12-2020

Resistance to Herbicides Conferred by Amaranthus palmeri Protoporphyrinogen IX Oxidase Mutations

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Resistance to Herbicides Conferred by *Amaranthus palmeri* Protoporphyrinogen IX Oxidase Mutations

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Cell and Molecular Biology

by

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Bachelor of Science in Agronomy, 2018

December 2020
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ABSTRACT

Palmer amaranth (*Amaranthus palmeri* S. Wats.) is one of the most problematic agronomic weeds to control in fields across Arkansas. Thus far, this species has evolved resistance to several herbicides, including protoporphyrinogen IX oxidase (PPO) inhibitors. The majority of PPO-resistant Palmer amaranth populations harbor a target-site mutation (substitution or deletion of amino acids). The objective of this thesis was to identify the level of fomesafen resistance conferred by *PPO2* mutations from Palmer amaranth. The experiments conducted aimed to (1) characterize the level of resistance conferred by the transgene Palmer amaranth *ppo2* carrying *ΔG210* mutation into the wild type rice (*Oryza sativa* cv. 'Nipponbare'); and (2) study the resistance level of Palmer amaranth plants having a single mutation (*ΔG210* or *G399A*) or a combination. For objective 1, 'Nipponbare' rice was transformed with Palmer amaranth *ppo2 ΔG210* gene via particle bombardment. The presence of the transgene in T_0 plants was confirmed, and seeds (T_1) were harvested. After selection with foliar treatment of fomesafen (0.78 kg ai ha^{-1}), T_1 plants carrying the mutation and showing low injury were maintained to produce T_2 seeds. Soil-based assay was conducted with T_2 seeds and the survivors were cultured to produce T3 seeds. Seeds from each surviving plant were kept as a separate line. The insertion of Palmer amaranth *ppo2 ΔG210* conferred resistance to fomesafen in rice. The data suggests that only homozygous transgenic plants had full resistance to fomesafen. For objective 2, one susceptible and six resistant accessions were used to conduct dose response assay with the PPO-herbicide, fomesafen, and to test cross resistance or multiple resistance. Selected survivors from these tests were genotyped for the two expected mutations. Homozygosity of *ΔG210* was correlated with high fomesafen resistance. At higher fomesafen rates, survivors carrying *ΔG210* in both alleles or accumulating *ΔG210+G399A* recovered better than heterozygous *ΔG210*

plants. Populations with higher frequency of individuals with these mutation profiles were also less sensitive to the other two PPO-herbicides tested, saflufenacil and trifludimoxazin.

ACKNOWLEDGMENTS

I would like to express my gratitude to some people that were essential in this period that I have been striving to achieve this milestone in my academic career. First of all, I would like to thank my advisor Nilda Roma-Burgos for believing in my capacity and giving me the opportunity to pursue a graduate degree at the University of Arkansas. I am humbly grateful for all the constructive criticism, honest opinions, and priceless guidance. She never expected less than my best and has always challenged my mind to think outside the box when facing challenges. I also would like to thank my co-advisor, Dr. Vibha Srivastava, for all her mentorship and certainly for her patience with a complete beginner in molecular endeavors. She has never let me walk away without a complete explanation to the hundred questions I had. I would like to thank Dr. Martin John Egan and Dr. Thallapuranam Krishnaswamy Suresh Kumar for accepting roles as my graduate advisory committee members, for their valuable inputs and their support.

I thank my fellow colleagues from the Weed Physiology group and Plant Biotechnology group: Eduardo Augusto, Diego Belapart, Yasmin Berchembrock, Dr. Flavia Botelho, Dr. Fernanda Caratti, Jeremie Kouame, Dr. Nelson Kruze, Felipe Machado, Matheus Machado, Dr. Soumen Nandy, Dr. Bhuvan Phathak, Shan Zhao, and Isabel Werle. In particular, I am deeply grateful for Dr. Gulabben Rangani and Dr. Lariza Benedetti for sharing their laboratory expertise and knowledge, and for being so patient while I was learning. I would not be able to achieve my results without your kindness, encouragement, and friendship. I also thank my fellow graduate students at the Cell and Molecular Biology program and at the Crop, Soil, and Environmental Sciences department for so many joyful moments. Even though this thesis was mainly conducted in the laboratory, I have conducted several field trials at the Clarksville, Fayetteville, Kibler and

Rohwer research stations. Therefore, I would like to express my gratitude to all people involved in these trials, in special Larry Earnest and Stephen Eaton. I have learned a lot from you.

I am also grateful for the training and mentorship I received as an undergraduate student from Dr. Carlos Eduardo Schaedler, Dr. Christopher Rouse, Dr. Eduardo Bohrer Azevedo, Dr. Elizete Radmann, Dr. Luciana Zago Ethur and Dr. Reiofeli Salas-Perez. Your passion for research and your work ethic have inspired me to always try to be a better scientist.

To my friends, Bruna Oliveira, Natalia Rodrigues, Samantha Segalin, Dr. Leonard Piveta, Maria Zaccaro, Dr. Franciene Villanova, Mary Jane Lytle, Teal Penka, Hannah Wright, Ana Carla Castanha, Mariana Polano, Juliana Oliveira, Caio Santos, Mila Pezoto, Renata Dornelles, Francisco Goulart, Taiane Ciocheta and Emanueli da Rosa, I am humbly blessed to have you in my life. Thank you for your thoughtful advices and camaraderie.

I would like to express my deepest gratitude to my friends and my whole family for all their support and understanding, I will never be able to thank you enough. In special, I would like to show my gratitude to my mother, Ana Maria de Carvalho-Hansbury, my grandparents, and my in-laws, Frank and Nina Moore, for always being there for me, no matter the situation. Most importantly, I want to thank my husband, Jason Martin Moore. He has been my daily source of strength and support.

DEDICATION

I dedicate this thesis to my mother Ana M. Carvalho-Hansbury, to my aunt Alzineia S. de Carvalho, to my brothers, and to my grandparents, Anedino and Suzana de Carvalho, and Benivaldo and Narciza dos Santos, for their endless support and love.

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INTRODUCTION

Introduction

Among the factors that may impact crop yield, weed competition is the one that generally causes the highest yield losses in many important crops around the world (Gharde et al. 2018; Oerke 2006). Archeological discoveries suggest that farmers have been using different methods to exterminate weeds from fields since nomads became settled farmers. For instance, hoes and other digging implements were discovered at archeological sites in China and Italy (Harvey 2010; Liu et al. 2014). Crop production improvement, product quality enhancement, and reduction of production costs are the primary reasons why weed control is a crucial operation in crop production (Harvey 2010; Liu et al. 2014; Radosevich et al. 2007).

Surveys conducted by Wychen in 2016 and 2017 denoted Palmer amaranth [*Amaranthus palmeri* (S.) Wats.] as the toughest weed to eradicate from corn, cotton, and soybean production systems in Arkansas. This weed has become resistant to several herbicides used in these crops. Therefore, its persistence and negative impact on crop production are almost inevitable. Thus far, different Palmer amaranth populations have evolved resistance to eight herbicide sites of action (Heap 2020; Ward 2013). One of the herbicides, to which Palmer has evolved resistance to, is fomesafen. Fomesafen is a diphenylether herbicide that controls monocot and dicot weeds by inhibiting the enzyme protoporphyrinogen oxidase (PPO) (Hao et al. 2011). The inhibition of this enzyme will lead to high peroxidative damage, and consequently, cellular death (Dayan and Watson 2011).

