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Distribution and validation of genotypic and phenotypic glyphosate and PPO-nhibitor resistance in Palmer amaranth (*Amaranthus palmeri*) from southwestern Nebraska

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Abstract

Failure to control Palmer amaranth with glyphosate and protoporphyrinogen IX oxidase (PPO)-inhibitor herbicides was reported across southwestern Nebraska in 2017. The objectives of this study were to 1) confirm and 2) validate glyphosate and PPO-inhibitor (fomesafen and lactofen) resistance in 51 Palmer amaranth accessions from southwestern Nebraska using genotypic and whole-plant phenotypic assay correlations and cluster analysis, and 3) determine which agronomic practices might be influencing glyphosate resistance in Palmer amaranth accessions in that location. Based on genotypic assay, 88% of 51 accessions contained at least one individual with amplification (>2 copies) of the 5-enolypyruvyl-shikimate-3-phosphate synthase (*EPSPS*) gene, which confers glyphosate resistance; and/or a mutation in the *PPX2* gene, either ΔG210 or R128G, which endows PPO-inhibitor resistance in Palmer amaranth. Cluster analysis and high correlation (0.83) between genotypic and phenotypic assays demonstrated that *EPSPS* gene amplification is the main glyphosate resistance mechanism in Palmer amaranth accessions from southwestern Nebraska. In contrast, there was poor association between genotypic and phenotypic responses for PPO-inhibitor resistance, which was attributed to segregation for PPO-inhibitor resistance within these accessions and/or the methodology that was adopted herein. Genotypic assays can expedite the process of confirming known glyphosate and PPO-inhibitor resistance mechanisms in Palmer amaranth from southwestern Nebraska and other locations. Phenotypic assays are also a robust method for confirming glyphosate resistance but not necessarily PPO-inhibitor resistance in Palmer amaranth. Moreover, random forest analysis of glyphosate resistance in Palmer amaranth indicated that *EPSPS* gene amplification, county, and current and previous crops are the main factors influencing glyphosate resistance within that geographic area. Most glyphosate-susceptible Palmer amaranth accessions were found in a few counties in areas with high crop diversity. Results presented here confirm the spread of glyphosate resistance and PPO-inhibitor resistance in Palmer amaranth accessions from southwestern Nebraska and demonstrate that less diverse cropping systems are an important driver of herbicide resistance evolution in Palmer amaranth.

Introduction

Palmer amaranth is indigenous to the southwestern United States and northern Mexico (Sauer 1957). Despite being previously described as an edible plant (Smith 1900), Palmer amaranth has long been documented as a serious weed problem in U.S. cropping systems (Hamilton and Arle 1958). Human-driven selection has strongly contributed to the rise of Palmer amaranth as a problematic weed. In the 1970s, when cotton (*Gossypium hirsutum* L.) picking became mechanized, machinery contributed to the spread of Palmer amaranth seeds across the southern United States (Sauer 1972). At that time, Palmer amaranth was considered the most successful weed of all dioecious *Amaranthus* species as it continued to spread across cotton fields (Sauer 1972). The spread of Palmer amaranth was facilitated by increased equipment movement across U.S. regions. In addition, the diversity of crops at the landscape level has decreased throughout the last century (Hiller et al. 2009). Modern agriculture in the United States is composed of a few dominant row crops planted in rotations, and Palmer amaranth has shown an extraordinary

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ability to infest such crops (Ward et al. 2013). Moreover, conservation agriculture is widely adopted in U.S. cropping systems, and Palmer amaranth tends to thrive in no-till fields due to its small seed size, which contributes to the rapid increase of infestations in crops (Ward et al. 2013). Currently, Palmer amaranth is the most economically damaging weed species infesting corn (*Zea mays* L.), cotton, and soybean (*Glycine max* L. Merr.) fields in the southern United States (Price et al. 2011; Ward et al. 2013).

The economic importance of Palmer amaranth is primarily related to its ability to evolve resistance to herbicides. The presence of herbicide-resistant Palmer amaranth in row crops leads to increased control costs (Ward et al. 2013). The history of herbicide resistance evolution in Palmer amaranth is a reflect of intense reliance and selection pressure from herbicides over time. In the 1990s, the first documented cases of herbicide resistance were against microtubule-inhibitor (Gossett et al. 1992), acetolactate synthase (ALS)-inhibitor (Horak and Peterson 1995), and photosystem II (PSII)-inhibitor herbicides (Heap 2020). After the introduction of glyphosate-resistant (GR) crops, weed management strategies shifted from the use of multiple herbicide sites of action (SOA) in a single season to reliance on single SOA POST herbicide (e.g., glyphosate) within and across growing seasons (Powles 2008). POST applications of glyphosate became widely used for weed management in soybean, cotton, and corn production, resulting in rapid evolution of GR Palmer amaranth (Culpepper et al. 2006). The spread of GR Palmer amaranth led to a reevaluation of the use of glyphosate as a sole means of weed control, and a push to diversify weed management strategies (e.g., additional herbicide SOA). The use of 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor, PPO-inhibitor, and long-chain fatty acid elongase–inhibitor herbicides increased in an attempt to manage GR Palmer amaranth. However, Palmer amaranth has also evolved resistance to these herbicide SOA (Heap 2020). New technologies such as auxin-resistant crops may be jeopardized by the newest reports of 2,4-D-resistant Palmer amaranth (Kumar et al. 2019) and the number of accessions with resistance to multiple herbicide SOA is also on the rise (Schwartz-Lazaro et al. 2017). Therefore, Palmer amaranth herbicide resistance evolution is rapidly limiting the chemical control options for weed management in corn, soybean, and cotton fields within U.S. cropping systems.

Thus far, Palmer amaranth has evolved resistance to eight herbicide SOA (Heap 2020), which is a major concern because weed management in conventional U.S. cropping systems is largely herbicide dependent. Factors relating to the intrinsic biology of Palmer amaranth have also contributed to its fast herbicide resistance evolution (Ward et al. 2013). Palmer amaranth grows up to 2 m tall with many lateral branches and produces thousands of seeds (Sauer 1955), making it a very competitive species with crops (Massinga et al. 2003; Morgan et al. 2001). Moreover, Palmer amaranth reproduces via obligate cross pollination, which increases the chances of herbicide-resistance alleles transferring via gene flow within and across populations (Gaines et al. 2012; Oliveira et al. 2018). The spread of GR Palmer amaranth across the southern and midwestern United States is occuring through both independent herbicide selection (Küpper et al. 2018) and seed dispersal (Farmer et al. 2017; Norsworthy et al. 2014). The recent migration of Palmer amaranth into the midwestern United States poses a serious threat to the sustainability of crop production in the region (Chahal et al. 2017; Kohrt et al. 2017). Palmer amaranth is now overlapping territory with another problematic dioecious *Amaranthus* species, waterhemp (*Amaranthus tuberculatus* syn. *rudis*; Oliveira et al. 2018). Therefore, the monitoring of Palmer amaranth infestations and diagnosis of herbicide resistance is extremely important to agricultural stakeholders.

