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Abstract

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Author summary

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*Text based on plos sample manuscript, see
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Introduction

Palmer amaranth (*Amaranthus palmeri* S. Watson) is an indigenous plant species from Southern North America [1]. Despite being an edible plant used to feed animals and native Americans [2], Palmer amaranth has long been documented as a serious weed problem in the United States (US) cropping systems [3]. Palmer amaranth produces thousands seeds and grows up to 2 m tall with many lateral branches [4], which made a very competitive species with crops [5,6]. In the 1970s, Palmer amaranth was considered the most sucessful weed of all dioecious *Amaranthus* species as it became widespread in cotton fields of Southern United States, especially when picking was mechanized [7]. Current, Palmer amaranth is the most economically damage weed species infesting corn, cotton and soybean fields of the Southern US [8,9].

The economical importance of Palmer amaranth is related to herbicide resistance. Palmer amaranth showed easily capacity to evolve resistance to herbicides. Factors related to intrinsic biology contributed for fast herbicide resistance evolution in Palmer amaranth [9]. For instance, as an obligate outcrosser species, Palmer amaranth warrant

cross polination, increasing the chances of hybridization and exchange of herbicide resistance alleles amongst *Amaranthus* species [10,11]. In addition, human-driven selection strongly contributed for the rise of Palmer amaranth as a problematic weed. In the US cropping systems, no-till is a standard practice amongst growers. Palmer amaranth thrived in no-till due to its small seed size, contributing for the rapid increase of Palmer amaranth individuals in field crops. Also, herbicide resistance in Palmer amaranth drastically increased when weed management strategy shift from multiple herbicide sites of action (SOA) to reliance on single post emergence herbicide (e.g., glyphosate) [12]. Thus far, Palmer amaranth evolved resistance to eight herbicide SOA [13], which is a concern as weed management in conventional US cropping systems is herbicide dependent.

The history of herbicide resistance evolution in Palmer amaranth is a result of intense selection pressure with herbicides. In the 1990s, it was first documented resistance to mitosis- [14], ALS- [15], and PSII-inhibitor herbicides [13]. With the introduction of glyphosate resistant (GR) crops, post emergence of glyphosate was the most used tool for weed management in soybean, cotton and corn, resulting in evolution of GR-Palmer amaranth [16], especially in cotton fields of Southern US. Palmer amaranth resistance to glyphosate spreaded across the Southern and Great Plains US through independent herbicide selection [17] and/or seed dispersal [18,19]. The spread of GR-Palmer amaranth leaded to rethink the use of glyphosate and to diversify weed management strategies (e.g., other herbicide SOA). The use of 4-hydroxyphenylpyruvate dioxygenase (HPPD)-, protoporphyrinogen oxidase (PPO)- and long chain fatty acids (LCFA)- inhibitor herbicides increased in a attempt to manage GR-Palmer amaranth. However, Palmer amaranth biotypes also evolved resistance to HPPD-, PPO- and LCFA-inhibitor herbicides [13]. New technologies such as auxin-resistant crops may jeopardize with newest report of 2,4-D-resistant Palmer amaranth [13]. Moreover, it is on increase the number of Palmer amaranth biotypes with multiple herbicide resistance [20,21]. Therefore, Palmer amaranth herbicide resistance evolution is shrinking the chemical control options for weed management in corn, soybean and cotton in US cropping systems.