The repetitive use of herbicides wields high selection pressure on weed populations which changes in size and diversity with time. In summary, the genetic composition of a population will change as a consequence of repeated treatments with the same class or family of herbicides, increases the frequency of resistant alleles. With the increase in the number of

resistant weeds, farmers have been diversifying their use of herbicide active ingredients again (Green and Owen 2010; Jasieniuk et al. 1996; Vencill et al. 2012). Thus far, around 260 weed species are resistant to one or several herbicides, with 13 species specifically resistant to PPO inhibitors (Heap 2020). The first discovered PPO-resistant weed was tall waterhemp (*Amaranthus tuberculatus*) which was reported in the United States in 2001, followed by wild poinsettia (*Euphorbia heterophylla*) in Brazil in 2004 (Shoup et al. 2003; Trezzi et al. 2005).

Resistance to this herbicide group is mainly attributed to target-site mutations. Patzoldt et al. (2006) discovered that deletion of the glycine codon at position 210 of the *PPO2* gene is responsible for conferring resistance to PPO herbicides in a tall waterhemp population. Later, this same mutation was identified as the resistance mechanism in a PPO-resistant Palmer population and in numerous other populations of both species (Evans et al. 2019; Lee et al. 2008; Salas et al. 2016; Wuerffel et al. 2015). This mutation causes an alteration in the binding domain of the mitochondrial PPO enzyme without significantly reducing the substrate binding affinity, which explains the natural selection of plants carrying this mutation (Dayan et al. 2010). The substitution of arginine with glycine or methionine at position 128 in Palmer amaranth also confers resistance (Giacomini et al. 2017; Salas-Perez et al. 2017). In 2019, a novel resistance-conferring mutation in the catalytic domain of *ppo2* was identified in this species. This new mutation is a substitution of glycine with alanine at position 399, reducing the affinity of PPO-inhibiting herbicides to the enzyme (Rangani et al. 2019).

Even though resistance to PPO inhibitors is mainly due to target-site mutations, non-target-site resistance to a PPO-inhibiting herbicide (fomesafen) has been observed in some populations (N.R. Burgos, unpublished) and was reported in Palmer population (Varanasi et al. 2018). Previously, a study conducted in Brazil with PPO-resistant wild poinsettia (*Euphorbia*

heterophylla) identified a different type of non-target-site resistance. The authors detected lower absorption of soil-applied fomesafen in resistant populations compared to the susceptible population (Trezzi et al. 2011).

As mentioned above, Palmer amaranth populations resistant to PPO-inhibiting herbicides in Arkansas are mainly due to target-site mutations. Investigations conducted with Arkansas populations showed that *ΔG210* is the predominant mutation among PPO-resistant accessions, followed by R128G. These populations showed varied resistance levels to PPO-herbicides despite carrying the same mutations (Salas-Perez et al. 2017; Varanasi et al. 2018). Further characterization of these mutations, alone or in combination, clarifies the level of PPO-resistance provided by their presence. Also, it may provide a suitable tool for genetic transformation in sensitive crops that would benefit from the introduction of PPO-herbicides in their weed management program.

Therefore, this research aimed to identify the level of fomesafen resistance conferred by *ppo2* mutations in Palmer amaranth populations. The objectives were to: 1) characterize the level of resistance conferred by the Palmer amaranth *ppo2* carrying *ΔG210* mutation into wild type rice (*Oryza sativa* cv. Nipponbare); 2) study Palmer amaranth populations *PPO2* having a single mutation (*ΔG210* or *G399A*) or combination of mutations to investigate the contribution of each mutation towards the herbicide tolerance level to PPO herbicides.

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CHAPTER II

REVIEW OF LITERATURE

Protoporphyrinogen IX Oxidase Inhibiting Herbicides

The first PPO-inhibiting herbicide to be commercialized was nitrofen back in the 1960s. Nitrofen, which is a diphenylether compound, was developed in the United States and rapidly adopted in Japan to use in rice (*Oryza sativa*) paddy fields due to its low toxicity to fish and its broad herbicidal spectrum (Matsunaka 1976). PPO inhibitors are classified as diphenyl ethers (fomesafen, acifluorfen, oxyfluorfen, lactofen), phenylpyrazoles (fluazolate), triazolinones (carfentrazone, sulfentrazone), oxadiazoles (oxadiazon), thiadizoles (fluthiacet), N-phenyl-phthalimides (flumioxazin, flumiclorac), oxazolidinedione (pentoxazone), or pyrimidinediones (butafenacil, saflufenacil). PPO-inhibiting herbicides can be used to control monocot and dicot weeds in pre- and postemergence applications on different crop systems like cotton, soybean, and corn (Hao et al. 2011).

The PPO enzyme (EC 1. 3. 3. 4) is the target of PPO-inhibiting herbicides (Duke et al. 1991). This enzyme catalyzes the reaction from transforming the substrate protoporphyrinogen IX (Protogen) into the product protoporphyrin IX (Proto IX) by removing six electrons from the substrate (Beale and Weinstein 1990). Since this enzyme is the last common enzyme in the tetrapyrrole pathway to synthesize heme and chlorophyll, the PPO-enzyme is necessary for crucial plant processes, such as light-harvesting, electron-transfer reactions, and photosynthesis. Tetrapyrrole compounds, which consist of four pyrroles (aromatic rings with four carbon and one-nitrogen molecule), are part of several biological pathways. Four classes of tetrapyrroles are present in plants: chlorophyll, heme, siroheme, and phytochromobilin. Three of these play a significant role in photosynthesis (hemes, chlorophylls, and bilins). Chlorophyll absorbs light and transfers light energy to other molecules, and heme is an ingredient in several physiological

processes such as respiration and photosynthesis. The biosynthesis of tetrapyrroles occurs mainly inside plastids (Larkin 2016; Moulin and Smith 2005; Tanaka and Tanaka 2007).

During the light reaction of photosynthesis, reactive oxygen species (ROS) are generated due to electron leakage, respiration, or light absorption that exceeds the transfer capacity. In sum, ROS will be generated when the cells exhibit stress-induced discrepancies in photosynthetic reactions, which is common in fluctuating environmental conditions. These ROS can cause severe cellular damage, but also signal the induction of oxidative stress responses. Tetrapyrroles are also essential in the oxidative stress response. These chemical compounds protect cells by contributing to the detoxification of ROS (Batoko et al. 2015; Busch and Montgomery 2015; Froyer et al. 2017; Mochizuki et al. 2010). The accumulation of the tetrapyrrole intermediate Mg-protoporphyrin IX triggers the communication between the cell nucleus to chloroplasts. Also, this intermediate closely interacts with several proteins linked with oxidative stress responses which will induce an increase in the nuclear expression when stress is applied (Kindgren et al. 2011; Strand et al. 2003).