Recent advances in high-throughput genome sequencing methods are expediting the elucidation and detection of herbicide resistance mechanisms in Palmer amaranth and other weed species. For glyphosate, the most common resistance mechanism in Palmer amaranth is *EPSPS* gene amplification (Gaines et al. 2011, 2019), whereas for PPO-inhibitor resistance, the major resistance mechanism is the *PPO2* glycine 210 deletion (ΔG210; Salas et al. 2016; Salas-Perez et al. 2017). Nevertheless, novel herbicide resistance mechanisms in Palmer amaranth are still being uncovered (Gaines et al. 2019), as evidenced by the recent documentation of two mutations in the *PPO2* enzyme in the R128 site of Palmer amaranth (Giacomini et al. 2017), and G399A, an amino acid substitution of glycine to alanine in the catalytic domain of *PPO2* at position 399 (Rangani et al. 2019). A few reports of nontarget-site resistance (NTSR) to glyphosate have been confirmed in Palmer amaranth (Dominguez-Valenzuela et al. 2017; Palma-Bautista et al. 2019) and one NTSR has been confirmed for PPO-inhibitor herbicides (Varanasi et al. 2018a). Detecting NTSR mechanisms in weed species is challenging because multiple genes can endow resistance (Ghanizadeh and Harrington 2017). Therefore, using genotypic assays might provide faster detection of known herbicide resistance mechanisms in Palmer amaranth, but genotypic assays fail to address novel resistance mechanisms, including metabolic resistance involving cytochrome P450 genes as well as unknown target-site resistance (TSR) mechanisms.

In Nebraska, corn and soybean growers strongly rely on glyphosate and PPO-inhibitor (e.g., fomesafen and lactofen) herbicides for weed management (Sarangi and Jhala 2018). In autumn 2017, growers in the southwest part of the state reported failure to control Palmer amaranth with glyphosate and PPO-inhibitor herbicides (R Werle, personal communication). Different strategies have been used for herbicide resistance confirmation, including herbicide application on suspected resistant plants under field conditions, harvesting suspected resistant plant seeds to conduct whole-plant and seed bioassays under control conditions (Burgos 2015), and/or collecting plant leaf tissue to assess herbicide resistance through biochemical and molecular techniques (Dayan et al. 2015; Délye et al. 2015). However, herbicide resistance confirmation can be labor-intensive, and growers typically hope for rapid screening results to make appropriate weed management decisions in the upcoming growing season. Therefore, the objectives of this study were to 1) confirm glyphosate and PPO-inhibitor resistance in 51 Palmer amaranth accessions from southwestern Nebraska via genotypic resistance assays, 2) validate the genotypic assay results using whole-plant greenhouse phenotypic assays of progenies in the same accession via correlation and cluster analysis, and 3) evaluate agronomic practices that may contribute to glyphosate resistance in Palmer amaranth accessions.

Materials and Methods

Plant Material and Growing Conditions

The study was performed with 51 arbitrarily selected Palmer amaranth accessions infesting crops across southwestern Nebraska. Each accession was collected from a single field. Location, agronomic practices, Palmer amaranth distribution, and density of each accession were recorded (Table 1). In August 2017, green leaf tissues were harvested from 5 random plants (parent) from each of

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Table 1. Agronomic and demographic information of Palmer amaranth accessions from southwestern Nebraska evaluated in this study.

Accession	County	Current crop	Previous crop[a]	Tillage	Irrigation	Weed distribution	Weed density[b]
Cha 1	Chase	Sorghum	Corn	Tilled	Rainfed	Widespread	Low
Cha 2	Chase	Corn	Wheat	Strip-till	Center pivot	Widespread	High
Cha 3	Chase	Corn	Fallow/Cornstalks	No-till	Center-pivot	Widespread	High
Cha 4	Chase	Soybeans	Fallow/Cornstalks	No-till	Center-pivot	Widespread	Low
Cha 5	Chase	Corn	Corn	Strip-till	Rainfed	Widespread	Low
Dun 1	Dundy	Wheatstubble	Other	Tilled	Rainfed	Widespread	Intermediate
Dun 2	Dundy	Corn	Sorghum	No-till	Rainfed	Widespread	Intermediate
Dun 3	Dundy	Other		Tilled	Center-pivot	Widespread	Intermediate
Dun 4	Dundy	Corn	Corn	No-till	Center-pivot	Edges	High
Dun 5	Dundy	Soybeans	Corn	Tilled	Center-pivot	Widespread	Low
Fro 1	Frontier	Corn	Sorghum	No-till	Rainfed	Edges	High
Fro 2	Frontier	Soybeans	Corn	Tilled	Rainfed	Edges	Low
Fro 3	Frontier	Soybeans	Wheat stubble	Tilled	Center-pivot	Widespread	High
Fro 4	Frontier	Sorghum	Fallow/Cornstalks	Tilled	Rainfed	Edges	Intermediate
Fro 5	Frontier	Soybeans	Corn	Tilled	Center-pivot	Edges	High
Hay 1	Hayes	Sorghum	Fallow/Cornstalks	Tilled	Center-pivot	Widespread	Intermediate
Hay 2	Hayes	Corn	Wheat stubble	No-till	Rainfed	Widespread	Intermediate
Hay 3	Hayes	Sorghum	Wheat stubble	Tilled	Center-pivot	Widespread	High
Hay 4	Hayes	Corn	Wheat stubble	No-till	Rainfed	Edges	Intermediate
Hay 5	Hayes	Sorghum	Wheat stubble	No-till	Rainfed	Widespread	High
Hit 1	Hitchcock	Corn	Fallow/Cornstalks	Tilled	Center-pivot	Edges	Low
Hit 2	Hitchcock	Soybeans	Corn	No-till	Rainfed	Widespread	Low
Hit 3	Hitchcock	Corn	Corn	No-till	Rainfed	Edges	High
Hit 4	Hitchcock	Sorghum	Wheat stubble	No-till	Rainfed	Edges	High
Hit 5	Hitchcock	Soybeans	Corn	No-till	Center-pivot	Edges	High
Kei 1	Keith	Other	Fallow/Cornstalks	Tilled	Center-pivot	Widespread	High
Kei 2	Keith	Corn	Fallow/Cornstalks	No-till	Center-pivot	Widespread	Intermediate
Kei 3	Keith	Soybeans		Tilled	Furrow	Widespread	High
Kei 4	Keith	Soybeans		No-till	Center-pivot	Widespread	Low
Kei 5	Keith	Other	Corn	Tilled	Center-pivot	Widespread	Low
Kei 6	Keith	Soybeans		No-till	Center-pivot	Widespread	High
Lin 1	Lincoln	Corn	Other	No-till	Center-pivot	Widespread	High
Lin 2	Lincoln	Soybeans	Corn	Tilled	Center-pivot	Widespread	Low
Lin 3	Lincoln	Soybeans		Tilled	Center-pivot	Widespread	Low
Lin 4	Lincoln	Corn		Tilled	Furrow	Widespread	High
Lin 5	Lincoln	Corn	Wheat stubble	No-till	Rainfed	Widespread	High
Log 1	Logan	Soybeans	Fallow/Cornstalks	Tilled	Center-pivot	Edges	Intermediate
Log 2	Logan	Other	Fallow/Cornstalks	No-till	Rainfed	Widespread	Intermediate
Log 3	Logan	Soybeans	Corn	Tilled	Center-pivot	Edges	High
Log 4	Logan	Soybeans	Corn	Tilled	Rainfed	Widespread	Low
Per 1	Perkins	Other	Sorghum	No-till	Rainfed	Widespread	Low
Per 2	Perkins	Soybeans	Corn	Strip-till	Center-pivot	Widespread	Intermediate
Per 3	Perkins	Fallow/Cornstalks	Corn	Tilled	Rainfed	Widespread	High
Per 4	Perkins	Soybeans	Corn	No-till	Center-pivot	Widespread	High
Per 5	Perkins	Other	Fallow/Cornstalks	No-till	Center-pivot	Widespread	Intermediate
Per 6	Perkins	Other	Fallow/Cornstalks	No-till	Center-pivot	Widespread	High
Red 1	Red Willow	Soybeans	Corn	No-till	Center-pivot	Edges	High
Red 2	Red Willow	Corn	Corn	Tilled	Center-pivot	Edges	High
Red 3	Red Willow	Wheat stubble	Wheat	No-till	Rainfed	Widespread	Intermediate
Red 4	Red Willow	Corn	Corn	No-till	Rainfed	Widespread	Low
Red 5	Red Willow	Fallow/Cornstalks	Corn	No-till	Rainfed	Widespread	High