In the US Midwest, corn and soybean growers strongly rely on EPSPS- (e.g., glyphosate) and PPO- (e.g., fomesafen) inhibitor herbicides for weed management. The recent migration of Palmer amaranth into the US Midwest poses a serious threat to the sustainability of crop production in that geography. Palmer amaranth is overlapping territory (parts of US Midwest) with another problematic dioecious *Amaranthus* species, waterhemp (*Amaranthus tuberculatus*). Thus, prevention and/or rapid diagnosis of herbicide resistance in Palmer amaranth has become a priority for agricultural stakeholders. The advances in high throughput genome sequencing methods are expediting the detection of herbicide resistance in Palmer amaranth and other weed species. For instance, glyphosate resistance mechanisms in Palmer amaranth is well studied. Most of Palmer amaranth biotypes have evolved resistance to glyphosate due to gene duplication [22,23]. Also, a *PPO2* glycine 210 (Gly210) deletion accounts for most of PPO resistance in Palmer amaranth [24,25]. Nonetheless, novel herbicide resistance mechanisms in Palmer amaranth are still uncovered [22]. This is the case of recently documented two new mutation in the *PPX2* enzyme in the R98 site of Palmer amaranth [26]. In addition, no Palmer amaranth biotypes with non-target site resistance mechanisms was confirmed for PPO and EPSPS-inhibitor herbicides. Therefore, using molecular assays might provide faster detection of known herbicide weed resistance in Palmer amaranth, but it fails to address herbicide resistance resulting from novel mechanisms.

In the fall of 2017, growers of South-central Nebraska reported failure to control Palmer amaranth with glyphosate and PPO inhibitor herbicides (Werle R, personal

communication), albeit, GR was found in only 6% of Palmer amaranth biotypes in Southwestern Nebraska in 2014 [27]. Documenting herbicide weed resistance with a single method may be difficult due to underestimated herbicide resistance mechanisms. Therefore, the objective of this study was (1) to confirm EPSPS- and PPO-resistance screening 51 Palmer amaranth biotypes from South-central Nebraska and validate using greenhouse and molecular assay methods, and (2) to evaluate agronomic practices that may contribute for EPSPS- and PPO-resistance in Palmer amaranth biotypes. We hypothesized the detection of resistant or susceptible Palmer amaranth populations are alike using greenhouse and molecular assays.

Material and Methods

Plant Material and Growing Conditions

The study was performed with 51 randomly selected Palmer amaranth biotypes infesting cropping system areas across eastern Nebraska (Figure 1). In August 2017, green leaf tissues of actively growing plant were harvest in each of the 51 Palmer amaranth biotypes, labeled and stored at -80 C to be used in molecular assays. Within the 51 Palmer amaranth biotypes, 19 randomly selected biotypes seedheads were harvest in September 2017, cleaned and stored at 5 C until the onset of the greenhouse experiments (Figure 1). Seeds were planted in 900 cm⁻³ plastic trays containing potting-mix (Pro-Mix®, Quakertown, PA, USA). Emerged seedlings (1 cm) were transplanted into 164 cm⁻³ cone-tainers. Palmer amaranth plants were supplied with adequate water and kept under greenhouse conditions at XX/XX C day/night temperature with XX% relative humidity. Artificial lighting was provided using metal halide lamps (600 Xmol m⁻² s⁻¹) to ensure 15 h photoperiod.

Herbicide resistance mechanism assays

Leaf tissue samples from 51 Palmer amaranth biotypes (five plants per population) were stored at -80°C until ready for genomic DNA extraction using a modified CTAB method [28]. DNA quality and quantity were checked on a Nanodrop 1000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and any samples with low DNA yields or high protein:DNA ratios were discarded and re-extracted. Previously described TaqMan qPCR assays were used to check for the presence of known PPO-inhibitor resistance mutations on the *PPX2* enzyme, including the glycine 210 deletion [29] and the R128G/M mutations [30]. Samples were also tested for increased numbers of EPSPS genomic copies using a previously described SYBR qPCR approach [31] in which EPSPS copy numbers were estimated based on comparison with a single-copy reference gene (CPS, carbamoylphosphate synthetase).

Greenhouse Assay

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 biotypes from eastern Nebraska to EPSPS- and PPO-inhibitor herbicides.

