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# Distribution of glyphosate-resistant *Amaranthus* spp. in Nebraska

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## Abstract

**Background:** Palmer amaranth (*Amaranthus palmeri* S. Wats.), common waterhemp (*Amaranthus tuberculatus var. rudis*), and redroot pigweed (*Amaranthus retroflexus* L.) are major weeds occurring in fields throughout Nebraska with recurrent grower complaints regarding control with glyphosate. The objective of this study was to investigate the frequency and distribution of glyphosate-resistant Palmer amaranth, common waterhemp, and redroot pigweed populations in Nebraska. The study also aimed to investigate how agronomic practices influence the occurrence of glyphosate resistance in the three Amaranthus species.

**Results:** Glyphosate resistance was widespread in common waterhemp (81% of the screened populations), few Palmer amaranth populations were glyphosate-resistant (6% of the screened populations), whereas no glyphosate-resistant redroot pigweed populations were identified in Nebraska. Weed species, geographic region within the state, and current crop were the most important factors predicting the occurrence of glyphosate resistance in fields infested with *Amaranthus* species in Nebraska.

**Conclusion:** The intensive glyphosate selection pressure exerted in soybean

(*Glycine max*) fields in eastern Nebraska is one of the major factors causing widespread occurrence of glyphosate resistance in common waterhemp in the state. The relatively low frequency of glyphosate-resistant Palmer amaranth in the state highlights the importance of the application timing and the adoption of multiple modes of action in weed management practices to delay the evolution of glyphosate resistance.

**Keywords:** *Amaranthus* spp., herbicide resistance, random forest, agronomic practices

## 1 Introduction

Palmer amaranth (*Amaranthus palmeri* S. Wats.), common waterhemp (*Amaranthus tuberculatus var. rudis*), and redroot pigweed (*Amaranthus retroflexus* L.) are major weeds occurring in fields throughout Nebraska. The three amaranths are C4 summer annual weed species members of the Amaranthaceae family and native to North America.[1–3] The *Amaranthus* species have a fast growth habit and are prolific seed producers, contributing to their success as troublesome weeds in cropping systems.[4] Seed production ranges from 400 000 to 1 000 000 seeds per plant in Palmer amaranth,[5] redroot pigweed,[6] and common waterhemp[7] under favorable environmental conditions. Redroot pigweed is a monoecious species, whereas Palmer amaranth and common waterhemp are dioecious.[1] The three *Amaranthus* species have an extended emergence window, which poses a challenge to their management.[7–9] Bensch *et al.* reported 79, 56, and 38% yield losses in soybean (*Glycine max*) with Palmer amaranth, common waterhemp, and redroot pigweed interference, respectively.[10] Corn (*Zea mays*) yield losses of up to 91%,[11] 43%,[8] and 34%[12] were reported with Palmer amaranth, common waterhemp, and redroot pigweed interference, respectively.

Glyphosate became a standard chemical option for management of amaranths and other weed species in US row crop production since 1996 as a result of the advent of genetically modified glyphosate-resistant (GR) crops.[13] Glyphosate is one of the most adopted herbicides worldwide because of its high efficacy, low toxicity to animals, and relatively low environmental impact.[14] Glyphosate is toxic to plants because it inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate pathway,[15] which is a biochemical pathway for the synthesis of the aromatic amino acids tyrosine,

phenylalanine, and tryptophan.[16] In a field study, Krausz *et al.* reported that glyphosate was effective at controlling amaranths, especially when plants were treated at early growth stages.[17] In 1995, prior to the advent of GR crops, glyphosate was applied in 6% of corn fields and in 20% of soybean fields in the USA, whereas in 2015, treated areas had increased to 77% and 97%, respectively.[18] The excessive reliance on glyphosate for weed control favored the occurrence of herbicide resistance.[19] According to Heap,[20] 37 GR weed species have been reported worldwide. Several Palmer amaranth and common waterhemp populations have evolved resistance not only to EPSP synthase inhibitors but also to herbicides that target acetolacte synthase (ALS), photosystem II, protoporphyrinogen oxidase (PPO), auxin receptors, microtubule assembly, and 4-hydroxyphenylpyruvate dioxygenase (HPPD) in the USA.[20] Redroot pigweed populations resistant to ALS and photosystem II inhibitors have also been reported in the USA.[20] The first cases of glyphosate resistance in Palmer amaranth and common waterhemp were identified in 2004 in Georgia[21] and Missouri,[22] respectively, whereas no case of GR redroot pigweed has been reported.[20] Interspecific hybridization with glyphosate resistance trait transfer has been reported in some *Amaranthus* species but not in redroot pigweed.[23] Bell *et al.* reported a common waterhemp population from Illinois with multiple resistance to herbicides that target EPSP synthase, ALS, PPO, and photosystem II.[24] Schultz *et al.* identified common waterhemp populations from Missouri showing resistance to glyphosate, ALS, PPO, photosystem II, and HPPD inhibitors.[25] Common waterhemp populations with resistance to herbicides that target ALS, HPPD, photosystem II, EPSP, and auxin receptors were reported in Nebraska.[26–28] Acetolacte synthase, HPPD, photosystem II, and GR biotypes of Palmer amaranth were also reported in Nebraska.[27,29] Jhala *et al.* reported a Palmer amaranth population with multiple resistance to herbicides that target HPPD and photosystem II in Nebraska.[30] GR Palmer amaranth was also reported in Arkansas,[31] Tennessee,[32] Mississippi,[33] North Carolina,[34] New Mexico,[35] and other states.[20]