[a]Empty cells in the "Previous crop" column indicate unidentified crops; "Other" indicate alfalfa, dry beans, or field peas.
[b]Low: <3 plants m^{-2}; Intermediate: 3–10 plants m^{-2}; High: >10 plants m^{-2}.

the 51 Palmer amaranth accessions, then labeled and stored at −80 C to be used in genotypic assays. Within the 51 Palmer amaranth accessions, a second sample of 19 arbitrarily selected accessions was obtained by collecting seeds (progeny) of 30 random plants from each accession in September 2017, then cleaned, and stored at 5 C until the onset of the whole-plant phenotypic assay. Seeds were planted in 900 cm^3 plastic trays containing potting-mix (Pro-Mix®, HP Mycorrhizae, Premier Tech Horticulture, Delson, QC, Canada). Emerged seedlings (1 cm) were transplanted into 164 cm^3 containers (Ray Leach "Cone-tainer" SC10®, Stuewe and Sons Inc, Tangent, OR). Palmer amaranth plants were supplied with adequate water and kept under greenhouse conditions at 28/20 C day/night temperature with 80% relative humidity. Artificial lighting was provided using metal halide lamps (600 mmol m^{-2} s^{-1}) to ensure a 15-h photoperiod.

Genotypic Herbicide Resistance Mechanisms Assays

Following the standard methodology from the University of Illinois Plant Clinic, three to five leaf tissue samples were collected from each of the 51 Palmer amaranth (parent) accessions collected from southwestern Nebraska. Genomic DNA extraction from leaf tissue samples were performed using a modified CTAB method (Doyle and Doyle 1987). DNA quality and quantity were checked on a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA) and any samples with low DNA yields or high

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protein-to-DNA ratios were discarded and reextracted. The *EPSPS* copy number (gene amplification) was estimated for each plant based on DNA extracted from tissue from a single leaf of Palmer amaranth. Samples were tested for glyphosate resistance via increased numbers of *EPSPS* genomic copies using a SYBR quantitative polymerase chain reaction (qPCR) approach (Chatham et al. 2015) in which *EPSPS* copy numbers were estimated based on comparison with a single-copy reference gene (*CPS*, for carbamoyl phosphate synthetase). Although the assay was originally developed for waterhemp, the same primers and assay conditions were confirmed to be suitable for Palmer amaranth (Nakka et al. 2017). The EPSPS primers are in a region of the gene that is highly conserved between waterhemp and Palmer amaranth. *Taq*Man qPCR assays were used to assess for the presence of two known PPO-inhibitor resistance mutations in the *PPO2* enzyme, including the glycine 210 deletion (Wuerffel et al. 2015) and the R128 glycine (R128G) and/or R128 methionine (R128M) mutations (Varanasi et al. 2018b).

Accessions with individuals containing more than two *EPSPS* copy numbers were considered GR, and individuals with the presence of ΔG210 or R128G/R128M mutations were considered PPO-inhibitor resistant. Other TSR and NTSR mechanisms were not tested.

Whole-Plant Phenotypic Assay of Progenies

This aspect of the research was conducted under greenhouse conditions in 2018 and 2019 at the University of Wisconsin-Madison to evaluate the sensitivity of 19 Palmer amaranth (progeny) accessions from southwestern Nebraska to glyphosate and PPO-inhibitor herbicides.

The experiments were conducted in a complete randomized design and the experimental unit was a 164 cm^3 cone-tainer with a single Palmer amaranth seedling. The study was arranged in a factorial design with Palmer amaranth progenies from 19 accessions and 3 herbicides with 20 replications and conducted twice (two experimental runs). Altogether, 2,280 Palmer amaranth seedlings were screened in the phenotypic assay. The arbitrarily selected 19 Palmer amaranth progenies were from Cha 3, Dun 3, Dun 4, Dun 5, Hay 1, Hay 3, Hay 4, Kei 2, Kei 3, Kei 5, Kei 6, Log 1, Log 2, Log 4, Per 2, Per 4, Red 2, Red 4, and Red 5 accessions (Table 1). Herbicides that were applied included glyphosate (Roundup PowerMAX®, Bayer Crop Science, Saint Louis, MO) at 870 g ae ha^{-1} plus 2,040 g ha^{-1} ammonium sulfate (DSM Chemicals North America Inc., Augusta, GA); fomesafen (Flexstar®, Syngenta Crop Protection, Greensboro, NC) at 226 g ai ha^{-1} plus 0.5 L ha^{-1} of nonionic surfactant (Induce®, Helena Agri-Enterprises, Collierville, TN); and lactofen (Cobra®, Valent US LLC Agricultural Products, Walnut Creek, CA) at 219 g ai ha^{-1} plus 0.5 L ha^{-1} of nonionic surfactant.

Herbicide treatments were applied to 8- to 10-cm-tall Palmer amaranth plants with a single-nozzle chamber sprayer (DeVries Manufacturing Corp., Hollandale, MN). The sprayer had an 8001 E nozzle (Spraying Systems Co., Wheaton, IL) calibrated to deliver 140 L ha^{-1} spray volume at 135 kPa at a speed of 2.3 km h^{-1}. Palmer amaranth accessions were visually assessed 21 d after treatment (DAT) as dead or alive. Plants within each accession-herbicide treatment were considered alive when prominent green tissue was observed in growing plants, whereas completely necrotic plants were considered dead.

Statistical Analyses

The statistical analyses presented herein were performed using R statistical software version 4.0.0 (R Core Team 2020).

Genotypic and Phenotypic Validation of Glyphosate and PPO-Inhibitor Resistance in Palmer Amaranth

The number of GR or PPO-inhibitor resistant Palmer amaranth individuals (parent plants) in the genotypic assays was converted to a percentage scale:

$$G = \frac{S}{T} * 100 \quad [1]$$

where G represents the percent of GR or PPO-inhibitor-resistant Palmer amaranth individuals, S is the total number of Palmer amaranth individuals that tested positive for genotypic herbicide resistance, and T is the total number of Palmer amaranth individuals (n = 3 to 5) screened for herbicide resistance in the genotypic assays. Fomesafen and lactofen are PPO-inhibitor herbicides; thus, G is same for both herbicides.

The number of surviving individuals in the phenotypic assay of Palmer amaranth progeny individuals were converted into a percentage scale:

$$P = \frac{X}{T} * 100 \quad [2]$$

where P represents the percent of surviving Palmer amaranth individuals after herbicide treatment in the phenotypic assay (glyphosate, fomesafen, or lactofen), X is the total number surviving Palmer amaranth individuals 21 DAT, and T is the total number of Palmer amaranth individuals (n = 40) treated with each herbicide. P (%) was determined only for the 19 accessions that were screened. Data from two experimental runs were combined.