The experiments were in a complete randomized design and the experimental unit was a cone-tainer with a single Palmer amaranth seedling. The study was arranged in a factorial design with 19 Palmer amaranth biotypes and three herbicides with 20 replications and repeated twice. The randomly selected 19 Palmer amaranth biotypes were named 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, Red 5 (Table 1). The herbicides were
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glyphosate (Round up PowerMax®, Bayer Crop Science, Saint Louis, MO, USA)
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applied at 870 g ae ha⁻¹ plus 2040 g ha⁻¹ ammonium sulfate (XXX); fomesafen
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(Flextar®, Syngenta Crop Protection, Greensboro, NC, USA) applied at 206 g ai ha⁻¹
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plus 0.5 L ha⁻¹ of non ionic surfactant (Induce®, Helena Agri-Enterprises, Collierville,
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TN, USA); and lactofen (Cobra®, Valent USA LLC Agricultural Products, Walnut
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Creek, CA, USA) applied at 280 g ai ha⁻¹ plus 0.5 L ha⁻¹ of non ionic surfactant.
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Herbicide treatment were applied to 8-10 cm tall Palmer amaranth seedlings with a
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single-tip chamber sprayer (DeVries Manufacturing Corp., Hollandale, MN, USA). The
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sprayer had an 8001 E nozzle (XXXXXXXX) calibrated to deliver 140 L ha⁻¹ spray
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volume at XXX kPa at speed of 2.3 km h⁻¹. Palmer amaranth biotypes were visually
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assessed at 21 days after treatment (DAT) as dead or alive. Plants were considered alive
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when proeminent green tissue was observed in growing plants but dead plants were
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complete necrotic without green tissue.
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Statistical Analysis

Molecular and greenhouse validation of EPSPS and PPO resistance in Palmer amaranth

The number of EPSPS- or PPO-resistant Palmer amaranth individuals in the molecular
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assays was converted into % of resistant compared to the total number of Palmer
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amaranth of each biotype screened for herbicide resistance:
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Equation 1:

$$M = \frac{S}{T} * 100$$

where M is the % EPSPS- or PPO-resistant Palmer amaranth individuals, S is the
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total number of Palmer amaranth individuals positive for herbicide resistance and T is
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the total number of Palmer amaranth individuals screened for herbicide resistance in
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molecular assays. Fomesafen and lactofen are PPO-inhibitor herbicides; thus, M is
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similar for both herbicides. Palmer amaranth individuals with > 2 EPSPS copy number
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were considered EPSPS-resistant.
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The number of alive Palmer amaranth individuals in the greenhouse assay were
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converted into % of alive seedlings compared to the total number of Palmer amaranth
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individuals of each biotype treated with herbicide (glyphosate, fomesafen or lactofen):
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Equation 2:

$$G = \frac{X}{T} * 100$$

Where G is the % alive Palmer amaranth individuals after herbicide treatment in
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greenhouse assay, X is the total number of alive Palmer amaranth individuals 21 DAT
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and T is the total number of Palmer amaranth individuals treated with herbicide. Data
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of two runs are combined due to the EPSPS- and PPO-resistance segregating nature
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Palmer amaranth biotypes used in this study.
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The correlation between R and G for each herbicide (glyphosate, fomesafen and
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lactofen) and between the two PPO inhibitor herbicides, fomesafen and lactofen, were
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performed with Pearson's analysis using *cor.test* function of R statistical software. The
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correlation value varies from -1 and 1, where 1 is the total positive correlation and -1
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the total negative correlation and 0 no linear correlation. The Pearson's analysis test
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the hypothesis of correlation between two variables is not equal to 0. If P -value > 0.05,
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there is no correlation or no significant relationship between two variables (correlation
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equals 0). For meeting the correlation criteria (e.g., two variables), it was used only
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Palmer amaranth biotypes treated with herbicide in the greenhouse assay.
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Random Forest

Random Forest is a powerful ensembling machine learning algorithm which combine multiple generated decision trees. The Random Forest procedure is described in deep by Breiman [32] and Biau [33]. Also, Random Forest has been used to described the incidence of crop disease [34] and glyphosate resistance in *Amaranthus* spp. [27]. In short,

The random forest analysis was performed with *randomForest* package of R statistical software to describe the influence of agronomic practices (e.g., tillage, irrigation, cropping-system) and weed demographics (e.g., density and distribution) on GR Palmer amaranth in South-Central Nebraska.

