# Regional Patterns of Herbicide Resistance Traits in Pigweed Escapees.

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## Introduction

Late-season pigweeds (*Amaranthus* spp) are threats to crop production. Just one pigweed can release 10,000 to 100,000 seeds during harvest, which builds the potential for devastating infestations during future growing seasons. Along with being prolific weeds, pigweed populations are becoming increasingly resistant to herbicides. The two focal pigweed species, waterhemp (*A. tuberculatus*) and Palmer amaranth (*A. palmeri*), display a dynamic repertoire of resistances, making them focal species for herbicide resistance (HR) research. Thus far, resistance to nine different modes of action have been confirmed with some individual pigweeds having stacked resistances for up to five modes of action. In the US, 14 states have surveyed pigweed populations to gain an understanding of how particular genotypes are linked to HR prevalence in field collected plants and progeny. Collectively, these surveys have found the distribution and type of HR pigweed populations vary across states, making it difficult to extrapolate management recommendations across state lines.

The National Agricultural Genotyping Center (NAGC) and weed scientists at North Dakota State University (NDSU) have initiated a statewide project to screen North Dakota pigweed populations for resistance to three herbicides: glyphosate, imazamox, and fomesafen. NAGC researchers genotyped field collected pigweeds and their greenhouse progeny to look for known markers within the *EPSPS, ALS,* and *PPO* genes that are associated with resistance to the three herbicides, respectively. NDSU researchers performed herbicide bioassay experiments within the greenhouse using seed (progeny) from the field collected pigweeds. The purposes of this public-private partnership were to: 1) verify the existence HR pigweed populations in North Dakota using both target-site genotyping and phenotyping experiments, and 2) compare within-individual genotyping and phenotyping results. We confirmed pigweed populations in North Dakota have the genetic markers associated with resistance to the three tested herbicides. Additionally, herbicide treatments indicated extensive resistance to glyphosate and imazamox throughout the 16 counties surveyed. As we genotype the remaining pigweeds from the greenhouse experiments, there are plans to expand the statewide assessment and provide genotyping as a service to assist in pigweed management at the farm and across North Dakota.

## Objectives:

*Objective 1: Genetic survey of pigweed populations in North Dakota for common target-site genotypes that confer resistance to glyphosate, PPO-inhibitor (fomesafen), and ALS-inhibitor herbicides (imazamox).*

*Objective 2: Compare whole-plant greenhouse and genotyping assays to determine the target-site basis for HR in pigweed escapees found in North Dakota fields.*

## Materials & Methods

*Field Collection* – Ten surveyors collected late-season pigweeds from 16 counties in North Dakota from September through November 2021 (Fig. 1). Surveyors were asked to locate five or more fields per county containing pigweeds that likely resisted herbicide treatments. Pigweed species in this study included Palmer amaranth, waterhemp, redroot pigweed, Powell amaranth, and tumble pigweed. In fields that contained multiple patches of pigweeds, surveyors selected two of the largest patches to sample and collected five mature seed heads per patch. Up to 10 seed heads per field were placed into a large, sealable envelopes and sent to NDSU Extension.

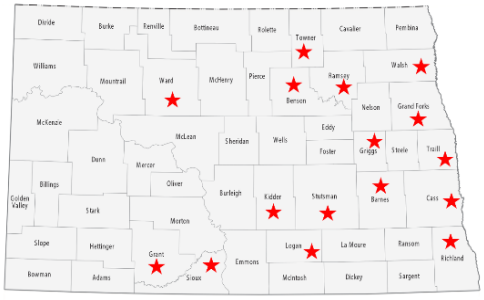


**Figure 1.** Red stars designate the 16 counties where late-season pigweeds were collected during the Fall of 2021.

*Greenhouse* – Seed heads were threshed, and remaining tissue from the field-collected plants were sent to NAGC for genetic analysis. The collected seeds were stratified at 4°C for at least five weeks prior to testing in the greenhouse. After stratification, we pooled seeds from the same field locations to sow in large communal trays with 16:8 hr day-to-night cycle and temperatures at 25-30°C. Plants were watered daily and fertilized weekly with 0.1 grams per plant of 20-20-20 fertilizer. After emergence (~ 10 days), we selected and transplanted individual plants into Cone-tainers (using same growing parameters) so they could reach the 2-to-3-inch growth stage. A single leaf was collected for genetic analysis prior to herbicide treatments and stored at -80°C until DNA extractions.