The G and P validation was performed with the 19 Palmer amaranth accessions treated with herbicide in the phenotypic assay (using progeny plants) as well as their respective genotypic (parent plants) assay results. We were interested to learn whether parental genotype within accessions correlated to their respective progeny phenotype. The correlation between G and P for each herbicide (glyphosate, fomesafen, and lactofen) and between the two PPO-inhibitor herbicides (fomesafen and lactofen) were performed with Pearson's analysis using the built-in *cor.test* function in R. The correlation value varies from −1 to 1, where 1 is the total positive correlation, −1 is the total negative correlation, and 0 indicates no linear correlation. Pearson's analysis tests the null hypothesis that correlation between two variables is equal to zero. If P-value > 0.05, the probability >5% that a correlation of some magnitude between two variables could occur by chance alone assuming null hypothesis is true; thus, there would be no correlation between variables.

Cluster Analyses

A clustering algorithm (k-means) was used to group the data based on G and P similarities of Palmer amaranth accessions studied herein. The k-means algorithm randomly assigns each individual data point to a cluster (Hartigan and Wong 1979). The k-means was performed using the built-in *kmeans* function in R. The number of clusters (k) was performed using the gap statistic method (Tibshirani et al. 2001). The number of k was estimated using *tidy*, *augment*, and *glance* functions from the tidymodels package in R (Kuhn and Wickham 2020). The appropriate number of k for a given dataset is estimated with the lowest total within-cluster sum of squares (W_k), which represents the variance within the

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clusters. The error measure W_k decreases monotonically as k increases, but from some k onward the decrease flattens markedly (Tibshirani et al. 2001). The location at which W_k bends to a plateau indicates the appropriate number of k.

Random Forest Analyses: Classification of Factors Influencing Glyphosate Resistance

Random forest is a powerful, ensembled machine-learning algorithm that generates and combines multiple decision trees in an attempt to obtain a consensus. The random forest procedure is described in detail by Breiman (2001) and by Biau and Scornet (2016). In short, the random forest analysis is largely based on two parameters: *ntree*, which is the number of decision trees; and *mtry*, the number of different predictors tested in each tree. For each decision tree, a subsample of observations from the data is selected with replacement to train the trees (bootstrap aggregating). These "in-bag" samples include approximately 66% of the total data and some observations may be repeated in each new training data set because this sampling occurs with replacement. The remaining 33% of the data are designated "out-of-bag" or OOB samples and are used in an internal cross-validation technique to estimate the model performance error. To evaluate the importance of an explanatory variable (or predictor), the random forest measures both the decrease in model performance accuracy as calculated by the OOB error and the decrease in the Gini index value. The Gini index value (mean decrease in accuracy) is the mean of a total variable decrease of a node impurity, weighted by the proportion of samples reaching that node in each individual decision tree. Therefore, variables with a large Gini index value indicates higher variable importance, and are more important for data classification. Random forest has been used to described the incidence of crop disease (Langemeier et al. 2016) and glyphosate resistance in *Amaranthus* spp. (Vieira et al. 2018).

The random forest analysis was conducted using genotypic results of 51 Palmer amaranth accessions (Table 1). The random forest was performed with the *randomForest* package in R software to describe the influence of *EPSPS* gene amplification (genotypic results), PPO-inhibitor resistance (genotypic results), location (county), agronomic practices (e.g., tillage, irrigation, current and previous cropping systems), and weed demographics (e.g., density and distribution) on glyphosate resistance in Palmer amaranth in southwestern Nebraska (Table 1). *EPSPS* gene copy number (genotypic results) was included as an explanatory variable to test the robustness of random forest because it is known to highly correlate with glyphosate resistance in Palmer amaranth (Gaines et al. 2019). For this analysis, the *ntree* parameter was set to 500, whereas *mtry* was set to 2 (default values).

Results and Discussion

Genotypic Confirmation and Phenotypic Validation of EPSPS- and PPO-Inhibitor Resistance in Palmer Amaranth

The individuals screened in the genotypic and phenotypic assays represent parents and their progeny, respectively. This methodology was chosen to simulate a real-farm scenario in which growers collect leaf samples from suspected herbicide-resistant accessions and mail them to the University of Illinois Urbana-Champaign Plant Clinic for molecular herbicide resistance confirmation; or to represent a situation in which a suspected herbicide-resistant seed sample is mailed to a state university weed science program for herbicide resistance confirmation through whole-plant bioassays. We were interested in the correlation and clustering analyses of these two approaches.

Table 2. Palmer amaranth accessions from southwestern Nebraska with *EPSPS* gene amplification and/or PPO resistance according to genotypic resistance assays in parent individuals.

	EPSPS gene amplification (No. of copies)				PPO resistance[a]		
Accession	Mean	Max.	Min.	% EPSPS resistant plants	Mutation	% PPO resistant plants	No. of Plants[b]
Cha 1	7	23	1	25		0	4
Cha 2	1	3	1	20		0	5
Cha 3	9	15	1	80		0	5
Cha 4	10	26	1	40	R128G	20	5
Cha 5	1	1	1	0	ΔG210	33	3
Dun 1	5	18	1	60	ΔG210	20	5
Dun 2	1	1	1	0	ΔG210	33	3
Dun 3	1	1	1	0	ΔG210	67	3
Dun 4	6	10	4	100		0	5
Dun 5	24	51	4	100	ΔG210	20	5
Fro 1	6	10	3	100	ΔG210	33	3
Fro 2	3	6	1	33	ΔG210	100	3
Fro 3	5	11	1	33	ΔG210	67	3
Fro 4	1	1	1	0	ΔG210	100	3
Fro 5	1	2	1	0		0	5
Hay 1	1	1	1	0	ΔG210	100	3
Hay 2	2	3	1	33	ΔG210	33	3
Hay 3	2	2	1	0	ΔG210	100	3
Hay 4	1	1	1	0	ΔG210	67	3
Hay 5	1	1	1	0		0	5
Hit 1	5	20	1	20		0	5
Hit 2	21	57	3	67		0	3
Hit 3	3	6	1	33	ΔG210	33	3
Hit 4	2	3	1	25		0	4
Hit 5	1	1	1	0	ΔG210	33	3
Kei 1	13	38	1	33	ΔG210	33	3
Kei 2	12	19	7	100	ΔG210	33	3
Kei 3	1	1	1	0		0	5
Kei 4	8	18	1	60		0	5
Kei 5	5	8	1	67	ΔG210	67	3
Kei 6	17	40	2	80		0	5
Lin 1	1	2	1	0	ΔG210	100	3
Lin 2	5	6	3	100	ΔG210	67	3
Lin 3	4	6	1	67	ΔG210	100	3
Lin 4	3	6	1	33	ΔG210	100	3
Lin 5	1	1	1	0		0	5
Log 1	34	57	1	67	ΔG210	33	3
Log 2	1	1	1	0		0	3
Log 3	4	7	1	67	ΔG210	33	3
Log 4	3	6	1	67	ΔG210	67	3
Per 1	1	1	1	0	ΔG210	33	3
Per 2	32	59	1	80		0	5
Per 3	1	1	1	0	ΔG210	33	3
Per 4	10	22	1	67		0	3
Per 5	1	2	1	0	ΔG210	33	3
Per 6	1	2	1	0	ΔG210	33	3
Red 1	2	3	1	33	ΔG210	33	3
Red 2	2	3	1	33	ΔG210	67	3
Red 3	2	6	1	20	R128G	20	5
Red 4	2	5	1	33	ΔG210	67	3
Red 5	1	2	1	0		0	5

[a]Empty cells in the "PPO resistance" column indicate no PPO-inhibitor resistance mutation detected.
[b]Number of plants screened in the genotypic herbicide resistance assay.

