through September. In 2022, healthy plants that survived multiple glyphosate applications during the growing season were collected from a sugar beet field at 40.6°N, 105.017°W, transplanted into 22-L pots, and taken to Colorado State University, where they were grown to maturity for seed collection. Samples 1 to 5 were collected from the west side of the field, whereas samples 6 to 10 were collected from the east side. All seeds were placed in 81.3-cm Miracle-Gro® Moisture Control® Potting Mix with Lambert LM-GPS (Scotts, Marysville, OH USA) until reaching the seedling stage in the greenhouse, where plants were watered once a day to maintain field capacity. For evaluating gene copy number variation, a known GR Colorado *B. scoparia* population with high *EPSPS* copy number (M32) (Westra et al. 2019) and a known glyphosate-susceptible Colorado inbred population (7710) were selected (Patterson et al. 2019; Pettinga et al. 2018; Preston et al. 2009). Genomic DNA was extracted from GR field populations that had survived a treatment with 900 g ai ha^{-1} glyphosate during the early growing season.

Extraction of Genomic DNA

Two young rosette leaves were collected from each individual plant at the 2.5-cm seedling height growth stage. Samples were immediately flash-frozen in liquid nitrogen and stored at −80 C until DNA extraction. Three biological replicates were used for DNA extraction from both parental and progeny lines. DNA extraction was carried out using the cetyl trimethylammonium bromide (CTAB) method (Doyle 1991), followed by quantification using a Thermo ND-1000 model Nanodrop spectrophotometer (Thermo Scientific, Wilmington, NC USA).

Quantitative Real-Time PCR for EPSPS Copy Number Determination

Quantitative real-time PCR (qRT-PCR) was employed to determine the *EPSPS* copy number variation. Ten nanograms per microliter (ng μl^{-1}) of genomic DNA from three biological replicates of each parental and progeny line were used for the qRT-PCR reactions. Additionally, three technical replicates were performed for each biological replicate to account for variation during the sample preparation for qRT-PCR. The reactions were performed using a Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) on a Bio-Rad CFX Real-time PCR System (Bio-Rad Technologies, Hercules, CA USA). The thermal cycling conditions included an initial 3-min denaturation step at 95 C, followed by 40 amplification cycles consisting of denaturation at 95 C for 15 s, and a combined annealing/extension step at 70 C for 30 s, as described elsewhere (Patterson et al. 2019; Wiersma et al. 2015).

Specific primer sets targeted the full-length Type I *EPSPS* segment (56.1 kb), the shorter Type II segment (32.9 kb), and a flanking mobile genetic element, *FAR1* (15 kb) (Table 1) (Patterson et al. 2019; Ravet et al. 2021). The acetolactate synthase (*ALS*) reference gene was used to normalize *EPSPS* copy number using the ΔΔCt method (Gaines et al. 2016; Wiersma et al. 2015). Briefly, the relative expression ratio (R) was calculated as $R = 2^{-(\Delta Ct(sample) - \Delta Ct(calibrator))}$ (Schmittgen and Livak 2008), where ΔCt(sample) represents the difference in threshold cycle (Ct) values between the target gene (*EPSPS*) and the reference gene (*ALS*) for a given sample, and ΔCt(calibrator) represents the average ΔCt value for the parental line used as the calibrator. The technical replicates were averaged first; and then biological replicates were averaged for the analysis, followed by calculation of standard deviation to assess variation within each treatment group.

Simple Sequence Repeat Genotyping Using Bio-analyzer

Simple sequence repeats (SSRs) were used to study inheritance in selected GR *B. scoparia* populations. From the set of 11 SSR primers mentioned in a study by Ravet et al. (2021), three specific markers were selected for further analysis because of their respective locations in the genome and because they showed allelic polymorphism in preliminary analysis of parental samples (Table 2). Specifically, SSR 1792 is on chromosome 4 (bp 103692085), SSR 2656 is on chromosome 6 (bp 46032154), and SSR 2896 is on chromosome 6 (bp 13718386). On the other hand, the mobile genetic element (MGE) is present as a single copy on chromosome 9 in the analyzed glyphosate-susceptible *B. scoparia* genome, while a nearly identical single-copy MGE is present next to *EPSPS* on chromosome 1 in the analyzed GR *B. scoparia* genome (Hall et al. 2025). Therefore, the SSR markers can be considered neutral with respect to glyphosate resistance and are not in linkage disequilibrium with the *EPSPS* gene duplication or the MGE.