Glyphosate resistance mechanisms in weeds include target-site resistance with mutations in the *EPSPS* gene, target-site gene amplification, and non-target-site resistance with active vacuolar sequestration, herbicide metabolism, and limited cellular uptake and translocation.[36,37] *EPSPS* gene amplification is the main glyphosate resistance mechanism in Palmer amaranth,[38] in which resistant biotypes produce

high levels of EPSPS as a result of the extra *EPSPS* gene copies, which act as a molecular "sponge" by binding glyphosate molecules.[19] The same resistance mechanism was reported in GR Palmer amaranth fromNebraska.[29] Glyphosate resistance mechanisms reported in common waterhemp populations include *EPSPS* gene amplification,[25,39–41] *EPSPS* target site mutation,[24,25,33] and non-target-site resistance mechanisms with reduced glyphosate uptake and translocation.[42] *EPSPS* target site mutation and non-target-site resistance mechanisms with reduced glyphosate uptake and translocation were also reported in Palmer amaranth,[43] albeit at a lower frequency when compared with common waterhemp. According to Sammons and Gaines, accumulation of multiple resistance mechanisms under glyphosate selection pressure, especially in cross-pollinated species, leads to enhanced glyphosate resistance levels.[37]

GR weeds such as Palmer amaranth and common waterhemp represent a challenge to cropping systems that rely on glyphosate for weed control.[44] Glyphosate-control failures on Palmer amaranth and common waterhemp are becoming a recurrent complaint among growers in Nebraska,[28,29] although it is not clear if the majority of the reports are attributable to glyphosate resistance or poor management practices, such as wrong application timing, inadequate dose, or improper application technique. A better understanding of the distribution of GR Palmer amaranth, common waterhemp, and redroot pigweed in Nebraska provides growers with important information on how to effectively manage the *Amaranthus* species in the state. Therefore, the objective of this study was to investigate the distribution of GR Palmer amaranth, common waterhemp, and redroot pigweed in Nebraska. Furthermore, the study aimed to investigate the impact of agronomic practices on the likelihood of glyphosate resistance in *Amaranthus* species.

## 2 Materials and Methods

### *2.1 Plant material*

Palmer amaranth, common waterhemp, and redroot pigweed seed samples were arbitrarily collected from 10–20 plants in 218 Nebraskan fields in the falls of 2013, 2014, and 2015. Seeds from within a

single field were identified as a population and agronomic variables (weed species, geographic region within the state, current field crop, irrigation, tillage practices, and location of sampled weeds in the field) were recorded along with Global Positioning System (GPS) coordinates for each population (Table 1). Seeds were stored at -20 °C for a minimum of 3 months to overcome dormancy. Seeds from each population were sown into plastic tubes (1 L) containing commercial potting mix, supplied with water and fertilizer as needed (UNL 5-1-4 at 0.2% v/v; Wilbur-Ellis Agribusiness, Aurora, CO, USA), and maintained in a greenhouse with controlled temperature and light conditions (30/20 °C day/night with a 16-h photoperiod).

**Table 1.** Amaranth populations collected from 218 fields in Nebraska in 2013, 2014 and 2015

| | *Percentage of populations*[a] | | |
|---|---|---|---|
| | *Palmer amaranth (95 populations)* | *Common waterhemp (100 populations)* | *Redroot pigweed (23 populations)* |
| Crops | | | |
| Alfalfa | 2.1 | | |
| Corn | 62.1 | 16.0 | 66.7 |
| Sorghum | 5.3 | | 4.8 |
| Soybean | 24.2 | 84.0 | 23.8 |
| Wheat | 3.2 | | 4.8 |
| Tillage | | | |
| No-till | 42.1 | 60.0 | 28.6 |
| Till | 51.6 | 35.0 | 71.4 |
| Irrigation | | | |
| Rainfed | 44.2 | 84.0 | 28.6 |
| Irrigated | 50.5 | 12.0 | 71.4 |
| Weed location within field | | | |
| Field borders | 41.1 | 23.0 | 19.0 |
| Inside fields | 53.7 | 76.0 | 76.2 |
| Nebraska geographic region | | | |
| Central | 24.2 | | 38.1 |
| East central | 5.3 | 42.0 | 4.8 |
| North central | 1.1 | 1.0 | 9.5 |
| Northeast | | 15.0 | 28.6 |
| Northwest | 4.2 | | 19.0 |
| South central | 15.8 | 1.0 | |
| Southeast | 8.4 | 41.0 | 4.8 |
| Southwest | 41.1 | | 4.8 |

a. Population percentages that do not add to 100% are caused by missing data.

### *2.2 Glyphosate dose–response study*

This study was conducted in the Pesticide Application Technology Laboratory, University of Nebraska-Lincoln West Central Research and Extension Center, in North Platte, NE. The Amaranth populations were subjected to a glyphosate (Roundup PowerMAX®; Monsanto Company, St. Louis, MO, USA) dose–response study, in which different rates of glyphosate (0, 39, 217, 434, 868, 1736, 3472, and 6935 g ae $ha^{-1}$) were applied to 10- to 12-cm-tall plants using a research spray chamber calibrated to deliver 93.5 L $ha^{-1}$ with an AI95015EVS nozzle (Teejet Spraying Systems, Wheaton, IL, USA) at 414 kPa. The experiment was conducted as a complete randomized design with four replications per treatment in which a single plant was considered as an experimental unit. Plant aboveground biomass was harvested at 21 days after treatment (DAT) and oven-dried at 65 °C to constant weight. The biomass data were converted into percentage of biomass reduction as compared with the untreated control.[28] A nonlinear regression model was fitted to the dry weight data using the *DRC* package in R software (R Foundation for Statistical Computing, Vienna, Austria).[45] The effective doses to reduce plant biomass by 50% and 90% ($GR_{50}$ and $GR_{90}$, respectively) were estimated for each population using a four-parameter log logistic equation:

$$y = c + \frac{d - c}{1 + \exp\left[b\left(\log x - \log e\right)\right]}$$

in which *y* corresponds to the biomass reduction (%), *b* is the slope at the inflection point, *c* is the lower limit of the model (fixed to 0%), *d* is the upper limit (fixed to 100%), and *e* is the inflection point ($GR_{50}$).[46] Resistance levels were calculated using the ratio of the $GR_{90}$ of each population to the glyphosate recommended label rate (868 g ae $ha^{-1}$). The experiment was replicated for common waterhemp and Palmer amaranth populations that were identified as putatively GR in the first experimental run. Data from the two experimental runs were combined.

### *2.3 Resistance map*

Palmer amaranth and common waterhemp resistance level data were displayed in an interpolated map format created in Esri® ARCMAP™

version 10.1 software (ESRI, Redlands, CA, USA). A new geostatistical database was created where population GPS coordinates were added and plotted. Map shapefiles of the Nebraska state boundary and county boundaries were added and a new layer was created with counties and collected populations combined (US Department of Commerce 2007). Counties where collections took place, and nearest adjacent counties, were selected and exported into a new data layer so that only collected counties would show interpolation data. Geostatistical analysis was performed using the ArcMap Geostatistical Wizard with the inverse distance weighting function. The source dataset was the collected population and the data field was the corresponding resistance level. Power was set to two and a standard neighborhood type was used with the maximum number of neighbors set at five and the minimum number of neighbors set at three. Inverse distance weighting was exported to a vector with a filled contour. A new layer was then exported by clipping the filled contour vector as the input features and the collected counties layer as the clipped features. Color classes were used in the filled contour to show an estimation of the resistance level of populations.

### *2.4 Random forest analysis*

The random forest algorithm is an ensemble classifier based on multiple classification and regression trees (CARTs), in which each tree is built using a randomly selected subset of training samples and variables.[47,48] By creating a large number of trees on bootstrap samples and averaging the outputs, the random forest algorithm yields a reliable variable importance classification.[48,49] The number of decision trees to be generated (*ntree*) and the number of variables to be selected and tested for the best tree node divisions (*mtry*) need to be specified in the model.[47] Approximately 66% of the samples (in bag) are used to train the trees, whereas the remaining samples (out of the bag) are used in an internal cross-validation technique to estimate the model performance error.[47,48] To evaluate the importance of a variable, the random forest measures the decrease in accuracy by means of the out of the bag (OOB) error and the Gini Index decrease when that variable is permuted while the others are kept constant.[50,51] The OOB error can also be used to estimate the model performance accuracy.[52]

The random forest analysis was performed with the *randomForest* package[50] in R software to identify the agronomic variables (weed species, geographic region within the state, crop, irrigation, tillage practices, and if weeds were located at field borders or inside fields) that contributed most to glyphosate resistance presence in fields infested with amaranths in Nebraska. The Nebraska's Agricultural Statistical Districts map[18] was utilized to define each population region (southeast, east central, northeast, south central, central, north central, southwest, and northwest). Populations with the upper limit of the 95% confidence interval of their estimated $GR_{90}$ >868 g ae $ha^{-1}$ (a commonly used label rate) were classified as having "practical" glyphosate resistance.[53] The *ntree* parameter (number of regression trees) was set to 5000, whereas the *mtry* (number of different predictors tested at each node) and the *nodesize* (minimal size of the terminal node) parameters were set to default values. Variable importance was measured with the Gini coefficient and a variable importance plot was constructed as described by Langemeier *et al.*[49]

## 3 Results and Discussion

Glyphosate rates tested herein were lethal to plants from redroot pigweed populations screened in this study (data not shown); therefore, no GR redroot pigweed populations were identified in Nebraska (Figure 1).

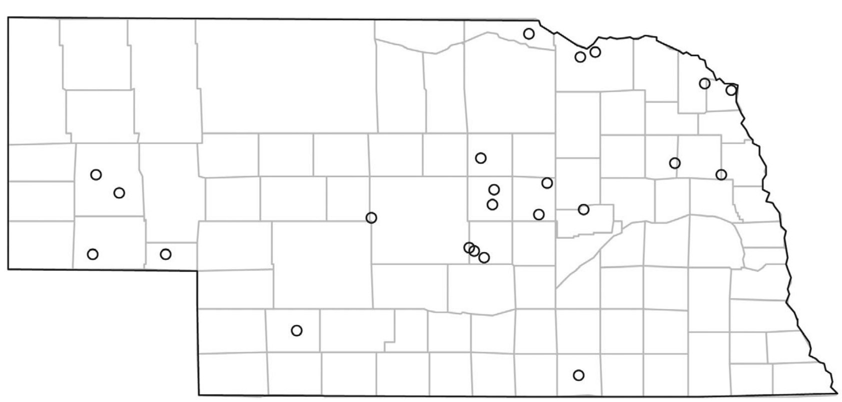

**Figure 1.** Distribution of glyphosate-susceptible redroot pigweed populations in Nebraska. A population was considered susceptible when the upper limit of the 95% confidence interval of its estimated $GR_{90}$ was less than the recommended glyphosate label rate (868 g ae $ha^{-1}$).