Glyphosate Resistance

Increased *EPSPS* copy number was detected in 63% of the 51 Palmer amaranth accessions analyzed (Table 2). Based on *EPSPS* gene amplification, our study showed that 10% of Palmer

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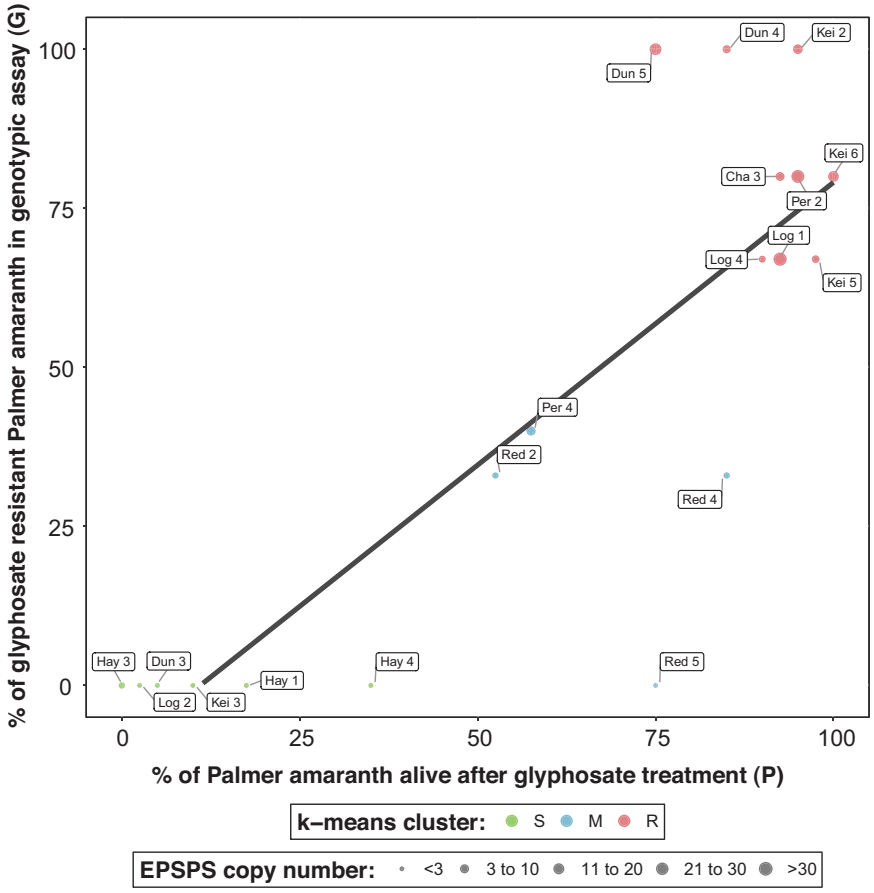

Figure 1. Validation between glyphosate resistance via genotypic (*EPSPS* gene amplification in parent) and phenotypic (glyphosate treatment in progeny) assays in Palmer amaranth accessions from southwestern Nebraska. Color-coded dots indicate three clusters for glyphosate resistance: susceptible (S), moderately resistant/susceptible (M), and resistant (R). Size-coded dots to represent the average *EPSPS* copy number for each Palmer amaranth accession.

Table 3. Correlation estimates between Palmer amaranth genotypic (parent) and phenotypic (progeny) results toglyphosate, fomesafen, and lactofen, and between phenotypic fomesafen and phenotypic lactofen results (PPO inhibitors).[a]

Herbicide	Correlation variables	Estimate	Lower CI	Upper CI	*t*-test value	P value
Glyphosate	*G* and *P*	0.83	0.60	0.93	6.15	0.0000
Fomesafen	*G* and *P*	0.52	0.09	0.79	2.53	0.0217
Lactofen	*G* and *P*	−0.05	−0.49	0.41	−0.20	0.8412
PPO inhibitors	*P*-fomesafen and *P*-lactofen	0.23	−0.25	0.62	0.98	0.3428

[a]Abbreviations: CI, confidence interval; *G*, genotypic (parent); *P*, phenotypic (progeny); PPO, protoporphyrinogen IX oxidase.

amararanth accessions had all individuals resistant to glyphosate, 53% were segregating for resistance, and 37% were susceptible to glyphosate (Table 2). Phenotypic analysis of 19 of these accessions confirmed the genotypic analysis data, in that a positive correlation (0.83; P-value = 0.0000) was observed between *G* and *P* assays (Figure 1 and Table 3). The high correlation between *G* and *P* for glyphosate resistance demonstrates that most Palmer amaranth accessions from southwestern Nebraska are resistant to glyphosate due to *EPSPS* gene amplification. The *EPSPS* gene amplification mechanism is widespread in Palmer amaranth (Gaines et al. 2019; Sammons and Gaines 2014). Gene amplification is an important evolutionary mechanism enabling weeds (Patterson et al. 2018) and other pests (Bass and Field 2011; Remnant et al. 2013) to evolve resistance to pesticides. Palmer amaranth was the first identified weed to evolve glyphosate resistance via *EPSPS* gene amplification (Gaines et al. 2010), followed by kochia (*Bassia scoparia* L. A.J. Scott), waterhemp, Italian ryegrass (*Lolium perenne* L. ssp. *multiflorum*), ripgut brome (*Bromus diandrus* Roth), goosegrass (*Eleusine indica* L.), windmill grass (*Chloris truncata* R. Br.), and smooth pigweed (*Amaranthus hybridus* L.; Patterson et al. 2018; Sammons and Gaines 2014). Other glyphosate resistance mechanisms have also been confirmed in Palmer amaranth, including a *Pro106* mutation in the *EPSPS* gene and reduced glyphosate absorption/translocation (Dominguez-Valenzuela et al. 2017; Palma-Bautista et al. 2019; Sammons and Gaines 2014).

The k-means strongly classified Palmer amaranth into three clusters, herein described as S (susceptible), M (moderately resistant/susceptible), and R (resistant) accessions (Figure 1). Palmer amaranth accessions classified as S showed no *EPSPS* gene amplification in the genotypic assay, but two accessions had individuals that survived glyphosate (870 g ae ha^{-1}) application (>15%) in the