The herbicide trials consisted of a randomized block of four seedlings (replicates) that were sprayed once with a single rate of a particular herbicide. We tested two rates (1X and 3X) of glyphosate, imazamox, and fomesafen herbicides per location. Appropriate adjuvants were included with the treatments (AMS with glyphosate, AMS + MSO with imazamox and fomesafen). Seedlings from the same location also included an untreated control group. In total, a maximum of 28 seedlings per location were included in the herbicide trials. Treatment response of seedlings from each source location was compared to susceptible controls at 21 days post-treatment. Plants were visibly rated on a scale of 0 to 100%, with 0 meaning no symptomology, and 100 representing complete plant death. At 21 days after treatment, we also classified plants as alive (and capable of reproducing) or dead. Any plant that received a rating of less than 95% at 21 days post-treatment was considered a survivor of that treatment.

*DNA Extractions* – Leaf tissue from the field collected plants (parent) and seedlings (progeny) were used in HR genotyping and sequencing assays. Briefly, a 4 x 4mm area of leaf tissue was added to a 96-well plate containing a single glass bead and homogenized using a GenoGrinder (Spex). DNA extractions were performed on a liquid handler Biomek NXP (Beckman) using NAGC's validated CTAB extraction protocols with Maxwell (Promega) reagents.

*Validated High-throughput Genotyping Assays* – We focused on high-throughput genotyping of two targets associated with HR in waterhemp and Palmer amaranth samples. These two targets included *EPSPS* copy number and *PPO-210* deletion, which are linked to HR to glyphosate and

fomesafen, respectively (Tranel, 2020). A group of both parent and progeny plants were tested using these two genotyping assays.

*Preliminary High-throughput Genotyping Assays* – Two additional genotyping assays for waterhemp and Palmer amaranth are nearing the final validation stages at NAGC. The first assay is a novel high-throughput assay for the EPSPS-106 marker that eliminates both the restriction digest and laborious gel analysis necessary for the current dCAPS genotyping method (Délye et al. 2014). The newly developed genotyping test identifies the substitution at amino acid 106 of the *EPSPS* gene associated with glyphosate resistance. The second assay identifies a polymorphism at amino acid residue 128 of the *PPO* enzyme, which can have two alternative amino acid changes (G and M) associated with resistance (Varanasi et al. 2018). The changes at amino acid 128 tend to be rarer in HR pigweed populations compared to the *PPO-210* deletion. Both of these assays have been tested in a subset of the parental and progeny samples.

*Sequencing* – Prior to the outcomes of the herbicide trials, we optimized a sequencing assay to search for genetic changes within the *ALS* gene associated with the fading efficiency of imazamox. In these samples, we developed primers for sequencing an 832-bp product of the coding region of the *ALS* gene, which contains known target-sites associated with imazamox resistance (Patzoldt & Tranel 2007). PCR products were sequenced in both directions at the University of Chicago DNA Sequencing & Genotyping Facility and raw sequencing files were analyzed using Geneious software.

## Results

*Survey* – The severe drought conditions in central and western ND reduced pigweed sightings, resulting in collections limited to the eastern one-third of North Dakota. Of interest, the majority of Ward County samples were Palmer amaranth, from locations where we were not aware of Palmer amaranth infestations. The surveyors collected seed heads from a total of 65 fields across the 16 counties. The pigweeds were collected from a variety of in-season crop fields including soybeans, dry beans, sunflower, wheat stubble, corn, and sugar beets.

*Greenhouse & Herbicide Trials* - The herbicide trials in the greenhouse included 1,796 seedlings. Pigweeds from Benson-02, Benson-04, Ward-04 and Ward-05 (County Name – Field Number) had low emergence so not all treatments were included for these locations.

**Table 1.** The number of herbicide resistant progeny for the three tested herbicides used in this study.

| Herbicide | Weak Resistance | Strong Resistance | Totals |
|---|---|---|---|
| Glyphosate | 2 | 189 | 191 |
| Fomesafen | 28 | 0 | 28 |
| Imazamox | 30 | 277 | 307 |
| **Totals** | 60 | 466 | **526** |

In total, 29% (526/1796) of the tested progeny were resistant to herbicides as indicated by the greenhouse trials (Table 1). Within the herbicide resistant category, imazamox resistance was the

most widespread (15 counties) across progeny as well as the distribution across North Dakota. Additionally, imazamox resistance was found across all five species included in the study. Glyphosate resistant was the second most widespread HR trait, mostly observed in waterhemp and Palmer amaranth progeny from 12 counties. Two redroot pigweed samples had weak glyphosate resistance from Traill County. Lastly, fomesafen resistance was observed in just 28 plants (waterhemp and Powell amaranth) across seven counties. Six counties had waterhemp populations resistant to all three herbicides.

*DNA Extractions* - To date, we have extracted DNA from 61% (1087/1796) of pigweed samples for genotyping analysis. For this report, we analyzed the genotyping results for waterhemp and Palmer amaranth samples because most of the previous research has focused on these two species.