There are two isoforms of the PPO enzyme: PPO1 and PPO2. PPO1 enzyme is compartmentalized in the chloroplast. PPO2 is compartmentalized in the mitochondria. However, in some plants such as Palmer amaranth and spinach, PPO2 is compartmentalized in the chloroplasts as well. Two nuclear-encoded genes (*PPO1* and *PPO2*) are responsible for producing the isoforms of the PPO enzyme (Dayan et al. 2018; Lermontova et al. 1997; Watanabe et al. 2001). Both isoforms are targets of PPO-inhibiting herbicides.

The application of PPO herbicides leads to an uncontrolled accumulation of Protogen in susceptible plants by inhibiting the PPO enzyme. The excess of the substrate is subsequently moved to the cytoplasm, where it is instantly oxidized by free oxygen present. This process will

form a highly photosensitive Proto IX product, which generates singlet oxygen molecules when it is exposed to light. The singlet oxygen molecules generated by light exposure will cause lipid peroxidation, membrane disruption, and cell disintegration, which will lead to cellular death. However, the level of damage triggered by this herbicide group depends on the quantity of light received and Proto IX accumulated by the plant (Becerril and Duke 1989; Duke et al. 1991; Hao et al. 2011; Jacobs et al. 1991).

Resistance History of PPO-Inhibiting Herbicides

Thus far, thirteen species from five families (Amaranthaceae, Asteraceae, Brassicaceae, Euphorbiaceae and Poaceae) are reported to be resistant to one or more PPO-herbicides (Heap 2020). Resistance to PPO-inhibiting herbicides can occur through target-site resistance (TSR) or non-target-site resistance (NTSR). TSR mechanisms consist of alterations or mutations (substitutions or deletions), overexpression, or amplification in the targeted gene. TSR mechanisms will prevent or decrease herbicide binding to the targeted binding site. Different from TSR mechanisms, NTSR to herbicides can develop as a consequence of the modification of one or several physiological processes. NTSR mechanisms can consist of the decrease of herbicide penetration or absorption due to alterations in cuticle properties or environmental stress; altered translocation away from the target protein; enhanced metabolism of the herbicide causing faster degradation (cytochrome P450s and glutathione *S*-transferases); and neutralization of toxic molecules generated as the result of the herbicide action (Délye et al. 2013; Jugulam and Shyam 2019; Powles and Yu 2010).

Common or tall waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer var. rudis (Sauer) Costea and Tardif] was the first documented PPO-resistant species in 2001. A population from

Kansas treated for several years with acifluorfen presented 34 times more resistance to acifluorfen or lactofen than the susceptible population**.** A cytochrome P-450 inhibitor was included to verify the presence of NTSR mechanism, but the results obtained in this experiment rejected this hypothesis (Shoup et al. 2003). A later study, with *A. tuberculatus* PPO-resistant populations from Illinois, identified the presence of a deletion of glycine at position 210 of *ppo2* (Patzoldt et al. 2005; Patzold et al. 2006). Over the years, other *A. tuberculatus* biotypes also showed PPO-resistance in different U.S. states and Canada (Bell et al. 2013; Evans et al. 2019; Lee et al. 2008; Thinglum et al. 2011; Wuerffel et al. 2015). Redroot pigweed (*Amaranthus retroflexus*) is another species among the Amaranthaceae family resistant to PPO-inhibiting herbicides. A fomesafen-resistant *A. retroflexus* population was identified in China. Gene sequencing revealed the substitution of arginine by glycine at position 128 of *ppo2* (Wang et al. 2020). Smooth pigweed (*Amaranthus hybridus*) was also reported to have PPO-resistant populations in Bolivia. However, little information is available about this report. PPO-resistance in Palmer amaranth (*Amaranth palmeri* S. Wats.) populations will be explored later in this review. Thus far, Amaranthaceae is the family with the highest number of PPO-resistant species in 5 different countries (Heap 2020)

The second PPO-resistant confirmed species was wild poinsettia (*Euphorbia heterophylla*) in 2004. Two populations, from fields where fomesafen and carfentrazone were regularly sprayed in Brazil, showed a level of resistance of 62- and 39-fold compared to the susceptible population (Trezzi et al. 2005). An experiment performed with uninjured leaves from the resistant and susceptible plants demonstrated that electric conductivity values were higher at some fomesafen concentrations in the plates with the susceptible samples. Therefore, the susceptible biotype displayed lower resistance against the penetration of PPO-inhibiting

herbicides indicating that NTSR mechanisms can be involved by coping with the oxidative stress caused by herbicide applications (Trezzi et al. 2011). Crosses between susceptible and resistant biotypes strongly suggests that PPO-resistance in *E. heterophylla* is conferred by a dominant nuclear gene (Brusamarello et al. 2016). Asian copperleaf (*Acalypha australis*), another Euphorbiaceae member, was confirmed resistant to fomesafen in China in 2011 (Heap 2020). Nevertheless, there is no elucidation of the resistance mechanism in *A. australis*.

Resistance to PPO-inhibiting herbicides occurrences in plants pertaining to Poaceae family was first reported in 2015. A wild oat (*Avena fatua*) population, a common weed in prairies in Canada, showed a sulfentrazone resistance level of 2-fold compared to the susceptible standard. Since this *A. fatua* population had never been exposed to this specific herbicide, target-site mutation was rejected. This population was already confirmed to be resistant to acetyl-CoA carboxylase and acetolactate synthase inhibitors. Therefore, it was suggested that resistance was a result of increased metabolism by cytochrome P450 enzymes selected in the previous herbicide use. Resistance to pyroxasulfone was also confirmed in this population (Mangin et al. 2016). In the same year, four rigid ryegrass (*Lolium rigidum*) populations were proven resistant to oxyfluorfen applications in Spain. Oxyfluorfen resistance in these populations varied between 5.01- and 20.10-fold in comparison with the susceptible population in a dose-response assay. A petri dish experiment with resistant and susceptible leaf discs was also performed to determine the amount of substrate (Protogen) in the presence of the herbicide. This experiment showed lower Protogen accumulation in resistant populations which means that neither PPO1 nor PPO2 were being inhibited by oxyfluorfen. The mechanism of resistance in this species has not been studied yet (Fernandez-Moreno et al. 2017).