qRT-PCR was performed utilizing genomic DNA diluted to a concentration of 5 ng μl^{-1} with EconoTaq PLUS master mix. The process starts with a 2-min denaturation step at 94 C, followed by denaturation at 94 C for 30 s, a 30-s annealing step at temperatures shown in Table 2, a 45-s extension step at 72 C for 37 cycles, and a final extension of 2 min at 72 C. The PCR products were then multiplexed using the three SSR markers (Table 2) and processed for fragment analysis using a 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA USA) at the biotechnology core facilities at the University of Nebraska (Lincoln, NE USA). A high-

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Table 2. List of simple sequence repeat (SSR) primers used for genotyping.

SSR name	Forward primer sequence (5′–3′)	Melting temperature Tm (C)	Reverse primer sequence (5′–3′)	Tm	Amplicon size	Motif repeat	Annealing temperature
					bp		C
1792	AACTAGTCGGATCGAGCCTT	58	AATCACACAACTCCGCAAGT	58.2	174	$(CCCAA)_n$	57
2656	AACCAAACCGCACTAAACTG	57.8	GCACAATAGAGAGGGCAAAA	58	277	$(TGGTT)_n$	62
2895	GTCATAGCCATCCCTTACCC	58.3	TATTGCCCTGTTCTTCAGGA	58.3	267	$(AGTTC)_n$	62

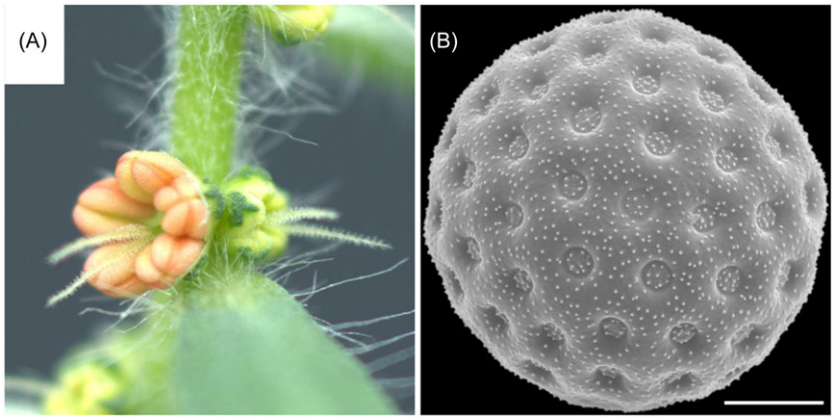

Figure 2. *Bassia scoparia* flower and pollen grain. (A) Photograph of a *B. scoparia* flower and (B) scanning electron micrograph of a *B. scoparia* pollen grain. Micrograph was obtained at 3,000× magnification and with 5 kV (line = 10 µm).

sensitivity DNA kit for the sizing and quantitation of fragmented DNA was utilized for sample preparation before fragment analysis (dsDNA 905 Reagent Kit, Agilent Technologies, Santa Clara, CA USA).

Statistical Analysis

Statistical significance of gene copy numbers was determined by ANOVA followed by Fisher's LSD test (Williams and Abdi 2010) at the $\alpha = 0.05$ level using module AGRICOLAE in RStudio (2024.04.2 Build 764) and R v. 4.3.1 (2023-06-16 ucrt).

The homogeneity of the gene CNV across years for parental and progeny *EPSPS* was assessed with the Bartlett's test (Bartlett and Fowler 1937) using the STATS module in R. This test was used to determine whether the data from the various collection years could be pooled. The null hypothesis (H0) assumes equal variances across groups, while the alternative hypothesis (Ha) states that at least two groups have unequal variances. Statistical analysis and means separation of the original data for each year are available in Supplementary Table 3.