*EPSPS-Copy & EPSPS-106 genotyping* - We genotyped 375 pigweeds for elevated *EPSPS* gene copies (Table 2). These samples included parent plants (if available) and the progeny from the greenhouse work. Within the genotyped pigweeds, relative *EPSPS* gene copies ranged from 1 - 15 (waterhemp) and 1 - 63 (Palmer amaranth). All counties had individuals with elevated gene copies. In a smaller subset (n = 138), we genotyped samples at the *EPSPS-106* marker and found this mutation in four waterhemp samples across Benson, Griggs, and Logan Counties. Thus, multiple target-site mechanisms (gene copies and site mutations) are contributing to glyphosate resistance in North Dakota pigweeds populations.

*PPO-210 deletion & PPO-128 genotyping* - We genotyped 381 pigweed samples for the deletion of the *PPO-210* amino acid that contributes to fomesafen resistance (Table 2). For *PPO-210*, we found the deletion in waterhemp samples originating from seven counties. The 210 deletion was observed in both the parent and progeny at an overall frequency of 21% (79/381) in genotyped samples. In a smaller subset (n = 88), we found no substitution at the *PPO-128* amino acid that would indicate resistance to fomesafen. We plan to continue the validation work and genotyping pigweeds at *PPO-128* to further characterize populations at this marker.

**Table 2.** Summary of waterhemp samples genotyped using the high-throughput assays of *EPSPS-Copy* and *PPO-210*.

| | **Percentage (number) of plants with elevated EPSPS gene copies (≥ 4 copies)** | | **Percentage (number) of plants with the PPO-210 deletion** | |
|---|---|---|---|---|
| **County** | **Parent** | **Progeny** | **Parent** | **Progeny** |
| Benson | 35 (7/20) | 8 (2/24) | 0 (0/20) | 8 (2/24) |
| Cass | - | 9 (4/43) | - | 5 (2/38) |
| Grand Forks | 95 (19/20) | - | 26 (8/29) | 75 (3/4) |
| Griggs | 90 (27/30) | 37 (49/132) | 43 (13/30) | 24 (32/132) |
| Ramsey | 60 (6/10) | - | 22 (2/9) | - |
| Stutsman | 100 (18/18) | 34 (19/56) | 10 (2/20) | 18 (10/56) |
| Logan | 67 (6/9) | - | 0 (0/10) | 66 (2/3) |
| Richland | 31 (4/13) | - | 8 (1/13) | 29 (2/7) |
| **Totals** | **73 (87/120)** | **35 (88/255)** | **20 (26/131)** | **21 (53/250)** |

*ALS gene sequencing* - We have begun targeted sequencing of pigweeds that displayed resistance to imazamox during herbicide trials. Within North Dakota pigweeds, we have confirmed two target-sites of interest, which include amino acid sites *ALS-574* and *ALS-653*. The *ALS-574* substitution was found in a resistant Palmer amaranth and the *ALS-653* has been confirmed in Powell amaranth, waterhemp, and tumble pigweed samples. This may be the first reported case of ALS-inhibitor resistant (imazamox) tumble pigweed in the world. The universality of the *ALS-653* mutation across pigweed species will need further evaluation before assay development.

*Genotype & Phenotype Comparisons*

*Glyphosate & EPSPS* - To date, we have 51 waterhemp samples that have both genotype and phenotype data for glyphosate resistance. Elevated *EPSPS* gene copies do not fully explain the mechanism for glyphosate resistance in these samples (Fig. 2). Knowing the exact number of gene copies that offer resistance has not been fully resolved and ranges in the literature from 1.4 copies to 4 in waterhemp (Chatham et al. 2015a; Chatham et al. 2015b). Waterhemp in Griggs and Benson Counties have the *EPSPS-106* substitution, so further genotyping using this assay is needed. Our study includes glyphosate resistant waterhemp that contain only one EPSPS gene copy, suggesting additional mechanisms including potential physiological (i.e. nontarget-site mechanisms) contributing to glyphosate resistance in pigweeds of North Dakota.