### *3.1 Palmer amaranth glyphosate dose–response*

Palmer amaranth is predominant in central and southwestern Nebraska, and 62.1% of the populations were collected in corn fields (Figure 2). The region has lower precipitation indices when contrasted with the eastern part of the state.[54] Ehleringer defined Palmer amaranth as a Sonoran desert weed species with efficient photosynthetic capacity and effective drought tolerance mechanism,[55] which explains the predominance of this species over other amaranths in the region. In contrast to grower complaints, only 6% of the Palmer amaranth populations screened in this study exhibited "practical" resistance to glyphosate (Figure 3). However, the authors recognize that this study represents a snapshot of what was occurring between 2013 and 2015 in Nebraska. Tabashnik *et al.* defines practical resistance as "field-evolved resistance that reduces pesticide efficacy and has practical consequences for pest control".[53] Some populations in this study had reduced sensitivity to glyphosate, with $GR_{90}$ ratios ranging from 18- to 27-fold difference in relation to the most susceptible population (highly sensitive to glyphosate), but with $GR_{90}$ estimates (upper limit of the 95% confidence interval) < 868 g ae $ha^{-1}$. Although the authors recognize that these populations may have individuals with genetically heritable reduced sensitivity to glyphosate and that intermediate levels of resistance may have continuum effects on weed management,[53] these populations were not classified as having "practical resistance". In addition, as *EPSPS* gene amplification is the most

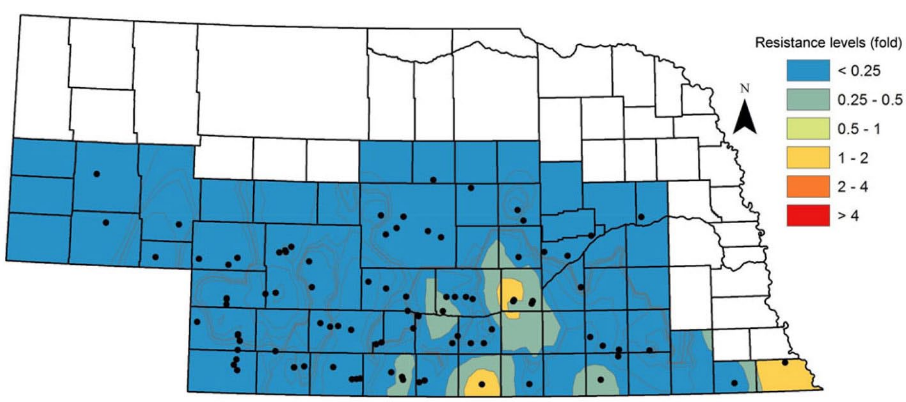


**Figure 2.** Distribution and glyphosate resistance level of Palmer amaranth populations in Nebraska. Resistance ratios were calculated as the ratio of the $GR_{90}$ of each population to the glyphosate label rate (868 g ae $ha^{-1}$).

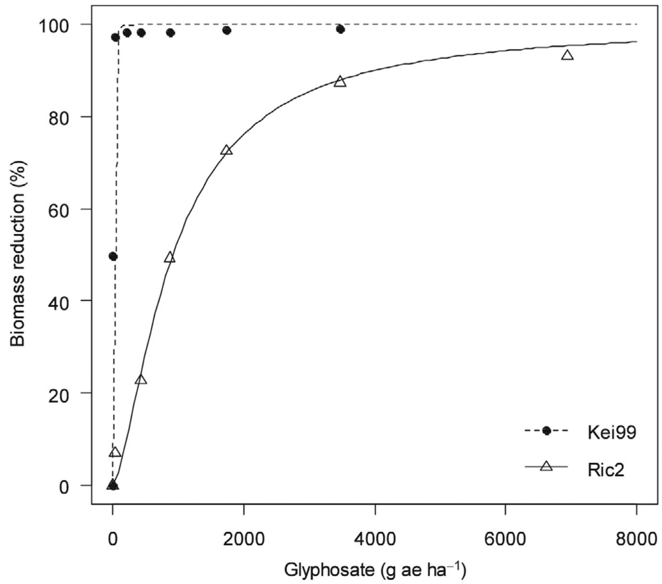


**Figure 3.** Biomass reduction of glyphosate-resistant (Ric2) and -susceptible (Kei99) Palmer amaranth populations from Nebraska at 21 days after treatment in a glyphosate dose–response bioassay conducted at the Pesticide Application Technology Laboratory, University of Nebraska-Lincoln West Central Research and Extension Center.

common glyphosate resistance mechanism in Palmer amaranth, and resistance levels correlate with *EPSPS* gene copy number,[38] the authors hypothesize that the populations with reduced sensitivity to glyphosate could have individuals with relatively low *EPSPS* copy numbers when compared with populations with higher resistance levels. Further studies with molecular characterization of the glyphosate resistance mechanisms of the populations with reduced sensitivity to glyphosate are required. Resistance ratios relative to the dose of 868 g ae $ha^{-1}$ ranged from 0.01- to 5.44-fold (Table 2). Culpepper *et al.* reported that 52% of Palmer amaranth populations collected in Georgia in 2005 and 2006 were resistant to glyphosate, whereas 17% of the populations collected in North Carolina had resistance to glyphosate.[56] Palmer amaranth escapes following glyphosate applications could be associated with the species biology, especially the extended germination period which poses a challenge for glyphosate application timing.[57] It has been reported that glyphosate control is reduced when plants are sprayed at later growth stages.[58,59] The environmental conditions of central and southwestern Nebraska (predominant