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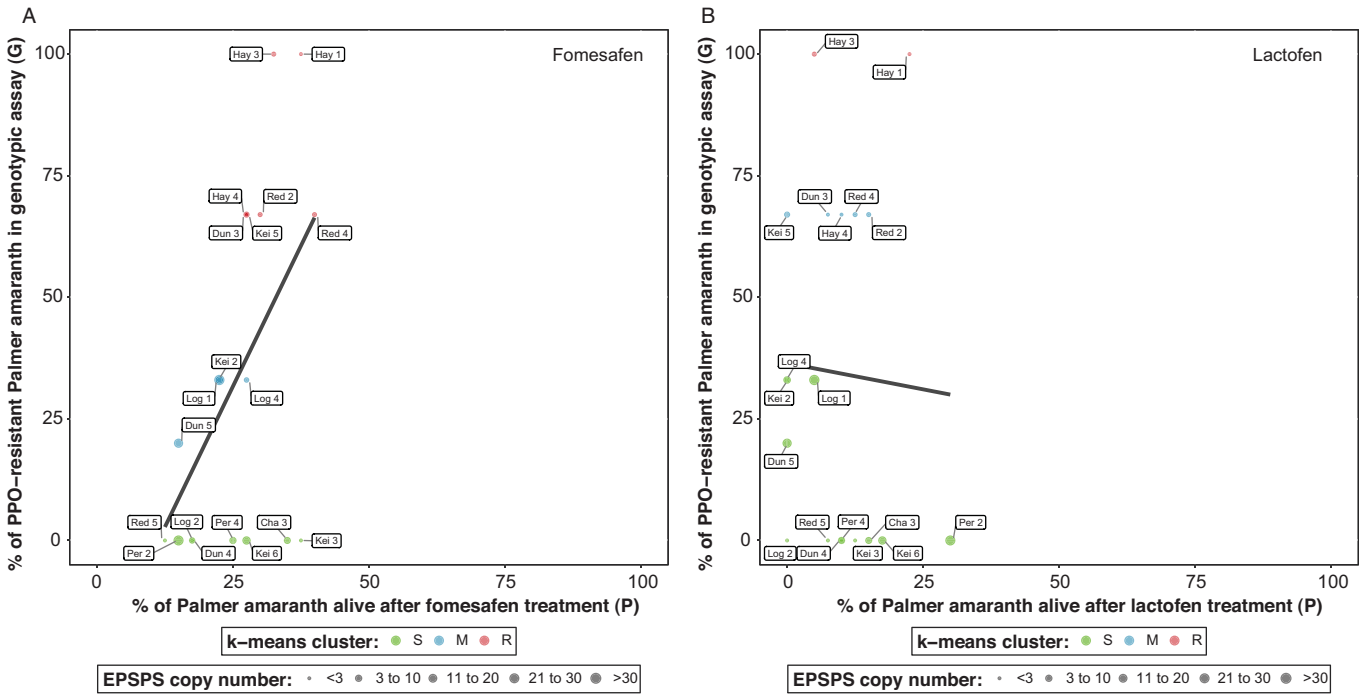

Figure 2. Validation between protoporphyrinogen IX oxidase (PPO) resistance via genotypic (ΔG210 mutation in parent) and phenotypic [fomesafen (A) and lactofen (B) treatment in progeny] assays in Palmer amaranth accessions from southwestern Nebraska. Color-coded dots to indicate three-cluster analysis for PPO-inhibitor resistance: susceptible (S), moderately resistant/susceptible (M), and resistant (R). Size-coded dots represent the average *EPSPS* copy number for each Palmer amaranth accession.

whole-plant phenotypic assay (Figure 1). For example, these two accessions showed low ($P = 18\%$, Hay 1) and moderate ($P = 35\%$, Hay 4) survival after glyphosate treatment in the phenotypic assays but four accessions (Dun 3, Hay 3, Log 2, and Kei 3) tested negative ($G = 0\%$) for glyphosate resistance in the genotypic assay and showed $P \leq 15\%$ in the phenotypic assays. In addition, nine accessions were correctly classified as R, having high G and P values (Figure 1). Two Palmer amaranth accessions were correctly classified as M (Red 2 and Per 4) but not the Red 4 ($P = 80\%$, $G = 33\%$) and the Red 5 accession, which showed high survival ($P = 75\%$) after glyphosate treatment despite having $G = 0\%$. Other glyphosate resistance mechanisms are likely present in accessions investigated herein. It remains unknown whether the Red 5 accession, which has no *EPSPS* gene amplification (G) but high number of progeny surviving glyphosate application (P), harbor additional resistance mechanisms, warranting further investigations.

Our results suggest that genotypic assays represents a robust tool for rapid detection of glyphosate resistance in Palmer amaranth accessions from southwestern Nebraska, and likely other geographic areas. The use of genotypic assays is possible largely due to the widespread occurrence of *EPSPS* gene amplification as the mechanism of glyphosate resistance in Palmer amaranth populations. Research on the molecular basis of *EPSPS* gene amplification in weed species is underway because additional work is needed to unveil this complex adaptative trait (Koo et al. 2018). The genetics of *EPSPS* gene amplification in weed species follows Mendelian inheritance in kochia (Jugulam et al. 2014), and non-Mendelian inheritance patterns in Palmer amaranth (Giacomini et al. 2019) and ripgut brome (Malone et al. 2016). More than 100 *EPSPS* gene copies have been documented in Palmer amaranth, whereas a maximum of 13 have been observed in kochia (Gaines et al. 2016; Kumar et al. 2015; Wiersma et al. 2015). The *EPSPS* gene copy variation in Palmer amaranth is a result of the extrachromosomal circular DNA (eccDNA) being transmitted to the next generation by tethering to mitotic and meiotic chromosomes (Koo et al. 2018), while in kochia, *EPSPS* copies are arranged in tandem repeats at a single locus (Patterson et al. 2018). Segregation for *EPSPS* copy number within Palmer amaranth families (F_1 and F_2) is transgressive, with individuals varying in *EPSPS* gene amplification levels even among clonal plants (Giacomini et al. 2019). Transgressive segregation for *EPSPS* in Palmer amaranth might explain the variable *EPSPS* copy numbers across individuals within accessions screened from southwestern Nebraska (Table 2). Gene amplification coupled with its prolific and dioecious nature are valuable traits for Palmer amaranth that help to increase its genetic complexity and allow it to adapt to current U.S. cropping systems.

PPO-Inhibitor Resistance

The genotypic assays showed nearly 70% of the 51 Palmer amaranth accessions from southwestern Nebraska were confirmed to be resistant to PPO-inhibitor herbicides (Table 2). Nearly 14% of Palmer amaranth accessions had all individuals resistant, 53% were segregating for resistance, and 33% had no mutation. In the phenotypic assays, fomesafen and lactofen treatments resulted in less than 40% survival within each Palmer amaranth accession (Figure 2). Thus, the correlation between G and P for PPO-inhibitor resistance in Palmer amaranth accessions was inconsistent (Table 3). While a higher G and P correlation (0.52; P-value = 0.0217) was observed for fomesafen (Figure 2A), no G and P correlation (−0.05; P-value = 0.84) was found for lactofen (Figure 2B). In addition, there was no correlation (0.23; P-value = 0.34) between fomesafen (P) and lactofen (P) in the phenotypic assay (Table 3). Application of fomesafen (226 g ai ha^{-1}) and lactofen (219 g ai ha^{-1}) provided high mortality ($P < 50\%$) in

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Palmer amaranth accessions from southwestern Nebraska, including accessions wherein 100% of individuals had a ΔG210 deletion (e.g., Hay 1 and Hay 3). Moreover, high Palmer amaranth mortality with PPO-inhibitor herbicides negatively influenced the clustering algorithm of Palmer amaranth as S, M, and R accessions (Figure 2, A and B). The k-means likely classified these accessions based on *G* results only. For example, Palmer amaranth accessions were classified as S, M, and R for fomesafen with $G = 0\%$, $20\% \leq G < 35\%$, and $G \geq 66\%$, respectively; however, *P* of all three clusters varied between 12% and 40% (Figure 2A). A similar trend was observed for lactofen (Figure 2B).