The latest species to be reported resistant to PPO-inhibiting herbicides was Sumatran fleabane (*Conyza sumatrensis*) in 2017. Pinho et al. (2019) collected seeds from *C. sumatrensis* populations that were not controlled by the field dose of saflufenacil. A greenhouse dose-response assay with saflufenacil was conducted using resistant and susceptible *C. sumatrensis* biotypes, and the authors determined that the resistant biotype was eight times more resistant to saflufenacil compared with the susceptible control. Thus far, the resistance mechanism in these Brazilian *C. sumatrensis* populations has not been elucidated. Different from *C. sumatrensis*, the mechanism involved in the resistance to PPO-inhibiting herbicides for common ragweed (*Ambrosia artemiisifolia*) was clarified. Similar to *C. sumatrensis*, *A. artemiisifolia* is part of the Asteraceae family and a problematic weed in agricultural lands. Seeds were collected from *A. artemiisifolia* survivors from a soybean field in Delaware in which flumioxazin and fomesafen applications failed. Dose-response assay was conducted with these seeds by spraying different rates of preemergence (saflufenacil and flumioxazin) and postemergence (acifluorfen, carfentrazone, flumiclorac, flumioxazin, fomesafen, lactofen, oxyfluorfen and pyraflufen) PPO-inhibiting herbicides. When compared to the susceptible standard, resistance to PPO-herbicides in the resistant biotype ranged from 80- to 3-fold for postemergence and from 22- to 10-fold for preemergence herbicides. Molecular investigation showed the substitution of arginine by leucine in position 98 (*R98L*) of the *ppo2* in the resistant biotype. The *A. artemiisifolia ppo2* gene, with or without the presence of *R98L*, was then inserted into the *Escherichia coli* system to determine whether the mutation was sufficient to confer resistance to acifluorfen. The *E. coli* plasmid containing *R98L* showed an acifluorfen resistance level of 31-fold in comparison to the plasmids where the mutation was absent (Rousonelos et al. 2012). The R98 locus in *A. artemiisifolia* is the

same as R128 in Palmer. The species *A. tuberculatus. A. palmeri* and *A. tuberculatus* have a 30-amino acid signal peptide in the *ppo2* gene (Rangani et al. 2019).

The occurence of a flixweed (*Descurainia sophia*) population resistant to the PPO-herbicide, carfentrazone, is also known. No further information is available about the resistance mechanism (Heap 2020). To date, goosegrass (*Eleusine indica*) is the only species to exhibit resistance to oxadiazon herbicide (Heap 2020; McElroy et al. 2017). Bi et al. (2020) identified the substitution of alanine to threonine at the 212th of the chloroplast isoform of the PPO-enzyme in resistant-goosegrass populations.

PPO-Resistant Palmer amaranth (*Amaranthus palmeri* S. Wats) Populations in Arkansas

Thus far, Palmer amaranth (*Amaranthus palmeri* S. Wats.) PPO-resistant populations in Arkansas are mainly due to the presence of mutations in the targeted protein. Initially, Salas et al. (2016) reported that PPO-resistant Palmer amaranth accessions in Arkansas had a deletion of a glycine at the position 210 which had previously conferred resistance in another plant from the Amaranthaceae family, tall waterhemp. In 2017, Salas-Perez et al. (2017) examined a total of 124 Palmer populations for resistance to foliar-applied fomesafen. The populations were collected between 2008 and 2015. As expected, few accessions from earlier years displayed resistance to fomesafen while 70% of the 2015 populations were resistant to this herbicide. Through an allele specific PCR assay, the authors detected the presence of PPO *ΔG210* in survivors from 47 accessions. This assay identified that 55% of survivors carried the deletion. Since a percentage of survivors did not carry the PPO *ΔG210*, RNA was extracted for obtaining full-length *PPO2* sequence. The substitution of arginine by glycine at 128 position (*R128G*) was identified in survivors of one specific accession. This specific population also assembled

survivors carrying PPO *ΔG210*. This same mutation was previously reported in other Palmer amaranth populations from Tennessee and Arkansas where the authors encountered a substitution of arginine by glycine or by methionine at 128 (*R128G* and *R128M*) (Giacomini et al. 2017).

Corroborating these findings, Varanasi et al. (2018a) surveyed the occurrence of resistance to PPO inhibitors among Palmer amaranth populations in Arkansas. The authors sprayed 227 accessions with fomesafen to determine the percentage of resistant plants per accession. Leaf tissue was collected from the survivors (167 accessions) and TaqMan qPCR was performed in order to identify the presence of *ΔG210*, *R128G*, or *R128M* gene mutations. The *ΔG210* was detected in 49% of the accessions sampled, followed by *R128G* substitution (27%).

A novel mutation was recently detected in the catalytic domain of the PPO2 enzyme in one fomesafen-resistant, Palmer amaranth field population (Rangani et al. 2019). An herbicide screening with foliar-applied fomesafen was conducted, and full-length sequences from susceptible and resistant plants were obtained through gene sequencing. The authors found a consistent substitution of glycine with alanine at position 399 (*G399A*) in all survivors. The resistant plants did not carry any other previously known mutations. A survey, with 35 previous screened fomesafen-resistant populations, exposed that around 14% field populations carried *G399A* mutation.

In Palmer, NTSR mechanisms conferring resistance to PPO inhibitors was first reported in Arkansas. Varanasi et al. (2018b) identified a PPO-resistant population that did not harbor any known resistance-conferring mutation. Around 200 seedlings from this accession were cultivated and later sprayed with fomesafen. Leaf tissue was collected from the survivors for DNA and RNA extraction. TaqMan allelic discrimination assay was conducted, and full-length PPX1 and

PPX2 sequences were obtained. Neither one of these two isoforms contained known resistance-endowing mutations. Also, a SYBR Green assay was conducted to measure if the resistant individuals were overexpressing the PPX2 gene in comparison with the susceptible. There was no difference in overexpression either. To verify if the resistance was conferred by a metabolic mechanism, cytochrome P450 (malathion) and glutathione S-transferase (NBD-Cl) inhibitors (with or without fomesafen) were applied on the seedlings obtained from the previous survivors. The herbicide was applied after the metabolic inhibitors. The application of malathion and NDB-Cl followed by fomesafen reduced the survival rates and the biomass of resistant plants in comparison to the application of fomesafen only. Therefore, the pre-treatment with cytochrome P450 and glutathione *S*-transferase inhibitors enhanced susceptibility to fomesafen applications. These two enzymes have a vital role in detoxification pathways, hence metabolism of toxins (including herbicides) (Anderson and Gronwald 1991; Powles and Yu 2010). These findings indicate the existence of non-target-site based mechanisms as the responsible for one PPO-resistant Palmer amaranth accession in Arkansas.

Transgenic Experiments with Plastidic Protoporphyrinogen IX Oxidase Enzyme

Resistance to peroxidizing herbicides in crops is a desired feature in weed control programs. The increasing of mitochondrial or plastidic PPO activity can confer herbicide resistance to chemical families pertained to this group (Lermontova and Grimm 2000; Jung et al. 2008b). Several approaches have been used to overexpress plastidic PPO enzyme in plants. Choi et al. (1998) inserted the *Bacillus subtilis* PPO gene into tobacco (*Nicotiana tabacum*) plants using CaMV 35S and Cab-promoter. Northern analysis confirmed that the *B. subtilis* PPO gene was expressed in the transformed plants. To identify if the expression was also conferring

herbicide resistance, a leaf disk assay was conducted. Tobacco tissues were placed in Petri plates containing 5 ml of 1% sucrose and 1mM of 2-(N-morpholino) ethanesulfonic acid with varied rates of oxyfluorfen. Transgenic tobacco plants under the CaMV 35S promoter were more resistant to oxyfluorfen than the susceptible and transgenic plants under the Cab-promoter. Overexpression of the *B. subtilis* PPO gene was also applied to transform rice plants (Ha et al. 2003). As in the tobacco transformation research, the transformed rice plants exhibited higher tolerance to oxyfluorfen than the wild type (WT) rice. A germination assay was performed to determine herbicide tolerance in seeds. Sterilized rice seeds (transgenic and WT) were placed in MS medium containing the antibiotic cefotaxime. Oxyfluorfen solution was added on the top of the medium at 1 μM. WT rice seeds has had its growth delayed while the transgenic lines have grown. The transgenic lines did not resist to oxyfluorfen applications greater than 2 μM which makes this approach impractical in field applications.