**Table 2.** Agronomic variables, estimation of $GR_{50}$ and $GR_{90}$, and resistance levels for selected Palmer amaranth populations from Nebraska. Resistance levels were calculated as the ratio of the $GR_{90}$ of each population to the glyphosate recommended label rate (868 g ae ha$^{-1}$)

| Population | County | Crop | Tillage | Irrigation | Weed location | $GR_{50}$ (g ae ha$^{-1}$)±SE | $GR_{90}$ (g ae ha$^{-1}$)±SE | Resistance level |
|---|---|---|---|---|---|---|---|---|
| Per15-2 | Perkins | Wheat | No | No | Field | 2.3±1.9 | 10.4±1.6 | 0.01 |
| Hay15-2 | Hayes | Sorghum | Yes | No | Field | 9.7±0.6 | 16.3±4.0 | 0.02 |
| Kei99 | Keith | Corn | No | Yes | Edges | 3.9±0.2 | 17.0±3.2 | 0.02 |
| Daw226 | Dawson | Corn | No | Yes | Edges | 5.2±0.6 | 20.4±10.1 | 0.02 |
| Per15-3 | Perkins | Corn | No | No | Field | 10.6±0.8 | 25.1±4.9 | 0.03 |
| Cust45 | Custer | Soybean | Yes | Yes | Field | 6.7±0.6 | 27.4±4.2 | 0.03 |
| Lin60 | Lincoln | Corn | Yes | Yes | Edges | 7.7±0.7 | 28.8±6.4 | 0.03 |
| Red157 | Red Willow | Corn | No | Yes | Field | 9.3±0.9 | 35.3±7.2 | 0.04 |
| Cha28 | Chase | Corn | No | No | Edges | 5.6±1.0 | 36.3±7.2 | 0.04 |
| Lin97 | Lincoln | Corn | No | Yes | Edges | 5.6±0.6 | 36.7±12.3 | 0.04 |
| Per33 | Perkins | Soybean | No | Yes | Edges | 6.2±0.5 | 52.7±13.9 | 0.06 |
| Paw6 | Pawnee | Soybean | No | No | Edges | 12.7±1.4 | 60.4±13.2 | 0.07 |
| Red163 | Red Willow | Corn | No | No | Field | 10.8±1.9 | 62.3±20.3 | 0.07 |
| Lin15-8 | Lincoln | Sorghum | Yes | No | Field | 13.2±3.8 | 188.7±90.4 | 0.22 |
| Hall13 | Hall | Soybean | Yes | No | Edges | 51.5±12.1 | 287.6±158.3 | 0.33 |
| Tha15-2 | Thayer | Alfalfa | No | No | Field | 80.3±19.0 | 982.8±451.7 | 1.13 |
| Buf15-1 | Buffalo | Soybean | Yes | Yes | Field | 122.64±26.3 | 2591.3±1168.3 | 2.99 |
| Frank4 | Franklin | Wheat | No | No | Field | 337.5±65.5 | 2623.0±1291.2 | 3.02 |
| Ric2 | Richardson | Soybean | No | No | Edges | 917.5±89.6 | 4021.2±1025.9 | 4.63 |
| Hal6 | Hall | Soybean | Yes | No | Field | 602.2±95.1 | 4724.9±1759.6 | 5.44 |

SE, standard error.

Palmer amaranth area) could also influence glyphosate performance. Glyphosate efficacy is reduced in several weeds under water stress and low-humidity conditions.[60–63] Adkins *et al*. reported that glyphosate efficacy on *Avena fatua* and *Urochloa panicoides* was reduced under water stress combined with high temperatures,[64] typical conditions found in central and southwestern Nebraska.

### 3.2 Common waterhemp glyphosate dose–response

Common waterhemp was predominantly found in eastern Nebraska, whereas no populations were found in the western part of the state (Figure 4). The majority of the common waterhemp populations were sampled in soybean fields (84%). The results indicate that GR common waterhemp is widespread in eastern Nebraska (Figure 5). Eighty-one percent of the common waterhemp populations screened in this study expressed “practical” resistance to glyphosate (Table 3). Similar

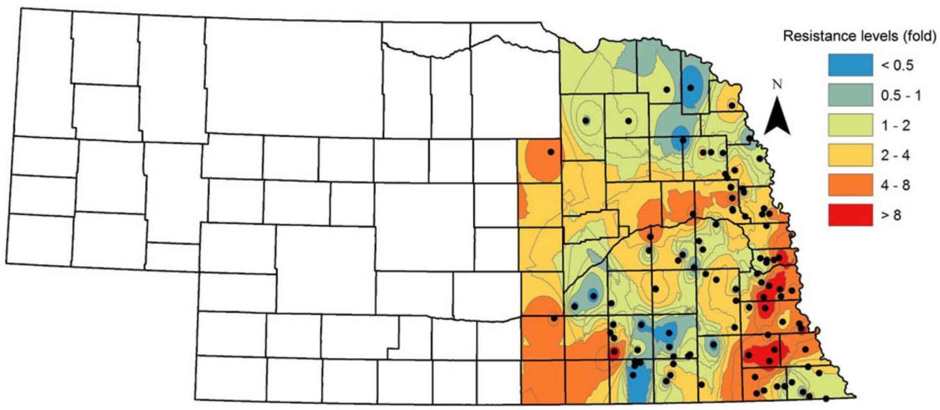


**Figure 4.** Distribution and glyphosate resistance level of common waterhemp populations in Nebraska. Resistance ratios were calculated as the ratio of the $GR_{90}$ of each population to the glyphosate label rate (868 g ae $ha^{-1}$).