Results and Discussion

Pollination Dynamics and Inheritance

As part of the Amaranthaceae family, *B. scoparia* is characterized by its small, inconspicuous greenish-white to pinkish flowers lacking showy petals or sepals arranged in dense clusters along slender stems (Friesen et al. 2009; Figure 2A). Additionally, it is considered an outcrossing species, because its flowers are protogynous, where stigmas are receptive 1 wk before dehiscence of anthers on the same flower (Friesen et al. 2009). Furthermore, its pollen grains are spheroidal with a diameter of 20 to 40 µm, having a granular surface due to 100 to 130 pores (Stallings et al. 1995a; Figure 2B), which is typical for a anemophilous pollen compared with pollen from biotically pollinated plants that is more than 200 µm (Harder 1998). These small spherical and dimpled pollen grains have structural similarities to a golf ball whose structure reduces air resistance and increases lift, maximizing their ability to remain suspended in the air (Ackerman 2000).

Being prone to wind pollination, *B. scoparia* relies on abiotic factors such as wind speed and direction for pollen dispersal and pollination (Chen et al. 2020). The absence of showy petals or nectar rewards suggests that *B. scoparia* is not dependent on insect pollinators for reproduction (Gill 2004).

Glyphosate Resistance Survey

Dispersal of herbicide-resistant alleles via pollen-mediated and/or seed-mediated means can drive the movement of herbicide-resistant populations across an agroecoregion. GR *B. scoparia* was first reported in Kansas in 2007 and is now causing problems in 10 western states (Heap 2024; Waite et al. 2013; Figure 3). This weed is highly fecund, releasing abundant amounts of pollen and producing large numbers of seeds that can be dispersed by wind as the mature plants tumble across a field. Earlier studies demonstrated that pollen-mediated dispersal is strongly dependent on prevailing wind direction during pollination (Beckie et al. 2016). Similarly, tumbling mature *B. scoparia* plants can produce100,000 seeds or more over 1 km with wind speed as low as 0.3 m s^{-1} (Beckie et al. 2016). These combined methods of dispersion are expected to lead to a rapid expansion of the resistance trait (Martin et al. 2020).

Interestingly, identification of GR *B. scoparia* populations has expanded primarily westward and against the predominant wind direction in this region of the United States (Figure 3; Supplementary

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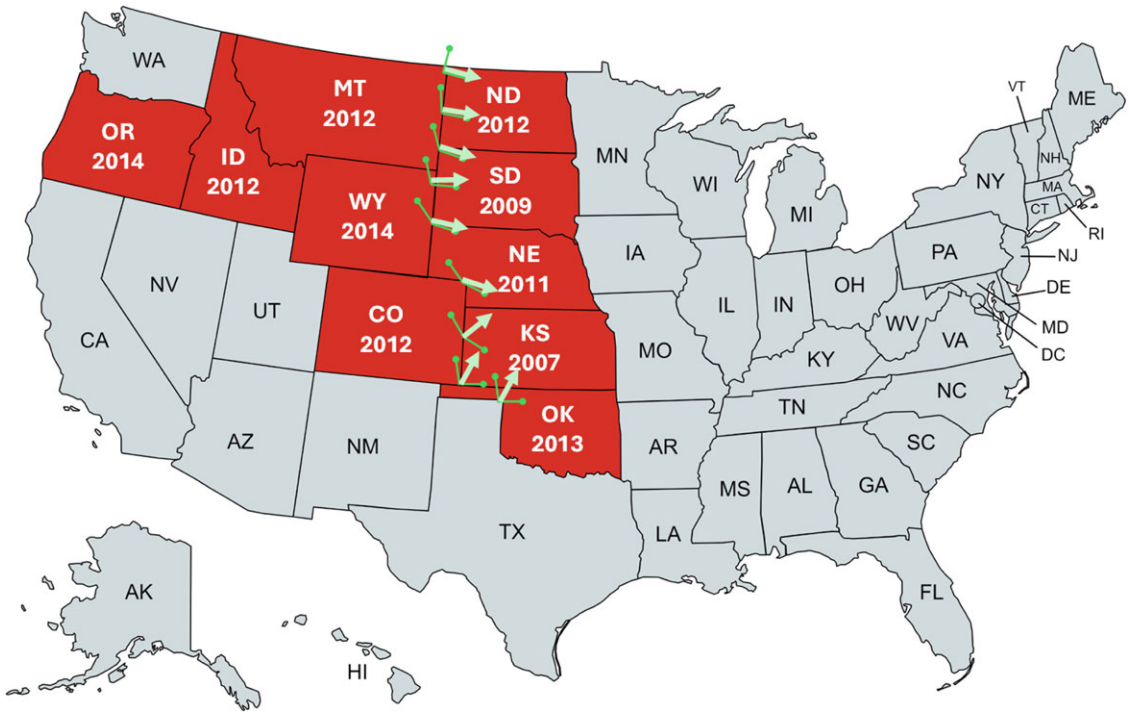