Instead of the PPO gene from *B. subtilis p*reviously used, Lermontova and Grimm (2000) inserted the plastid-located PPO gene of *Arabidopsis thaliana* to overexpress the PPO enzyme in tobacco. Southern-blot analysis was carried to confirm the presence of the construct. Northern-blot analysis showed the expression levels of *A. thaliana* PPX1 and *N. tabacum* PPX2. The expression of PPX2 did not change with the insertion of the plastid-located PPO of *A. thaliana*. Foliar application of acifluorfen and leaf assay with varied acifluorfen concentrations were conducted. In both tests, the transgenic plants presented almost no injury while the WT plants displayed large necrotic areas in their leaves. A germination assay showed that transgenic positive seeds could germinate at higher acifluorfen concentration (300 nM) while 200 nM of acifluorfen completely blocked the WT germination. Crude chloroplast was extracted from leaves of the transgenic lines and WT to measure the activity of the PPO enzyme. Their results

indicated that the increased activity of PPO (five times higher in transgenic plants) prevented the accumulation of the substrate protoporphyrinogen, thus neutralizing the phytotoxicity caused by the application of acifluorfen.

Volrath et al. (1999) modified the *PPO1* from *Arabidopsis thaliana* generating some mutants tolerant to the PPO herbicide, butafenacil. Subsequent studies with these mutants showed that the combination of the substitution of tyrosine by methionine at the position 426 (Y426M) and of serine by leucine at the position 305 (S305L) in the gene conferred high tolerance to butafenacil without losing the enzyme functionality (Hanin et al. 2001). Maize (*Zea mays*) genetically modified with this double mutation through *Agrobacterium*-mediated transformation exhibited high tolerance to butafenacil as well. Even though multiple gene copies were not required to induce high tolerance, it was observed that the most tolerant mutated maize plants were prone to have numerous copy numbers of the mutant (Li et al. 2003).

Also working with mutants, Kataoka et al. (1990) identified, in *Chlamydomonas reinhardtii* (one type of green alga), a mutant strain (rs-3) responsible for conferring elevated tolerance to PPO-inhibiting herbicides in this alga. Further characterization showed that this mutation resulted from the substitution of a valine by a methionine at 291 position in the PPX1 gene (Randolph-Anderson et al. 1998). This mutation caused resistance to a potent PPO herbicide, oxyfluorfen. Therefore, it could potentially represent a suitable option for suppressing sensitive weeds in algal production (Bruggeman et al., 2014).

Transgenic Experiments with Mitochondrial Protoporphyrinogen IX Oxidase Enzyme

Mitochondrial *PPO2* overexpression has also been employed to achieve tolerance to diphenylether herbicides although at a lower level. Transgenic rice (M4), expressing *Myxococcus*

xanthus PPO protein with high PPO activity in the chloroplast and mitochondria, was confirmed to be resistant to PPO-inhibiting herbicides from various chemical families, including diphenylether (acifluorfen and oxyfluorfen), oxadiazole (oxadiazon) and triazolinone (carfentrazone-ethyl) (Jung et al. 2004; Jung et al. 2008a). Further research on M4 revealed that in addition to being resistant to PPO-inhibiting herbicides, M4 also has a higher drought tolerance (after 7 days of drought stress exposure) compared to the WT. This line exhibited higher water content, lower injury (foliar) and less oxidative damage that can explain its enhanced drought tolerance (Jung et al. 2010; Yun et al. 2013).

Lee et al. (2004) developed transgenic rice overexpressing human PPO. They aimed to increase the mitochondrial activity of this enzyme, consequently providing resistance to PPO-inhibiting herbicides. The overexpression of this protein successfully conferred resistance to the herbicide oxyfluorfen in germination. However, further research with this same transgenic rice conducted by Jung et al. (2008b) indicated that the overexpression of human PPO enzyme deregulated the tetrapyrrole pathway. This abnormal pathway resulted in an accumulation of Proto and Mg-porphyrin which induced high formation of the reactive oxygen species, and lower contents of chlorophyll and heme. The mature transgenic plants exhibited severe necrotic spots and growth retardation during development.

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CHAPTER III

CHARACTERIZATION OF THE *ΔG210* MUTATION FROM PALMER AMARANTH (*Amaranthus palmeri*) IN RICE (*Oryza sativa*)

Abstract

Palmer amaranth (*Amaranthus palmeri* S. Wats.) has evolved resistance to eight herbicide modes of action, including protoporphyrinogen IX oxidase (PPO) inhibitors. The majority of PPO-resistant Palmer populations in Arkansas harbor the *PPO2 ΔG210* mutation. This study aimed to determine if the presence of the Palmer amaranth *ppo2* carrying the *ΔG210* mutation would confer resistance to fomesafen applied in rice (*Oryza sativa* cv. 'Nipponbare'). Transgenic rice overexpressing the Palmer amaranth *ppo2 ΔG210* gene was generated via particle bombardment. The presence of the transgene in T_0 plants was confirmed, and seeds (T_1) were harvested. T_1 seedlings were foliar treated with 0.78 kg ha^{-1} fomesafen to select resistant T_1 plants. T_1 plants containing the construct, showing low injury from fomesafen, were grown to produce T_2 seeds. In a soil-based assay on T_2 seeds, fomesafen caused 92% and 27% germination reduction in wild type (WT) and T_2, respectively. All T_2 survivors carried the *ppo2* transgene. T_2 survivors of the soil-based assay showed a wide range of injury (30 to 95%). All T_2 plants carrying the transgene had 155 to 1144-fold increase in gene expression in *ppo2* gene expression when compared to WT and T_2 plants, which were negative for the transgene. The injury level did not correlate with gene expression level or gene copy number. T_3 progenies of survivors from soil-applied fomesafen carried the Palmer amaranth *ppo2* transgene. It can be assumed that only *ΔG210* homozygous plants are able to survive preemergence application of fomesafen. In an agar-based dose-response assay, fomesafen severely inhibited the root growth of all WT seedlings, but not the root growth of T_2 seedlings, with a few exceptions. Therefore, the Palmer amaranth *ppo2- ΔG210* confers resistance to fomesafen in rice and the mutation needs to be present in both alleles to attain full resistance. This research also supports the principle that herbicide-resistant genes from weeds can be used as transgene to develop herbicide-resistant crops.

Introduction

Mutations occur naturally. Mutations within a gene modifies the gene by changing, deleting, or duplicating one or more nucleotides. This type of mutation may change the stability, activity, location, or interactions of a gene's encoded protein or RNA product. Even though the majority of the mutations are classified as having negative effects, a few of them are quite advantageous (Alberts et al. 2014; Lodish et al. 2007). The capacity of some plants to tolerate one or several herbicides is among one of the advantages that a mutation may provide. The existence of mutations conferring herbicide resistance is a major factor in why several herbicidal compounds have been losing their efficacy in controlling some weeds (Heap 2020).