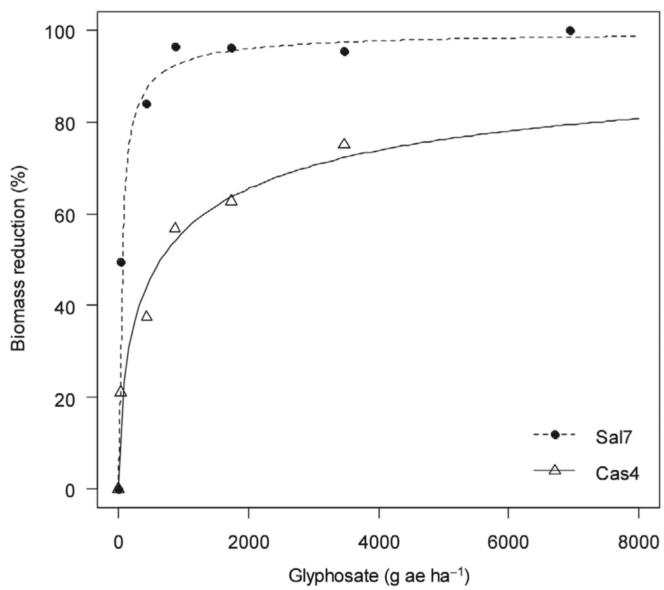


**Figure 5.** Biomass reduction of glyphosate-resistant (Cas4) and -susceptible (Sal7) common waterhemp populations from Nebraska at 21 days after treatment in a glyphosate dose–response bioassay conducted at the Pesticide Application Technology Laboratory, University of Nebraska-Lincoln West Central Research and Extension Center.

results were reported in Missouri, where 58% of the screened common waterhemp populations survived the glyphosate label rate.[25] Chatham *et al.* reported that 28% of the common waterhemp populations screened throughout Illinois in 2010 were GR.[39] They indicated that the

**Table 3.** Agronomic variables, estimation of $GR^{50}$ and $GR_{90}$, and resistance levels for selected common waterhemp populations from Nebraska. Resistance levels were calculated as the ratio of the $GR_{90}$ of each population to the glyphosate recommended label rate (868 g ae $ha^{-1}$)

| Population | County | Crop | Tillage | Irrigation | Weed location | $GR_{50}$ (g ae $ha^{-1}$)±SE | $GR_{90}$ (g ae $ha^{-1}$)±SE | Resistance level |
|---|---|---|---|---|---|---|---|---|
| Dix12 | Dixon | Corn | No | No | Field | 60.4±6.1 | 190.8±56.0 | 0.22 |
| But1 | Butler | Soybean | No | No | Field | 79.8±5.1 | 360.4±50.7 | 0.42 |
| Sal3 | Jefferson | Soybean | No | No | Field | 70.2±6.7 | 383.7±86.1 | 0.44 |
| Ric9 | Richardson | Soybean | No | No | Field | 89.9±14.7 | 505.6±169.8 | 0.58 |
| Sau10 | Saunders | Soybean | Yes | No | Edges | 133.1±14.8 | 747.6±130.4 | 0.86 |
| Ant77 | Antelope | Soybean | Yes | Yes | Field | 144.2±20.8 | 757.8±164.6 | 0.87 |
| Ric11 | Richardson | Soybean | Yes | No | Field | 131.1±16.7 | 890.3±167.1 | 1.03 |
| Cedar3 | Cedar | Corn | Yes | Yes | Edges | 185.8±45.5 | 924.5±357.6 | 1.07 |
| Lan9 | Lancaster | Corn | No | No | Edges | 81.8±18.7 | 1008.9±435.8 | 1.16 |
| Jef12 | Saline | Soybean | No | No | Field | 161.7±30.2 | 1176.0±321.7 | 1.35 |
| Dod1 | Dodge | Corn | Yes | No | Field | 152.4±33.9 | 1282.5±422.0 | 1.48 |
| Gag8 | Gage | Corn | No | No | Field | 65.2±18.1 | 1609.8±667.5 | 1.85 |
| Cum7 | Cuming | Soybean | No | No | Field | 198.5±26.1 | 1789.4±349.3 | 2.06 |
| Dod13 | Dodge | Corn | Yes | No | Field | 182.6±47.2 | 2345.7±783.5 | 2.70 |
| Sew1 | Seward | Soybean | Yes | Yes | Field | 590.5±58.4 | 2853.9±608.2 | 3.29 |
| Polk1 | Polk | Soybean | No | Yes | Field | 869.1±68.4 | 4230.3±836.4 | 4.87 |
| Joh13 | Johnson | Soybean | No | No | Field | 459.9±78.0 | 5820.8±1763.5 | 6.71 |
| Cas9 | Cass | Soybean | No | No | Edges | 375.2±96.1 | >6935 | >8.0 |
| Cas4 | Cass | Soybean | Yes | No | Field | 653.1±153.7 | >6935 | >8.0 |
| Oto11 | Otoe | Soybean | No | No | Field | 994.7±277.7 | >6935 | >8.0 |

SE, standard error.

relatively low percentage of glyphosate resistance in common waterhemp despite major complaints from growers could be attributed to poor management practices and not to glyphosate resistance.