Figure 3. States with reported glyphosate-resistant (GR) *Bassia scoparia* populations (and year of first report) based on data from Heap (2024). The map was generated with MapChart (https://www.mapchart.net/usa.html). Light green arrows indicate 20-yr (January 2001–December 2020) predominant wind direction during flowering ranged from northeast to southeast, and small lines represent the range of wind direction during that period of time. Data from National Aeronautics and Space Administration (NASA) Langley Research Center Prediction of Worldwide Energy Resource (POWER) Project (https://power.larc.nasa.gov/).

Figure 4. Impact of *Bassia scoparia* on agroecosystem. (A) glyphosate-resistant (GR) *B. scoparia* plants growing in a Colorado sugar beet field. (Photo by André Araujo.) (B) Path taken by a GR *B. scoparia* plants tumbling across a field, dropping seeds that later emerge and grow as GR weeds. (Photo by PW).

Table 1). Indeed, analysis of the wind pattern from 2001 to 2020 reveals that this region received wind from the west or southwest with an average wind speed around 5 m s^{-1} (Supplementary Table 1). Therefore, the westward movement of GR *B. scoparia* over long distances may not be solely the result of wind dispersal. On the other hand, the lack of eastward expansion may be due to the wetter midwestern conditions that are not conducive to good *B. scoparia* survival and expansion. Consequently, the emergence of GR *B. scoparia* in other states may be the result of seed transport by farming equipment or birds or the result of independent selection of new resistant biotypes (Kumar and Jha 2015b).

GR *B. scoparia* poses a great challenge to agricultural weed management strategies, particularly in regions where this weed species has become widespread (Kumar et al. 2019; Figure 4A). GR *B. scoparia* seeds can spread during harvesting or while tumbling across large areas, resulting in movement of GR *B. scoparia* in the field and beyond (Figure 4B).

A total of 51 *B. scoparia* samples were collected from Colorado and assessed for glyphosate resistance frequency (Supplementary Table 2). Most of the samples were collected from the fallow phase of wheat (*Triticum aestivum* L.) or corn (*Zea mays* L.) cropping systems. However, some samples were also from roadsides and field margins, with a few from alfalfa (*Medicago sativa* L.) fields. Due to the reliance on glyphosate for weed management in no-till fallow, 68% of the progenies had some GR individuals.

Overall, glyphosate resistance frequency in the samples collected (Supplementary Table 2) ranged from 0% to 63%. Mapping the *B. scoparia* resistance survey data on 51 accessions collected in 2015 and screened in 2016 indicates GR *B. scoparia* georeferenced clustering within an 80-km radius of this survey (Figure 5). A 80 km