Among the herbicides that had their overall efficacy decreased due to point mutations are the inhibitors of the enzyme protoporphyrinogen IX oxidase (PPO, EC 1.3.3.4). These herbicides kill susceptible plants by inhibiting the catalysis of the oxidation of the protoporphyrinogen IX into protoporphyrin (Porra and Falk 1964; Poulson and Polglase 1975). In plants, the PPO enzyme may be nuclear-encoded in two forms. The first, PPO1, is compartmentalized in the chloroplast. The second, PPO2, is compartmentalized in the mitochondria and, in a few species, also in the chloroplast (Lermontova et al. 1997; Watanabe et al. 2001). In a susceptible organism, the two forms of the PPO enzyme in both organelles will be inhibited by PPO-inhibiting herbicides. This inhibition will induce an unrestrained accumulation of the substrate (protoporphyrinogen IX) which will be later moved to the cytoplasm where it reacts readily with free oxygen. This oxidation process produces a highly photosensitive protoporphyrin IX which will generate singlet oxygen when exposed to light. These singlet oxygen molecules will cause lipid peroxidation, cellular membrane disruption, disintegration of cells, loss of carotenoids and

chlorophyl (bleaching effect), and, consequently, cellular death (Dayan and Duke 1996; Duke et al. 1991; Lermontova et al. 1997; Matringe et al. 1989; Orr and Hess 1982).

To date, a total of 13 species evolved resistance to PPO herbicides. One of these is Palmer amaranth (*Amaranthus palmeri* S. Wats.) (Heap 2020). Palmer amaranth is recognized among people working in the agricultural industry as one of the major weeds in the southeastern region of the United States (Wychen 2016; Wychen 2017). The PPO-resistance mechanism is widely studied in the Amaranthaceae family, of which Palmer amaranth is a member. The first case of PPO-resistant Palmer amaranth plants was detected in a retroactive screening of a 2011 population in Arkansas (Salas et al. 2016). A previously identified PPO-resistance conferring mutation in tall waterhemp (*Amaranthus tuberculatus*) was detected in these Palmer amaranth plants. This mutation consisted of the deletion of a glycine at the 210th position in the *ppo2* gene, also known as ΔG210 (Lee et al. 2008; Patzoldt et al. 2006). A substitution of arginine to glycine or methionine at the 128th position in the *ppo2* (*R128G* or *R128M*) was the second mutation encountered in PPO-resistant populations (Giacomini et al. 2017; Salas-Perez et al. 2017; Varanasi et al. 2018). The latest identified resistance-conferring mutation is the substitution of glycine at the 399th position in the *ppo2* to alanine (Rangani et al. 2019).

One way to precisely obtain the contribution of a particular mutation in the whole-plant herbicide resistance is by expressing it in a heterologous system. Researchers have shown that the presence of the above-cited mutations in *PPO2* will reduce the ability of PPO herbicides to inhibit the targeted enzyme, consequently reducing the control of resistant weeds (Huang et al. 2020; Patzoldt et al. 2006; Rousonelos et al. 2012). However, these experiments were conducted using experimental models, such as *Escherichia coli* or *Arabidopsis thaliana*. Thus far, there is no information regarding the level of tolerance to PPO-herbicides conferred by the presence of

any of these specific mutations in a complex and economically important plant system. Therefore, this study aimed to determine if the presence of the Palmer amaranth *ppo2* carrying the *ΔG210* mutation would confer resistance to fomesafen applied pre- or postemergence in rice (*Oryza sativa* cv. 'Nipponbare').

Materials and Methods

Plant Transformation with Palmer amaranth *ΔG210 - ppo2.* A transgenic rice plant containing the Palmer amaranth *ppo2* mutant gene, Gly_{210} deletion (*ΔG210*), was previously generated (Figure 1). The plasmid pRP7 (6816 bp size), containing maize ubiquitin-1 promoter and *nos* terminator, was digested with the enzymes Xma I and Sac I to remove *gus* gene region. The *gus* region was replaced by Palmer amaranth *ppo2* containing *ΔG210* mutation (pACL1 plasmid). Explants from rice seedlings (*Oryza sativa* cv. 'Nipponbare') were transformed with pACL1 and a marker gene vector (pHPT) by particle bombardment (Rangani and Langaro, unpublished). After selection in hygromycin and root/shoot growth, a single transgenic event was recovered that regenerated a single T_0 plant. This T_0 plant was transplanted into commercial soil (Sunshine® Premix No. 1; Sun Gro Horticulture, Bellevue, WA), and maintained in the greenhouse located inside the Rosen Alternative Pest Control Center at the University of Arkansas and grown to maturity.

Leaf tissues were collected and stored on ice or in -80°C until DNA extraction. Genomic DNA was extracted following a modified version of the CTAB protocol established by Doyle and Doyle (1987). The genomic DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). To verify the presence of the transgene, the *ppo2* in these plants was PCR-amplified using transgene-specific primers. The PCR reaction mixture (20 µl)

consisted of 10 µl 2X Emerald Amp® MAX PCR Master Mix, 1 µl of forward (kpnApxF: 5'-ggggtacccgggTAAACTGATCTTATGTTAATTC-3') and reverse (sphApxR: 5'-ggaattcgagctcgcatgcTTACGCGGTCTTCTCATCCATC-3') 5 µM primers, 7 µl of sterile water and 1 µl of genomic DNA (~ 100 ng). Genomic DNA from WT and T_0 plants (once verified) were used as negative and positive controls, respectively. A PCR reaction consisted of 2 min at 95°C, followed by 40 cycles of 1 min denaturation (95°C), 1 min annealing (58°C), and a 2 min extension (72°C). A final extension step (72°C) occurred for 10 min after the completion of the cycles. The PCR products were stored at 4°C. The products were separated by gel electrophoresis using a 0.8% agarose gel using gel red dye. A 1 kb ladder was used to determine the size of the products.

The presence of the transgene in the T_0 plant was confirmed by PCR (Figure 2). The PCR product was purified using a GeneJET gel extraction kit (Thermo Fisher Scientific, Grand Island, NY) following the company's instructions. The purified sample was sequenced at Eurofins Genomics, Louisville, KY. Using Sequencher 5.4.6 software (Gene Codes Corporation, Ann Arbor, Michigan, USA), the DNA sequence from T_0 was aligned and compared to susceptible and resistant containing *ΔG210* sequences provided in Salas et al. (2016) confirming the presence of the deletion (Figure 3).