Palmer amaranth accessions Dun 5 (20%), Kei 2 (33%), Kei 5 (67%), and Log 4 (33%) were segregating for PPO-inhibitor resistance in the genotypic assay (*G*, Figure 2B); however, the progeny of these accessions were sensitive to lactofen treatment ($P = 0\%$). In contrast, Palmer amaranth accessions Cha 3, Kei 6, Per 2, and Red 5 tested negative for ΔG210 or R128G mutations ($G = 0\%$) but more than 15% of the individuals survived both fomesafen and lactofen treatment. Also, Palmer amaranth accessions Kei 3, Per 4, and Dun 4 showed 38%, 25%, and 18% survival, respectively, after fomesafen treatment but less than 15% survival after lactofen treatment. It has been shown that a mutated PPO enzyme has reduced affinity for several PPO-inhibitor herbicides in Palmer amaranth (Schwartz-Lazaro et al. 2017); however, it has been difficult to determine Palmer amaranth resistance based on field survival because sensitive plants could tolerate PPO-inhibitor herbicides (Lillie et al. 2020). In addition, PPO-inhibitor herbicide efficacy on PPO-resistant Pamer amaranth control can be influenced by application time (Copeland et al. 2019). Therefore, confirmation of Palmer amaranth resistance to PPO-inhibitor herbicides using phenotypic assays is complex and needs further investigation.

In the 34 PPO-inhibitor-resistant Palmer amaranth accessions tested herein, 32 presented the the ΔG210 in the *PPX2* gene, while the R128G mutation was confirmed in two Palmer amaranth accessions. The phenotypic validation for PPO-inhibitor resistance presented here is limited by the high mortality of Palmer amaranth individuals in the whole-plant assays. This could be explained by 1) the number of individuals sampled for the *G* assay study may have been too low for the objective of validation; 2) the herbicide rate used herein resulting in high individual mortality; 3) greenhouse conditions were ideal and plants faced no environmental stress during and following application of PPO-inhibitor herbicides, which is different for plants under field conditions in southwestern Nebraska; and 4) plant size strongly impacts the level of resistance, with smaller plants being less resistant than larger plants (Coburn 2017). It is likely that Palmer amaranth individuals used herein were smaller than usual because of the small volume of the cone-tainers, which limits root and shoot development. Hence, the whole-plant bioassays failed to confirm resistance to PPO-inhibitor herbicides in Palmer amaranth accessions from southwestern Nebraska, making genotypic assays a necessary step for resistance confirmation.

Random Forest: Classification of Factors Influencing Glyphosate Resistance

The final OOB error rate of the random forest analysis was 13.33%, meaning that >86% of OOB samples were adequately classified by the model. Results showed *EPSPS* gene amplification as the top predictor (Figure 3A). This highlights the robustness of the approach, with GR Palmer amaranth accessions in southwestern Nebraska containing this mechanism of resistance. In 2014, a survey with *Amaranthus* spp. in Nebraska confirmed widespread glyphosate resistance for waterhemp (81%) but not for Palmer amaranth (6%; Vieira et al. 2018). Vieira et al. (2018) demonstrated the spread of waterhemp in eastern Nebraska, and Palmer amaranth in south central Nebraska, which partly overlaps territory with Palmer amaranth accessions surveyed herein (Figure 4). The rapid glyphosate resistance evolution in Palmer amaranth accessions from southwestern Nebraska raised questions about whether resistant accessions were introduced via seed/gene flow or they arose independently. Although we did not test this specific hypothesis, the random forest analysis did shed some light on glyphosate resistance evolution in Palmer amaranth in that part of Nebraska. The random forest analysis ranked (high to low) *EPSPS* gene amplification > county > current crop > previous crop > Palmer amaranth density > tillage > irrigation > Palmer amaranth distribution > PPO-inhibitor resistance as the factors influencing the presence of glyphosate resistance in Palmer amaranth of southwestern Nebraska (Figure 3A).

County was the second most important factor for the presence of GR Palmer amaranth. All counties presented at least one Palmer amaranth individual with *EPSPS* copy number >2. The lowest number of GR accessions was found in Hayes (Hay) and Perkins (Per) counties with one (out of five) and two (out of six), respectively (Figure 3B). County influence on *EPSPS*-inhibitor resistance in Palmer amaranth is likely related to crop diversity as current and previous crops strongly influenced the presence of glyphosate resistance in Palmer amaranth accessions. Five Palmer amaranth accessions (Dun 4, Dun 5, Fro 1, Kei 2, and Lin 2) demonstrated 100% resistance (grouped as glyphosate-resistant) and those accessions were all found in fields where current corn or soybean crops were preceded by corn or sorghum (Figure 3C). The high incidence of Palmer amaranth accessions with 100% resistance to glyphosate in less diverse cropping systems suggests the influence of repeated glyphosate applications. In contrast, *EPSPS* gene amplification was not detected in 19 Palmer amaranth accessions (grouped as glyphosate-susceptible), from which only two accessions were found in corn and soybean rotations (Fro 5 and Hit 5; Table 2). The majority of glyphosate-susceptible Palmer amaranth accessions were found in rotations of corn, sorghum (*Sorghum bicolor* L.), wheat (*Triticum aestivum* L.), fallow, soybean, and other crops (e.g., alfalfa [*Medicago sativa* L], dry bean [*Phaseolus vulgaris* L], and field peas [*Pisum sativum* L]; Figure 3C). Therefore, the occurrence of GR Palmer amaranth accessions is reduced in rotations with more diversified crops, most likely due to crop and herbicide rotations with lower reliance on glyphosate. For example, according to a survey, 2,4-D and metsulfuron-methyl are the most used POST herbicides in grain sorghum and wheat crops, respectively, in Nebraska (Sarangi and Jhala 2018). Crop diversity exerts a different selection pressure on weed communities, including planting, canopy closure timing, and harvest date, which help reduce the dominance of single weed species (Andrade et al. 2017). Despite not having long-term herbicide application records for the areas we sampled from, it has been demonstrated that overreliance on a single or few herbicide SOA in areas with low crop diversity contributed to resistance (Hicks et al. 2018). In addition, it has been shown that herbicide mixture (multiple SOA in one application) is more effective in delaying herbicide weed resistance than herbicide rotation (multiple applications, each with a single SOA; Beckie and Reboud 2009, Evans et al. 2016). However, without herbicide mixtures and