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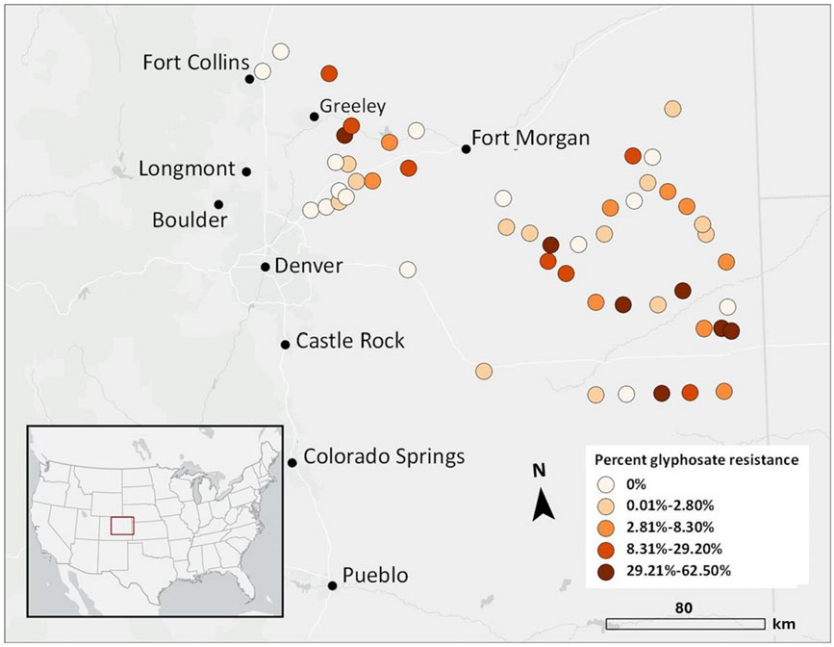

Figure 5. Georeferenced collection sites. Each collection site was mapped using Arc Catalogue and ArcGis (v. 10.2.1) (Maguire 2008) to visualize spatial patterns of glyphosate resistance across Colorado and facilitate comparisons over time.

radius was selected as a practical limit for sample collection logistics. Identifying GR *B. scoparia* clusters is important to focus weed control efforts in areas with the highest GR *B. scoparia* pressure. Resources can be directed toward these areas to prevent further spread and ensure effective weed management.

Glyphosate Resistance in Bassia scoparia

The mechanism of glyphosate resistance in *B. scoparia* is associated with *EPSPS* gene CNV (Gaines et al. 2016; Godar et al. 2015; Jugulam et al. 2014). While sensitive *B. scoparia* individuals have a single *EPSPS* copy, GR *B. scoparia* plants with multiple copies of *EPSPS* have reduced sensitivity to glyphosate and can survive and reproduce in glyphosate-treated fields, leading to the proliferation of resistant populations and exacerbating weed management challenges (Lim et al. 2021).

The origin of the *EPSPS* gene duplication event and the evolution of glyphosate resistance are attributed to an MGE containing a *FAR1*-like transposon (Hall et al. 2025). Amplified *EPSPS* copies are typically positioned in tandem in the GR *B. scoparia* genome. Researchers have performed FISH (fluorescence *in situ* hybridization) analysis to study these tandem repeats and found the sizes of repeat to be ~45 kb and ~66 kb (Jugulam et al. 2014). There are two dominant repeats upstream and downstream of CNV boundaries known as Type I, which has a full-length 56.1-kb repeat, and Type II, which has a smaller 32.9-kb repeat, as well as a large MGE of ~15 kb interspersed in the repeat structure (Patterson et al. 2019). A typical MGE containing AR1 DNA binding, zinc finger, and SWIM domains is present both upstream and downstream of the CNV repeats in GR populations. Unequal crossing-over events, facilitated by a transposable element insertion, have resulted in amplification of the *EPSPS* gene. A recently discovered repetitive element called Muntjac, with a mutator Don-Robertson transposase that has transcription factor-like activity, provides perspectives of GR *B. scoparia* via trans-duplication processes (Dupeyron et al. 2019; Hall et al. 2025). In *B. scoparia*, the larger Type I *EPSPS* locus contains seven other co-duplicated genes, whereas the smaller Type II *EPSPS* (32.9 kb) repeat contains only four genes (Patterson et al. 2019). Because the gene duplication of *EPSPS* involves a structural variant, the number of *EPSPS* gene copies inherited by progenies may differ from the numbers in the parents due to unequal crossing over during meiosis (Hall et al. 2025; Jugulam et al. 2014).

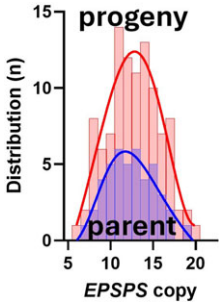

Figure 6. Distribution of *EPSPS* copy number in parent (blue) and progeny (red) samples.