Response of T_1-*ΔG210* Plants to Foliar-Applied Fomesafen. To confirm whether the transgene provided fomesafen resistance in rice, a foliar assay was conducted in the greenhouse. T1 seeds were pre-germinated on Petri dishes containing Murashige and Skoog (MS) medium (Murashige and Skoog 1962) (Appendix A). After shoot and root emergence from the hull, the seedlings were transferred to 15-cm-diameter pots filled with commercial potting soil (Sunshine® Premix No. 1;

Sun Gro Horticulture, Bellevue, WA). At V3 (third-leaf collar visible; Counce et al. 2000), the plants were sprayed with 0.78 kg ha^{-1} fomesafen (Flexstar®, Syngenta Crop Protection, Greensboro, NC), with 0.5 %v/v non-ionic surfactant (Induce, Helena Chemical, Collierville, TN). This dose corresponds to twice the maximum allowed dose in soybean fields (Anonymous 2020). This herbicide is not labeled in rice. After treatment, the plants were returned to the greenhouse and watered 48 h later, and as needed. Twenty-eight T_1 plants (1 plant per pot) were sprayed. The experimental units were arranged in a completely randomized design. Wild type (WT) rice plants were used as the susceptible reference.

Plant injury (%) was evaluated at 2 weeks after treatment (WAT) on a rating scale of 0 to 100%, where 0 = no injury and 100 = dead plant without green tissue (Burgos et al. 2013; Frans et al. 1986). The respective nontreated checks of WT and T_1 were used for comparison. To distinguish T_1 plants with high resistance from the ones with low resistance, injury data of T_1 and WT plants were analyzed using hierarchical clustering in JMP Pro v.15 (SAS Institute, Cary, NC.). Leaf tissues were collected from T_1 plants and the presence of the transgene was verified by PCR as described previously. A scatter plot was prepared in SigmaPlot version 14.0 (Systat Software, San Jose, CA) to visualize the correlation between transgene presence and plant injury level.

T_1 plants containing the construct, and showing low injury, were cultured to produce seeds T_2 seeds. To have enough seeds to conduct the physiological tests, a pool of seed was created from 18 T_1 plants.

Sensitivity of Wild Type Rice to Fomesafen. Although all WT plants showed higher injury (30 to 60%) than the transgenic plants, the foliar application of fomesafen did not control WT plants

completely. To avoid any ambiguity, dose-response assays were conducted to assess the sensitivity of WT plants to fomesafen. The first dose-response assay consisted of postemergence application of fomesafen. The experiment was conducted in a completely randomized design with four replications and nontreated checks as control. Each replication consisted of one pot with 5 plants. WT seedlings were grown in 11x11-cm pots until the V3 stage and sprayed with 390 (1x), 780 (2x), 1170 (3x), 1560 (4x), and 3120 (8x) g ha^{-1} fomesafen. The herbicide was sprayed with 0.5% v/v of non-ionic surfactant in a spray chamber equipped with an air-propelled motorized boom, fitted with 1100067 nozzles (Teejet, Wheaton, IL) calibrated to deliver 187 L ha^{-1}. The treated plants were assessed visually relative to nontreated check plants at 2 WAT using a scale of 0 to 100%, where 0 = no herbicide injury and 100 = complete control.

Since rice is not highly sensitive to the foliar application of fomesafen, a second dose-response assay was conducted with preemergence application of fomesafen to field soil medium. The experiment was separated by doses with four replications and a nontreated check as control. A replication consisted of one 12.2- by 9.5- by 5.7-cm flat filled with a 1:1 ratio of field soil and commercial potting soil. Following protocol used by Brabham et al. (2019), the flats with soil were soaked in water and allowed to drain to field capacity prior to planting and spraying to ensure the incorporation of the herbicide into the soil. Eight seeds were placed in each flat. Immediately after planting, the pots were sprayed 0.125x, 0.25x, 0.5x, 1x, 2x of the recommended dose (390 g ai ha^{-1} fomesafen). Treatments were sprayed in a spray chamber equipped with an air-propelled motorized boom, fitted with 1100067 nozzles (Teejet, Wheaton, IL) calibrated to deliver 187 L ha^{-1}. At 2 WAT, pots were assessed to determine germination reduction (%) based on the number of germinated plants in nontreated control.

Data from the foliar and soil assays were analyzed by regression using the "drc" package in R 3.5.1 (Ritz et al. 2015). The curves generated to soil and foliar assay were plotted in the same graph. The model used (three-parameter Weibull I) is defined by:

$$Y = c + (d - c)exp\,[-exp\big(b(log(dose) - \log\,(ED50)\big)]$$

where *Y* is the herbicide injury (%), *c* and *d* are standard parameters determining the lower and upper limits of the dose-response curve, and *b* is the slope around ED_{50} which is the dose level giving 50% of response of *Y* (Holland-Letz et al. 2019; Ritz 2010). The herbicide dose to cause 50% injury (ED_{50}) was calculated for the two application methods.

Response of T_2-*ΔG210* Seed to Soil-Applied Fomesafen. A soil-based assay was conducted to determine if the presence of the transgene would correlate with higher germination capacity and lower seedling injury from soil-applied fomesafen. Flats (12.2- by 9.5- by 5.7-cm) were filled with a 1:1 ratio of field soil and commercial potting soil. Prior to planting, the flats were presoaked and allowed to drain. Eight T2 or WT seeds were planted in each flat. After planting, the flats were sprayed with 390 g ha^{-1} fomesafen (maximum labeled dose in soybean). Treatments were applied with a CO_2-pressurized backpack sprayer (Teejet, Wheaton, IL) sprayer attached to a handheld boom fitted with one 8002 XR even flat fan nozzle calibrated to deliver 187 L ha^{-1}. The experiment was arranged in a completely randomized design with three replicates and two runs. Each flat is one replication. Nontreated checks were included.

Seedling emergence count and visible injury of each emerged seedling (%) were evaluated at 3 WAT. Height (cm), number of tillers, and number of panicles were recorded at the reproductive stage. Germination reduction (%) relative to nontreated checks was calculated using the formula:

Germination reduction (%)

$$= \frac{Germination\ of\ nontreated - Germination\ of\ treated}{Germination\ of\ nontreated} x\ 100$$

The injury data were subjected to analysis of variance (ANOVA) using GLIMMIX function in SAS v. 9.4 (SAS Institute, Cary, NC 27513). The run x treatment was not significant; therefore, the data from two runs were combined, resulting in eight replications. Since the germination reduction data did not fit a normal distribution via Shapiro-Wilk test, beta distribution was assumed for this response analysis (Gbur et al. 2012). Student's t test ($p < 0.05$) was used to compare means if the treatment effect was significant. Injury per survivor (%) data were analyzed using hierarchical clustering in JMP Pro v.15 (SAS Institute, Cary, NC.). The data obtained for height, number of tillers and number of panicles at reproductive stage were grouped by the clusters created with the injury per survivor. Then, the data were subjected to analysis of variance (ANOVA) using the JMP Pro v.15 (SAS Institute, Cary, NC.). Phenotypic measurements from nontreated WT plants were used for comparison. Fisher's protected LSD ($p < 0.05$) was used for comparison if means were significant.

Leaf tissues were collected from survivors to verify the presence of the transgene, perform gene expression analysis, and obtain the number of gene copies (described below). Leaf tissues from the nontreated T_2 plants were also collected to verify the frequency of transgene presence (%) in the T_2 generation and relate this with the number of survivors from the soil-based assay. The survivors were cultured to produce T_3 seeds.