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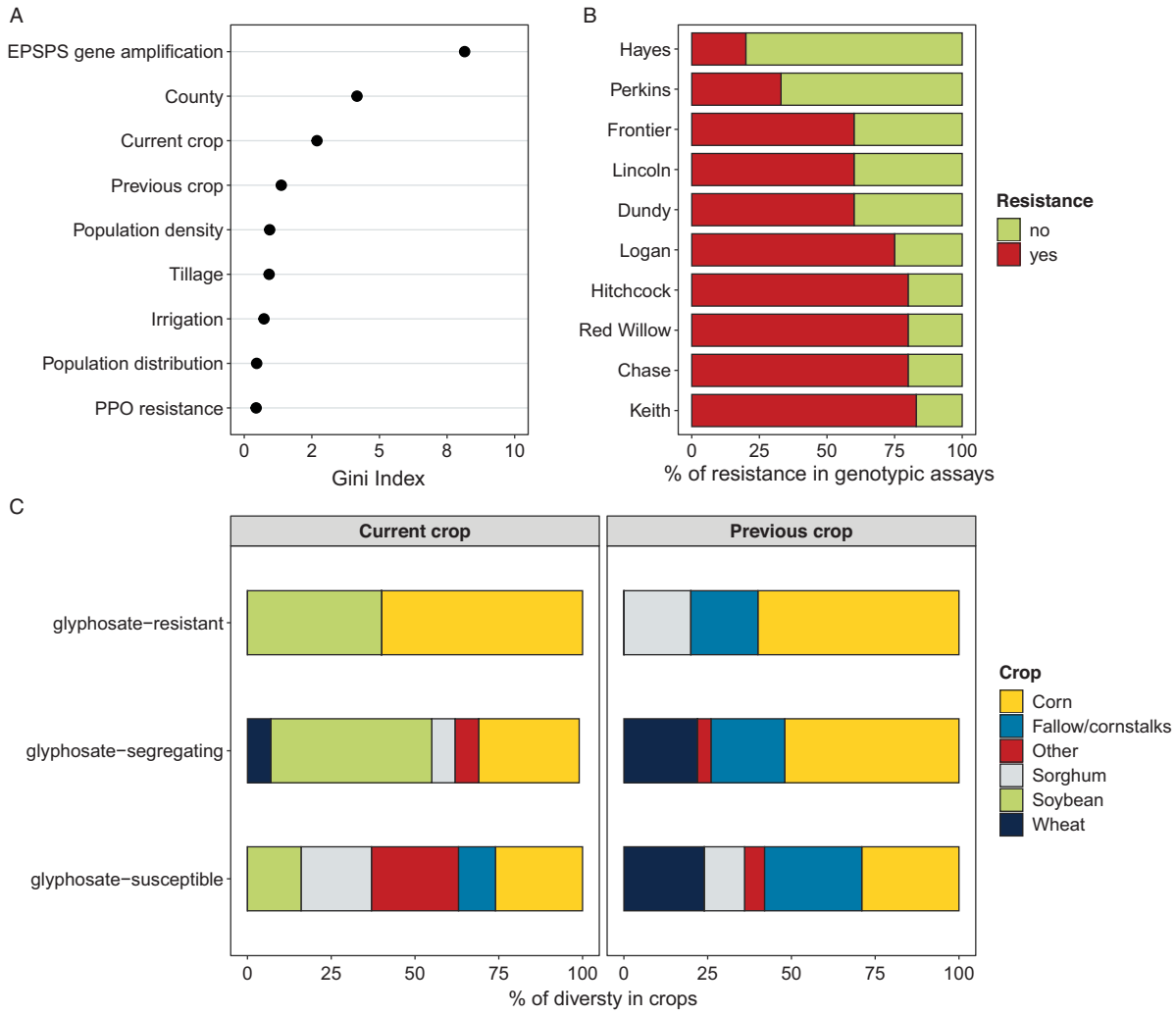

Figure 3. Random forest analysis of likelihood of glyphosate resistance (*EPSPS* gene amplification >2) in parental Palmer amaranth accessions from southwestern Nebraska. Variables are ordered by importance measured using the Gini coefficient (A). Percentage of glyphosate resistance (genotypic assay) in Palmer amaranth across 10 counties in southwestern Nebraska (B). Percentage of diversty in current and previous crop where the Palmer amaranth accessions was detected in southwestern Nebraska. Based on genotypic resistance assay, accessions are grouped into glyphosate-resistant, glyphosate-segregating, and glyphosate-susceptible, representing Palmer amaranth with all resistant, mixture of resistant and susceptible individuals, and all susceptible individuals, respectively. Other crops are represented by alfalfa, dry bean, and field pea (C).

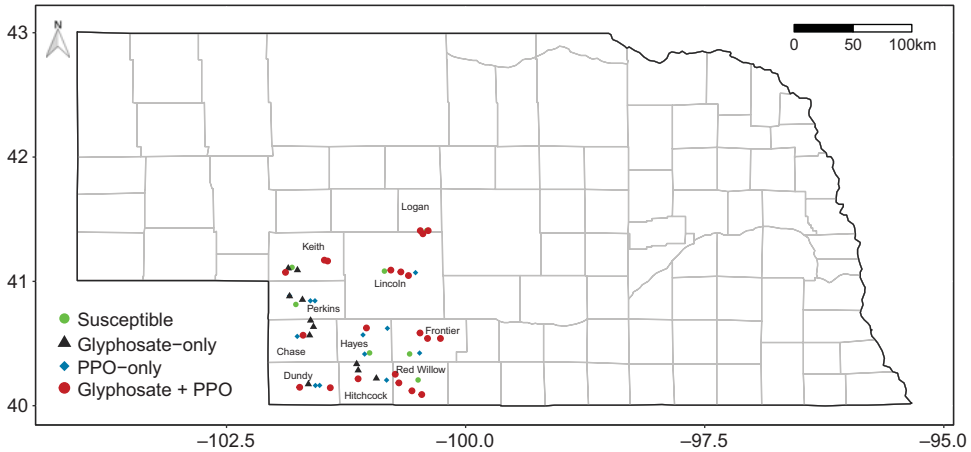

Figure 4. Presence of glyphosate and/or protoporphyrinogen IX oxidase-inhibitor resistance based on genotypic resistance assay in 51 parental Palmer amaranth accessions from southwestern Nebraska. County names are listed within their territory.

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rotations, increased crop diversity alone is not enough to minimize herbicide resistance evolution in weed species.

The random forest analysis suggested that PPO-inhibitor resistance had no influence on glyphosate resistance, indicating that PPO-inhibitor resistance mutations (ΔG210 or R128G) and *EPSPS* gene amplification are not associated. An emerging concern in weed science is the ability of some species to stack genes for multiple herbicide resistance in a single accession. Accessions of Palmer amaranth have been reported to be resistant to HPPD-inhibitor herbicides (Jhala et al. 2014), PSII-inhibitor herbicides (Jhala et al. 2014), and glyphosate herbicides (Chahal et al. 2017) in Nebraska. According to our genotypic assay results, multiple resistance (glyphosate and PPO-inhibitor herbicides) was present in 41% of Palmer amaranth accessions in southwestern Nebraska (Table 2); while 6%, 11%, and 13% of accessions were susceptible to both herbicides, resistant to glyphosate only, and resistant to PPO-inhibitor only, respectively (Figure 4). The 19 Palmer amaranth accessions evaluated in the whole-plant phenotypic assay were also resistant (>80% of individuals within each accession) to an ALS-inhibitor herbicide (imazethapyr at 70 g ai ha^{-1}; data not shown). Thus, it is likely that two- and three-way resistance exists in most Palmer amaranth accessions from southwestern Nebraska.

Practical Implications

Herein we documented the distribution of GR and PPO-inhibitor-resistant Palmer amaranth accessions in southwestern Nebraska (Figure 4). Rapid genotypic assays are important for detection of known mutations to support growers in making future weed management decisions. Glyphosate resistance via *EPSPS* gene amplification was highly correlated to whole-plant phenotypic assay results, mostly due to the spread of *EPSPS* gene amplification as the mechanism of resistance in Palmer amaranth accessions. These result supports the use of either genotypic or phenotypic assays for confirmation of glyphosate resistance in southwestern Nebraska. PPO-inhibitor resistance was also present in several accessions, but phenotypic results were less correlated with the confirmed PPO-inhibitor resistance mutations, showing the complexity of resistance confirmation with whole-plant phenotypic assays, warranting the use of genotypic assays for PPO-inhibitor resistance confirmation. Still, weeds will continue to evolve resistance to herbicides, and whole-plant phenotypic assays are fundamental for detecting populations with novel herbicide resistance mechanisms. For example, the G399A PPO mutation was not known at the time this research was conducted, and may be present in some accessions studied herein. Moreover, the GR Palmer amaranth accessions were found in crops where glyphosate was likely applied, suggesting resistance evolution was mostly due to an over-reliance on glyphosate. Great progress has been made toward understanding the molecular basis of herbicide resistance in Palmer amaranth, but the continuous spread of herbicide resistance to new locations is evident. Thus, increased crop rotation and diversity, rotation of herbicide mixtures, and adoption of innovative nonchemical control strategies are necessary for Nebraska and other geographic locations to minimize selection and spread of herbicide-resistant weed populations.

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