Results

Molecular and greenhouse validation of EPSPS and PPO resistance in Palmer amaranth

A strong correlation (0.83; P -value=0.00) between molecular (M) and greenhouse (G) assays was found when Palmer amaranth biotypes were tested for EPSPS resistance (Figure 2 and Table 2). Seven Palmer amaranth biotypes tested negative glyphosate resistance ($M=0\%$) in the molecular assay but three biotypes showed low (18%, Hay 1), moderate (35%, Hay 4) and high (75%, Red 5) survival after glyphosate treatment (Figure 2). The other four biotypes (Dun 3, Hay 3, Log 2 and Kei 3) that tested negative ($M=0\%$) for EPSPS resistance in the molecular assay showed less than 15% glyphosate survival (G).

Fomesafen and lactofen provided less than 40% Palmer amaranth survival in the greenhouse assays (Figure 3). The correlation between M and G for PPO resistance in Palmer amaranth biotypes were controversial (Table 2). While a high M and G correlation (0.52; P -value=0.02) was observed for fomesafen (Figure 3A), no M and G correlation (-0.05; P -value=0.84) was found for lactofen (Figure 3B). Palmer amaranth biotypes Dun 5, Kei 2, Kei 5 and Log 4 are segregating for PPO resistance in molecular assay (M) but individuals in these biotypes were sensitive to lactofen treatment ($G=0\%$, Figure 3B). However, these biotypes were less sensitive to fomesafen. For example, nearly 30% of individuals pertaining to Log 4 biotype survived fomesafen treatment (Figure 3A). In contrast, Palmer amaranth biotypes Cha 3, Kei 6, Per 2 and Red 5 tested negative for molecular PPO-resistance ($M=0\%$) but over 15% of biotypes survived both fomesafen or lactofen treatment. Also, Palmer amaranth biotypes Kei 3, Per 4 and Dun 4 showed 38, 25 and 18% survival after fomesafen treatment but less than 15% for lactofen. Correlation between fomesafen and lactofen greenhouse assay (G) did not occur (0.23; P -value=0.34; Figure 4)

Random Forest

Discussion

The high correlation between greenhouse and molecular assays demonstrate that most of GR Palmer amaranth populations of south-central Nebraska is due to EPSPS gene amplification. However, we do not fully accept our hypothesis of GR validation in greenhouse and molecular assays as a Palmer amaranth population (Red 5) showed no EPSPS gene amplification but survived (75%) glyphosate application (870 g ae ha^{-1}). Palmer amaranth was the first identified weed to evolve glyphosate resistance via EPSPS gene amplification [35], followed by kochia (*Kochia scoparia*), waterhemp

(*Amaranthus tuberculatus*), Italian ryegrass (*Lolium multiflorum*) and smooth pigweed (*Amaranthus hybridus*) [36]. The EPSPS gene amplification is the only uncovered GR mechanism in Palmer amaranth [22,36], albeid other GR mechanism has arisen, including Pro106 mutation or reduced glyphosate absorption/translocation [36]. It remains unknown whether the Palmer amaranth populations (Red 5 and Hay 4) have low level resistance to glyphosate (above 1-fold rate) or a novel GR Palmer amaranth mechanisms, which warrant further investigations. A study with kochia showed a positive correlation between the level of glyphosate resistance and number of EPSPS copy number [37]; therefore, supporting the hypothesis of novel GR mechanisms in Palmer amaranth.

The genetic in GR Palmer amaranth fallows a non-Mendelian single gene pattern of inheritance of EPSPS copy number [38,39]. Also, Palmer amaranth showed transgressive segregation for EPSPS copy number, with individuals varying EPSPS amplification levels within clonal plants [40]. The transgressive inheritance segregation in Palmer amaranth might explain the variable EPSPS copy number in individuals within populations screened from south-central Nebraska (Table 2). Moreover, most of Palmer amaranth populations from south-central Nebraska is still segregating for GR. In 2014, a survey with *Amaranthus* spp. in Nebraska showed a widespread GR in waterhemp (81%) but not in Palmer amaranth (6%) [27]. Based on EPSPS molecular assay, our study showed that 10% of Palmer amaranth populations pursuit all individuals resistant to glyphosate, 53% segragating for resistance and 37% susceptible to glyphosate. Therefore, GR Palmer amaranth in on increase on Nebraska cropping systems.