EPSPS and FAR1 Copy Number Variation

The homogeneity of the gene CNV across years in parental and progeny for *EPSPS* CNV was assessed with Bartlett's test in a location under constant glyphosate selection. This test was used to determine whether there were significant differences in these populations between years under conditions of glyphosate selection. Bartlett's test revealed no statistically significant difference in variance for *EPSPS* copy number across the three collection years (2018, 2020, and 2022) in the parental lines (Bartlett's K-squared = 1.948, df = 2, P-value = 0.3776) and progenies (Bartlett's K-squared = 0.45504, df = 2, P-value = 0.7965). The pooled data had normal distribution in both parents and progeny (Figure 6), with an average 12 to 13 *EPSPS* copy numbers in both parents and progenies. While the number of

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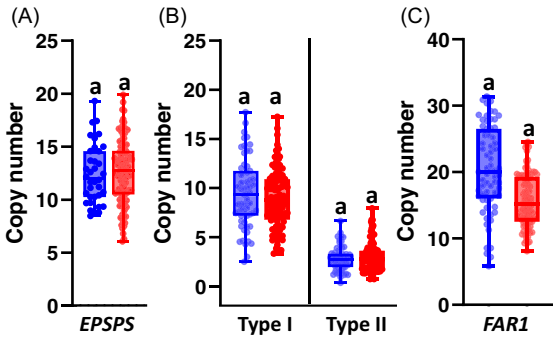

Figure 7. *EPSPS* gene copy number. (A) Total *EPSPS* copy number comparison between parents (blue) and progeny (red) for the years 2018, 2020, and 2022. (B and C) Pooled parent and pooled progeny Type I, Type II *EPSPS* and *FAR1* copy numbers. Each data point represents individual samples. Means with same letters are not statistically different at the $\alpha = 0.05$ level, using Fisher's protected LSD test.

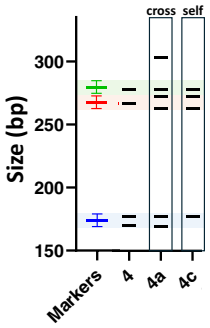

Figure 8. Example of an simple sequence repeat (SSR) analysis for assessing whether progenies were the result of a self-pollinating or outcrossing event. The amplicon sizes for the selected SSR markers were 174, 267, and 277. The range considered as matching the markers was ±5-bp, as shown by bars.

copies ranged from 7 to 19 and 6 to 20 in the parent and progeny, respectively, both populations include 95% of the distribution.

There was no difference (P-value = 0.769) between the *EPSPS* copy number of parental and progeny populations (Figure 7A). This suggests that the *EPSPS* copy number distribution was constant in the parental and the progeny populations over the 2018 to 2022 study period (Figure 7A).

The copy number variations of the Type I and Type II *EPSPS* segments and the *FAR1* transposable element were investigated. The range in copy numbers of Type I *EPSPS* was similar in the parental and progeny lines (Figure 7B). While the same is true with Type II *EPSPS*, this smaller duplication event is not as common as the Type I duplication (Figure 7B), which accounts for most of the *EPSPS* copies. This provides evidence for a stable parental *EPSPS* copy number being maintained under selection at a population level and is consistent with the trait maintaining Hardy–Weinberg equilibrium even under selection (Rousset 2008). It appears that a stable CNV with at least 6 to 7 copies is sufficient for full resistance, and thus there was no selection to further increase the CNV over consistent selection pressures in these glyphosate-treated sugar beet fields. Finally, there were more *FAR1* copy numbers than the total *EPSPS* copy numbers (Figure 7A and 7C), suggesting that *FAR1* is also present in other parts of the genome (Hall et al. 2025). Interestingly, based on qRT-PCR markers, Ravet et al. (2021) defined three genotypes of *EPSPS* gene duplication. Our samples from all 3 yr fall into the category of genotype I, characterized by an

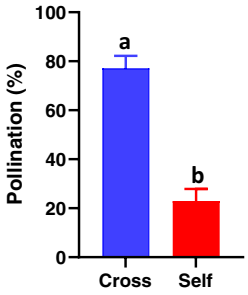

Figure 9. Percentages of progeny resulting from outcrossing (blue) and self-pollinating (red) events ($n = 58$). Error bars are ±1 SE of the mean. Means with different letters are statistically different at the $\alpha = 0.05$ level, using Fisher's protected LSD test.

increase in *EPSPS*, Type I and II repeats, and MGE copy numbers corresponding to more than 10 *EPSPS* copies (Ravet et al. 2021).