Twelve percent of the populations had $GR_{90}$ ratios ranging from 2- to 3-fold difference in relation to the most susceptible population, but with the upper limit of the 95% confidence interval of their estimated $GR_{90}$ <868 g ae $ha^{-1}$. As previously described for the Palmer amaranth results, populations with reduced sensitivity to glyphosate were not classified as having "practical resistance" in this study. The authors hypothesize that these populations may have individuals with genetically heritable reduced sensitivity to glyphosate with relatively low *EPSPS* copy number in relation to populations with higher resistance levels. Moreover, common waterhemp populations with reduced sensitivity to glyphosate could have different glyphosate resistance mechanisms, such as *EPSPS* target site mutation (Pro106Ser) and/or non-target-site resistance which results in reduced glyphosate uptake and

translocation. The Pro106Ser *EPSPS* mutation has been reported in several common waterhemp populations throughout the USA.[24,25,39,42] It has been suggested that this mutation is usually associated with low levels of glyphosate resistance, where even though plants have reduced sensitivity to glyphosate, they do not survive higher rates of the herbicide.[24] Further studies with molecular characterization of the glyphosate resistance mechanisms of these common waterhemp populations with reduced sensitivity to glyphosate are required.

### *3.3 Random forest analysis*

Random forest is considered a powerful machine learning classifier because of its nonparametric nature, high classification accuracy, and capability of estimating variable importance .[51] The OOB error of this random forest model corresponded to 11.47%, which means that >88% of the OOB samples were correctly classified by the model. Weed species was the best predictor for the presence of glyphosate resistance in *Amaranthus* species in Nebraska, followed by geographic region within the state and current crop. This, however, is just a snapshot of where things were between 2013 and 2015. Follow-up surveys are needed to further determine the current distribution and frequency of glyphosate resistance within the state. The least important factors were tillage practice and weed location within the field (Figure 6). Six percent of the Palmer amaranth populations were confirmed GR, 81% of the common waterhemp populations were GR, whereas no GR redroot resistant populations were identified. The dioecious reproduction characteristic of Palmer amaranth and common waterhemp combined with the high potential of pollen-mediated gene flow are considered major factors in the spread of glyphosate resistance for these species.[65] The multiple glyphosate resistance mechanisms reported in common waterhemp, such as *EPSPS* target site mutation (Pro106Ser) and non-target-site resistance mechanisms with reduced glyphosate uptake and translocation, could contribute to the higher frequency of glyphosate resistance in common waterhemp when compared with Palmer amaranth. Although both glyphosate resistance mechanisms were also reported in a Palmer amaranth population from Mexico,[43] the literature suggests that both mechanisms are more frequent in common waterhemp.

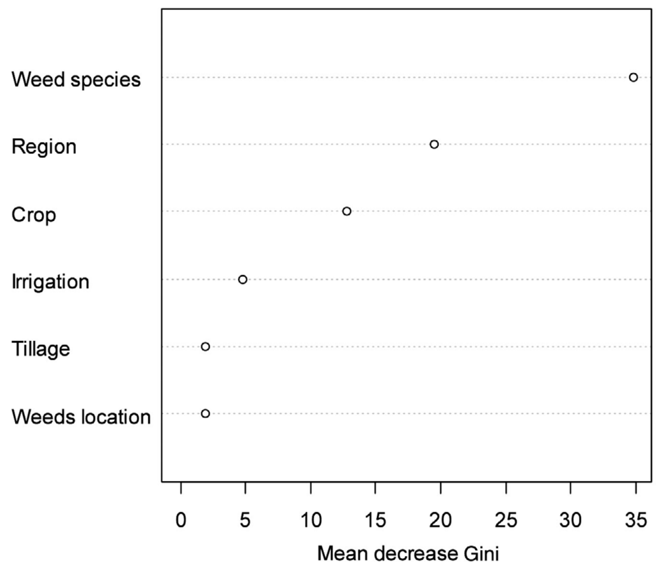


**Figure 6.** Random forest analysis of likelihood of glyphosate resistance in *Amaranthus* species in response to agronomic strategies and geographic location within Nebraska. Variables are ordered by importance measured using the Gini coefficient.

The majority of the GR common waterhemp populations were collected in eastern Nebraska, where approximately 85% of these were collected in soybean fields. Interestingly, two of the six GR Palmer amaranth populations identified in the study were also collected in eastern Nebraska, whereas four populations were collected in central and southcentral Nebraska, regions with common waterhemp presence. Glyphosate resistance in common waterhemp is also widespread in Missouri and Iowa,[20] states with borders with eastern Nebraska.

It was estimated that 13 million ha of soybean fields were planted in Nebraska in 2016, with 76% located in eastern Nebraska.[18] The planted area for corn in the same year corresponded to 24 million ha, whereas 56.5% was located in the eastern/southeastern/northeastern part of the state, 27.1% in the central/north central/south central part, and 16.4% in the southwestern/ northwestern part (Table 4). The National Agricultural Statistics Service of the United States Department of Agriculture (USDA-NASS) estimated that a total of 3408 tons of herbicide active ingredients were applied in soybean in Nebraska during 2016, and 75% of the total amount was glyphosate.[66] Conversely, it was estimated that a total of 12,567 tons of herbicide active ingredients were applied in corn in Nebraska in the same year, and 38% of

**Table 4.** Soybean and corn planted area in Nebraska in 2016[a]

| Nebraska region | Soybean (%) | Corn (%) |
|---|---|---|
| Central | 8.2 | 11.9 |
| East central | 28.9 | 21.9 |
| North central | 3.5 | 4.4 |
| Northeast | 24.9 | 18.4 |
| Northwest | 0.1 | 4.6 |
| South central | 9.1 | 10.8 |
| Southeast | 22.4 | 16.2 |
| Southwest | 2.9 | 11.8 |
| Total area (million ha) | 12.85 | 24.34 |

a. USDA National Agricultural Statistics Services, 2017 (https://quickstats.nass.usda.gov/).