The GR evolution in Palmer amaranth might occur independently or introduced to new locations [17]. The random forest analysis suggested that current and previous crop are the top two factors influencing GR Palmer amaranth in south-central Nebraska.

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Table 1. Demographic list of *Amaranthus palmeri* biotypes with respective Nebraska County location and agronomic practices

Biotype	County	Current crop	Previous crop	Tillage	Irrigation	Weed distribution	Weed density
Cha 1	Chase	sorghum	corn	tilled	rainfed	spread	Low
Cha 2	Chase	corn	wheat	strip-till	center pivot	spread	high
Cha 3	Chase	corn	fallow/cornstalks	no-till	centerpivot	spread	high
Cha 4	Chase	soybeans	fallow/cornstalks	no-till	centerpivot	spread	low
Cha 5	Chase	corn	corn	strip-till	rainfed	spread	low
Dun 1	Dundy	wheatstubble	other	tilled	rainfed	spread	intermediate
Dun 2	Dundy	corn	sorghum	no-till	rainfed	spread	intermediate
Dun 3	Dundy	other		tilled	centerpivot	spread	intermediate
Dun 4	Dundy	corn	corn	no-till	centerpivot	edges	high
Dun 5	Dundy	soybeans	corn	tilled	centerpivot	spread	low
Fro 1	Frontier	corn	sorghum	no-till	rainfed	edges	high
Fro 2	Frontier	soybeans	corn	tilled	rainfed	edges	low
Fro 3	Frontier	soybeans	wheatstubble	tilled	centerpivot	spread	high
Fro 4	Frontier	sorghum	fallow/cornstalks	tilled	rainfed	edges	intermediate
Fro 5	Frontier	soybeans	corn	tilled	centerpivot	edges	high
Hay 1	Hayes	sorghum	fallow/cornstalks	tilled	centerpivot	spread	intermediate
Hay 2	Hayes	corn	wheatstubble	no-till	rainfed	spread	intermediate
Hay 3	Hayes	sorghum	wheatstubble	tilled	centerpivot	spread	high
Hay 4	Hayes	corn	wheatstubble	no-till	rainfed	edges	intermediate
Hay 5	Hayes	sorghum	wheatstubble	no-till	rainfed	spread	high
Hit 1	Hitchcock	corn	fallow/cornstalks	tilled	centerpivot	edges	low
Hit 2	Hitchcock	soybeans	corn	no-till	rainfed	spread	low
Hit 3	Hitchcock	corn	corn	no-till	rainfed	edges	high
Hit 4	Hitchcock	sorghum	wheatstubble	no-till	rainfed	edges	high
Hit 5	Hitchcock	soybeans	corn	no-till	centerpivot	edges	high
Kei 1	Keith	other	fallow/cornstalks	tilled	centerpivot	spread	high
Kei 2	Keith	corn	fallow/cornstalks	no-till	centerpivot	spread	intermediate
Kei 3	Keith	soybeans		tilled	furrow	spread	high
Kei 4	Keith	soybeans		no-till	centerpivot	spread	low
Kei 5	Keith	other	corn	tilled	centerpivot	spread	low
Kei 6	Keith	soybeans		no-till	centerpivot	spread	high
Lin 1	Lincoln	corn	other	no-till	centerpivot	spread	high
Lin 2	Lincoln	soybeans	corn	tilled	centerpivot	spread	low
Lin 3	Lincoln	soybeans		tilled	centerpivot	spread	low
Lin 4	Lincoln	corn		tilled	furrow	spread	high
Lin 5	Lincoln	corn	wheatstubble	no-till	rainfed	spread	high
Log 1	Logan	soybeans	fallow/cornstalks	tilled	centerpivot	edges	intermediate
Log 2	Logan	other	fallow/cornstalks	no-till	rainfed	spread	intermediate
Log 3	Logan	soybeans	corn	tilled	centerpivot	edges	high
Log 4	Logan	soybeans	corn	tilled	rainfed	spread	low
Per 1	Perkins	other	sorghum	no-till	rainfed	spread	low
Per 2	Perkins	soybeans	corn	strip-till	centerpivot	spread	intermediate
Per 3	Perkins	fallow/cornstalks	corn	tilled	rainfed	spread	high
Per 4	Perkins	soybeans	corn	no-till	centerpivot	spread	high
Per 5	Perkins	other	fallow/cornstalks	no-till	centerpivot	spread	intermediate
Per 6	Perkins	other	fallow/cornstalks	no-till	centerpivot	spread	high
Red 1	Red Willow	soybeans	corn	no-till	centerpivot	edges	high
Red 2	Red Willow	corn	corn	tilled	centerpivot	edges	high
Red 3	Red Willow	wheatstubble	wheat	no-till	rainfed	spread	intermediate
Red 4	Red Willow	corn	corn	no-till	rainfed	spread	low
Red 5	Red Willow	fallow/cornstalks	corn	no-till	rainfed	spread	high