The inheritance of glyphosate resistance was investigated using three specific markers SSRs (2656, 2896, and 1792) across the parent and progeny from the samples collected in 2018, 2020, and 2022. SSR marker analysis requires some interpretation of the peaks. In this study, peaks within ±5-bp from an SSR marker were considered to match that marker, whereas those with a size beyond the ±5-bp threshold were considered different (as illustrated in Figure 8). Plant samples with amplicon size outside this threshold were considered to be outcrossing events. For example, the parent (4) had amplicons at 170, 177, 267, and 278 bases. One of its progenies (4a) had amplicons at 169, 177, 263, 272, 278, and 303, which indicates an outcrossing event; another progeny (4c) had 177, 263, 272, and 278 amplicons, which indicates either a self-pollinating event or an outcrossing event between individuals with identical genotypes for these markers.

Consequently, the outcrossing rate was approximately 78%, whereas the estimated self-pollination rate was 22% (Figure 9). However, the self-pollination rate may be an overestimation, as it may combine true self-pollination events as well as outcrossing events that yielded progenies reporting the same markers as the parents for the three SSR markers evaluated.

GR *B. scoparia* biotypes have a transposon-associated CNV of *EPSPS*. On average, parents and progenies have 12 to 13 copies of *EPSPS*, and most of these duplicated genes have the larger Type I genetic structure. Inheritance of the resistant trait is stable under constant selection, with the number of copies being similar in the parents and progenies over years, suggesting that populations have sufficient resistance to glyphosate and there has not been selection for greater copy numbers. The wide range in the distribution of the number of *EPSPS* copies can be accounted for by the fact that *B. scoparia* favors outcrossing, which is encouraged by its protogynous flower structure, with stigmas being receptive several days before anthers release their pollen. Additionally, the open configuration of the flower promotes wind dispersal of *B. scoparia*'s small spheroidal anemophilous pollen grains. The high fecundity and wind dispersal of the GR trait through pollen likely promote the local spread of GR *B. scoparia* plants (Geddes and Pittman 2022). However, the westward spread of GR *B. scoparia* from Kansas to Oregon is against prevailing wind, suggesting that these seeds are moved by other means, and poses significant challenges for weed management in agricultural systems. Strong winds can easily spread *B. scoparia* seeds in multiple directions, highlighting the pattern of seed dispersal from tumbling mother

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plants (Stallings et al. 1995b). Effective monitoring of pollen movement could be beneficial in controlling the distribution of resistant traits across fields. Unchecked seed dispersal of this tumbling weed has potential for contributing to further herbicide-resistance development.

The elevated *EPSPS* gene copy number imparting glyphosate resistance in *B. scoparia* is stably inherited across generations and growing seasons. This indicates that once resistance is established in a *B. scoparia* population, it is likely to persist without continued glyphosate application. Thus, resistance management requires long-term, integrated approaches. The discovery of a high outcrossing rate (78%) under field conditions highlights the rapid potential for gene flow among *B. scoparia* populations. Combined with the weed's protogynous flowering structure, wind-dispersed pollen, and ability to disperse seeds over long distances, these traits facilitate the widespread dissemination of resistance. Immediate management strategies should focus on preventing seed production and minimizing pollen escape from known GR populations. This can be achieved by using preemergence soil-residual herbicides with different modes of action, rotating crops to disrupt *B. scoparia* life cycles, and enhancing crop competitiveness through narrow row spacing or cover crops. Given that many GR populations possess *EPSPS* copy numbers above the threshold for field-level resistance, reliance on postemergence glyphosate alone is no longer viable. Therefore, integrating herbicides with different modes of action, especially preemergence herbicides with good residual activities, and crop rotation may prevent seed deposition and infestation of GR *B. scoparia* in soil (Kumar and Jha 2015a, 2015b).

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2025.10049

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