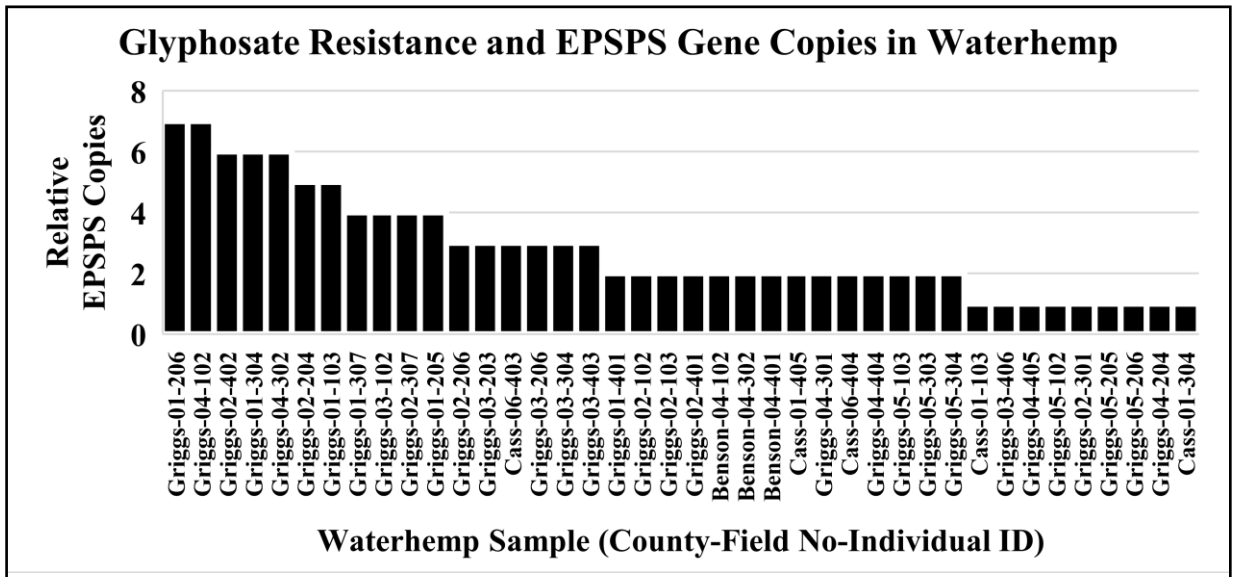


**Figure 2**. Subset of 51 waterhemp samples that were genotyped and phenotyped for glyphosate resistance. Samples originated from Benson, Cass, and Griggs Counties and were sorted by their relative number of EPSPS gene copies, which ranged from 1 to 7 copies.

*Fomesafen and PPO-210* - Due to the low prevalence of resistance in our herbicide trials, we have genotypes for 21 waterhemp samples that showed fomesafen resistance. For these samples, 66% (14/21) of the plants contained at least one copy of the *PPO-210* deletion. The remaining 7 resistant waterhemp samples will be genotyped at the *PPO-128* marker. There were seven Powell amaranth samples that displayed resistance to fomesafen, which need additional sequencing at the PPO gene to identify markers associated with resistance.

## Conclusion

This is the first North Dakota study to screen for HR in pigweed populations with two complementary methods: 1) genotyping pigweeds at target-sites associated with HR, and 2) phenotyping by controlled herbicide treatments in the greenhouse. We found pervasive resistance to imazamox and glyphosate across pigweed populations. At the county level, glyphosate and fomesafen resistance in waterhemp populations could be determined by both phenotyping and genotyping. However, individual level comparisons of glyphosate resistance require genotyping additional markers to look for other target-site mechanisms to explain herbicide trial outcomes. Similarly, additional sequencing of the ALS gene in resistant individuals is needed to determine the predominant genetic mechanisms contributing to imazamox resistance. The challenges and complexity of characterizing HR with both genotyping and phenotyping was expected (see Tranel 2020) and we plan to continue genotyping and sequencing pigweeds to better understand the genetic mechanisms that drive HR in North Dakota.

High-throughput genotyping assays provide several advantages when performing large scale HR surveys. Genotyping can be performed on the pigweeds collected directly from the field and does not require greenhouse work. For ND counties where field collected plants (i.e., parent) and progeny were genotyped (Table 2), we found genetic evidence of glyphosate (elevated EPSPS gene copies) as well as fomesafen (PPO-210 deletion) resistance. Along with reducing the demand for greenhouse space, genotyping provides faster results because it does not require seed germination, greenhouse space, or applying herbicides to plants. At NAGC, genotyping at the *EPSPS* and *PPO* can be performed in less than one day and high-throughput testing allows for a large collection (e.g., 95+ plants) to be simultaneously genotyped. Lastly, an individual pigweed can be simultaneously tested for genetic markers associated with multiple herbicides, unlike greenhouse trials that cannot test additional herbicides once the plant succumbs to an effective herbicide. In our study, several individual waterhemp plants from Griggs, Stutsman, and Grand Forks Counties contained both elevated EPSPS copies and the PPO-210 deletion, suggesting multiple HRs for these individuals.

Despite advantages of genotyping, greenhouse research will remain an important approach for HR testing in pigweeds. Herbicide trials help associate novel target-site mechanisms and identify resistance in cases where mechanisms are not fully understood, such as fomesafen resistance in Powell amaranth or metabolic mechanisms for glyphosate resistance. Our study combines the benefits of both genotyping and phenotyping methods to describe where HR pigweed populations exist in North Dakota and demonstrate how genotyping can help inform weed management on the farm.

## References

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