Table 2. List of Palmer amaranth biotypes with EPSPS gene amplification and PPO resistance in molecular assays.

Biotype	EPSPS gene amplification				PPO resistance		# Plants
	Average	Max	Min	% EPSPS resistant plants	Mutation	% PPO resistant plants	
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	R128 het	20	5
Cha 5	1	1	1	0	PPX210	33	3
Dun 1	5	18	1	60	PPX210	20	5
Dun 2	1	1	1	0	PPX210	33	3
Dun 3	1	1	1	0	PPX210	67	3
Dun 4	6	10	4	100		0	5
Dun 5	24	51	4	100	PPX210	20	5
Fro 1	6	10	3	100	PPX210	33	3
Fro 2	3	6	1	33	PPX210	100	3
Fro 3	5	11	1	33	PPX210	67	3
Fro 4	1	1	1	0	PPX210	100	3
Fro 5	1	2	1	0		0	5
Hay 1	1	1	1	0	PPX210	100	3
Hay 2	2	3	1	33	PPX210	33	3
Hay 3	2	2	1	0	PPX210	100	3
Hay 4	1	1	1	0	PPX210	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	PPX210	33	3
Hit 4	2	3	1	25		0	4
Hit 5	1	1	1	0	PPX210	33	3
Kei 1	13	38	1	33	PPX210	33	3
Kei 2	12	19	7	100	PPX210	33	3
Kei 3	1	1	1	0		0	5
Kei 4	8	18	1	60		0	5
Kei 5	5	8	1	67	PPX210	67	3
Kei 6	17	40	2	80		0	5
Lin 1	1	2	1	0	PPX210	100	3
Lin 2	5	6	3	100	PPX210	67	3
Lin 3	4	6	1	67	PPX210	100	3
Lin 4	3	6	1	33	PPX210	100	3
Lin 5	1	1	1	0		0	5
Log 1	34	57	1	67	PPX210	33	3
Log 2	1	1	1	0		0	3
Log 3	4	7	1	67	PPX210	33	3
Log 4	3	6	1	67	PPX210	67	3
Per 1	1	1	1	0	PPX210	33	3
Per 2	32	59	1	80		0	5
Per 3	1	1	1	0	PPX210	33	3
Per 4	10	22	1	67		0	3
Per 5	1	2	1	0	PPX210	33	3
Per 6	1	2	1	0	PPX210	33	3
Red 1	2	3	1	33	PPX210	33	3
Red 2	2	3	1	33	PPX210	67	3
Red 3	2	6	1	20	R128 het	20	5
Red 4	2	5	1	33	PPX210	67	3
Red 5	1	2	1	0		0	5

Table 3. Demographic list of *Amaranthus palmeri* biotypes with respective Nebraska County location and agronomic practices

Herbicide	Correlation variables	Estimate	CI lower	CI higher	t	P-value
Glyphosate	<i>M</i> and <i>G</i>	0.83	0.60	0.93	6.15	0.00
Fomesafen	<i>M</i> and <i>G</i>	0.52	0.09	0.79	2.53	0.02
Lactofen	<i>M</i> and <i>G</i>	-0.05	-0.49	0.41	-0.20	0.84
PPO inhibitors	<i>G</i> -Fomesafen and <i>G</i> -Lactofen	0.23	-0.25	0.62	0.98	0.34