this amount was glyphosate. These herbicide use statistics highlight the over-reliance on glyphosate and the intensive glyphosate selection pressure exerted on weeds in eastern Nebraska, especially in soybean fields. It is also possible to infer that, although growers rely on glyphosate for weed control in corn, they are also utilizing different modes of action such as atrazine (22% of total applied herbicide active ingredients) and other pre-emergent herbicides such as chloroacetamides (29% of the total applied herbicide active ingredients). Evans *et al.* reported in a classification and regression tree analysis that glyphosate resistance was more likely in common waterhemp populations from fields in Illinois with frequent glyphosate applications and fewer modes of action per year.[67] The data provided by USDA-NASS help clarify why glyphosate resistance is not widespread in western Nebraska (e.g., majority of the planted area in this region corresponds to corn, a crop in which producers adopt more diverse herbicide programs). Moreover, the region has a predominance of Palmer amaranth and little to no presence of common waterhemp.

Pollen-mediated gene flow could be a major factor contributing to the widespread occurrence of glyphosate resistance in eastern Nebraska. Sarangi *et al.* reported that the GR trait in common waterhemp from Nebraska was highly mobile and its pollen-mediated dispersal was influenced by distance and wind.[68] The authors reported up to 9% gene flow occurring in plants at 50m from the pollen source, whereas the variability in gene flow increased with increasing distance from the source. Several other factors could also influence pollen dispersal,

such as isolation distance, geographic barriers, crop canopy, recipient plant size, environmental conditions, and pollen competition.[65,69] Additional studies are required to understand how these factors could influence pollen-mediated gene flow with glyphosate resistance dispersal in *Amaranthus* ssp. Sarangi *et al.* highlighted that management strategies adopted by growers are focused on delaying herbicide resistance evolution over a small area, but they lack efficiency in preventing large-scale movement of herbicide resistance through pollen-mediated gene flow.[68]

This observation could also address why tillage practices were not considered important in predicting glyphosate resistance in the random forest model, as only 31% of the surveyed soybean fields in eastern Nebraska had tillage practices. Tillage can be considered as an additional weed management tool to control GR weeds,[14] but may only be effective for certain weed species. Some studies suggest that tillage practices combined with herbicide programs could potentially delay herbicide resistance evolution in specific situations.[70] However, it seems unlikely that tillage practices would mitigate glyphosate resistance evolution in common waterhemp from eastern Nebraska, as the GR trait is widespread and highly mobile through pollen-mediated gene flow in the species.

Although pollen-mediated glyphosate resistance transfer from Palmer amaranth to common waterhemp,[23] and gene introgression from common waterhemp to Palmer amaranth were reported,[71] the relatively low frequencies of the interspecific hybridization between species combined with their geographic distribution in the state seem to contribute to the delay in the glyphosate resistance evolution in Palmer amaranth in Nebraska. It is important to mention that the few GR Palmer amaranth populations reported in the study were present in areas with GR common waterhemp presence. This observation may indicate that glyphosate resistance in Palmer amaranth in Nebraska could be associated with pollen-mediated glyphosate resistance transfer from common waterhemp. Further studies are necessary to test this hypothesis.

The random forest analysis detected a minor importance of irrigation practices in the prediction of glyphosate resistance presence in fields with amaranths in Nebraska. This observation is probably a result of a confounding factor regarding the irrigation distribution in the state, where the majority of the irrigated fields are located in western

Nebraska as a consequence of the reduced precipitation in this region. Only 25% of the surveyed irrigated fields were present in eastern Nebraska, the region with widespread glyphosate resistance.

Interestingly, the random forest analysis indicated that the location of weeds within each site (field borders or inside fields) did not have importance in the prediction of glyphosate resistance in amaranths from Nebraska. The results indicate that the glyphosate resistance was also identified in plants that were collected in field borders and roadsides. This corroborates the results reported by Bagavathiannan and Norsworthy, who found only 3% of a total of 215 Palmer amaranth populations that were collected from roadsides in Texas to be susceptible to glyphosate.[72] The authors suggested that growers should implement appropriate control strategies to manage roadside populations, especially if they are close to agricultural fields.

## 4 Conclusion

The results reported in this study help clarify the glyphosate resistance status of *Amaranthus* species in Nebraska. It can be concluded that the intensive glyphosate selection pressure exerted in eastern Nebraska, especially in soybean fields, is the major factor responsible for the widespread occurrence of glyphosate resistance in common waterhemp in the state. It can be inferred that pollen-mediated gene flow may play an important role in the dispersal of glyphosate resistance in common waterhemp in eastern Nebraska. The relative low frequency of GR Palmer amaranth in the state highlights the importance of using multiple modes of action for weed management practices, as the majority of the corn fields in western Nebraska had glyphosate-susceptible Palmer amaranth biotypes and were likely treated with multiple effective modes of action. The recurrent complaints regarding Palmer amaranth glyphosate control in the state were likely associated with delayed applications and the extended germination window of the species. Furthermore, the presence of GR Palmer amaranth populations in areas with common waterhemp presence, mainly in southern Nebraska, may indicate the potential risk of glyphosate resistance dissemination to Palmer amaranth populations in western Nebraska through pollen-mediated gene flow, although this hypothesis needs to be further tested.

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