Agar-Based Germination Assay with T_2 Seeds. To further evaluate the tolerance of the transgenic rice line to fomesafen, an agar-based germination assay with different fomesafen concentrations was conducted. Following the protocol by Nishimura et al. (2006), rice seeds (WT

and T_2) were dehulled then surface-sterilized in 70% ethanol for 30 s, submerged in 30% sodium hypochlorite with sodium dodecyl sulfate (SDS), and shaken for 30 min. The seeds were then rinsed five times with sterile water, and left to dry on a sterile surface.

The sterile seeds were placed in round Petri dishes containing 25 mL of half-strength MS medium supplemented with aliquots from the stock to attain 0, 5, 10, 20, 40, 60, 80 and 100 µM of fomesafen. The stock solution was prepared by dissolving 50 mg technical grade fomesafen (Sigma Aldrich, St. Louis, MO) in 250 µL acetone. The control also received 100 µL acetone to verify potential toxicity of acetone alone. Each Petri dish was divided in two; one-half contained 5 seeds of T_2 and the other half had 5 seeds of WT.

The plates were incubated at 26°C in a plant growth room at the Rosen Alternative Pest Control Center, University of Arkansas. Root growth (%) was evaluated visually at 2 WAT relative to a nontreated check. Data were analyzed by regression using the "drc" package in R 3.5.1 (Ritz et al. 2015). The model used (a three-parameter log-logistic) is defined by:

$$Y = \frac{d}{1 + \exp\{b[\log(x) - \log(ED50)]\}}$$

where *Y* is the root growth (%), d is the upper horizontal asymptote, x is the fomesafen dose, and *b* is the slope around ED_{50} which is the dose level giving 50% of response of *Y* (Ritz 2010). The herbicide dose to cause 50% of root growth reduction (ED_{50}) was calculated. Leaf tissues of T2 survivors from 20-100 µM treatments were collected for DNA extraction. The presence of the transgene in transgenes showing root growth was verified by PCR using the primer pair and PCR conditions described in Section "Plant Transformation with Palmer amaranth *ΔG210 - ppo2*".

Gene Expression Analysis of Transgene Carrying Palmer amaranth *ppo2* gene in T_2 survivors from Soil-Applied Fomesafen. For RNA extraction, leaf tissues were collected in liquid nitrogen and stored at -80°C. RNA was extracted following a modified version of the extraction protocol developed by Hongbao et al. (2008) using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA). The RNA extracted was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). To remove potential genomic DNA, the samples were treated with DNase (Invitrogen, Carlsbad, CA, USA). After DNase treatment, RNA was re-quantified and converted to cDNA using qScript® cDNA SuperMix kit (Quanta BioSciences, San Diego, CA).

T_2 survivors of soil-applied fomesafen showed different levels of injury. To further understand this wide variation, quantitative real-time PCR (qPCR) was performed to determine if Palmer amaranth *ΔG210-ppo2* gene expression differs among these plants. Fifteen plants representing the whole range of injury levels were used to conduct this experiment. RNA from WT and T_0 plants were included as negative and positive control, respectively. A T_2 sample without the transgene was also included as a second negative control. Blank controls consisting of primers without DNA were included.

The qPCR reaction mixture (10 µl) consisted of 5 µl iTaq Universal SYBR® Green SuperMix (BioRad, Hercules, CA), 1 µl of cDNA, 0.5 µl of 5 µM forward and reverse primers (Table 1), and 3 µl of nuclease free water. The qPCR was conducted using a CFX96 Real-Time PCR machine (BioRad, Hercules, CA) using the following conditions: 2 min at 95°C, followed by 40 cycles of 30 s denaturation at 95°C, 1 min annealing at 59°C, 1 min extension at 65°C, followed by melt-curve analysis. Each sample was analyzed in two technical replicates. Melt curve analysis was used to verify the specificity of cDNA products. Primers were designed to

target Palmer amaranth *ppo2* containing the deletion. The Ct values were normalized against native *PPO2* from rice (OsPPO2), ubiquitin (ubiQ) and eukaryotic elongation factor1-alpha (eEF-1α). Primer sequences are given in Table 1.

The fold-change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method where ΔCt = [Ct target gene – Ct of the internal control gene], ΔΔCt = [ΔCt targeted sample – ΔCt wild type] and $2^{-\Delta\Delta Ct}$ = gene expression fold compared to WT (Livak and Schmittgen 2001). Scatter plots correlating the fold-change by individual to its respective injury level at 3WAT, height, number of tillers and number of panicles at the reproductive stage were generated.

Gene Copy Number Verification in T_2 survivors from Soil-applied Fomesafen. Genomic DNA was extracted from leaf tissue using a modified version of the extraction protocol by Edwards et al. (1991) and quantified using a NanoDrop spectrophotometer. A quantitative real-time polymerase chain reaction (qPCR) was used to determine the transgenic *ppo2* copy number relative to the native rice *PPO2*. The same primer pairs qPPO2F4 x qPPO2R4 and OsPPO2F2 x OsPPO2R2 (Table 1), previously used in the gene expression assay, were used in this study. For the qPCR, 10 µL reactions consisting of 5 µL iTaq Universal SYBR® Green SuperMix, 0.5 µL forward and reverse primers (5 µM), 1 µL gDNA (100 ng/ µL) and 3 µL nuclease-free water. The qPCR was conducted using the same settings used in Section "Gene Expression Analysis of Transgene Carrying Palmer amaranth *ppo2* gene in T_2 survivors from Soil-Applied Fomesafen". The amplification was done in two technical replicates. Blank controls consisting of primers without DNA were included. To calculate the genomic copy number of transgenic *ppo2* relative to native *PPO2*, F4R4 Ct value was compared to the endogenous gene (OsPPO2 Ct value) to normalize F4R4 Ct values of each reaction [ΔCt = (Ct *A. palmeri PPO2* – Ct of *O. sativa*

PPO2)]. Using a modified approach based on the method used by Ingham et al. (2001), gene copy number was calculated as $2^{-\Delta Ct}$. Graphs correlating copy number to injury, gene expression and morphological traits were generated.

Detection of Presence of Transgene Among Selected T_3 Lines. To verify the presence of transgene among the lines, nine to ten seedlings from three T_2 survivors of soil-applied fomesafen with low injury (under 50%) and two with high injury (90%) rates were grown in commercial potting soil for DNA extraction. Leaf tissues were collected, and DNA was extracted as described previously. The transgene was PCR-amplified to confirm its presence. The PCR reaction was conducted as described previously. DNA from WT and T_0 plants was used as negative and positive controls, respectively.

Results and Discussion

Response of T_1-*ΔG210* Plants to Foliar-Applied Fomesafen. No plant was completely controlled with foliar application of fomesafen. The foliar injury ranged from 0 to 60% among T_1 and from 30 to 60% among WT plants (Figure 4). The hierarchical cluster analysis generated two groups of plants based on their injury levels (Table 2). Cluster 1 was characterized as "highly tolerant" and cluster 2 as "minimally tolerant". Sixty-eight percent (68%) of T_1 plants were highly tolerant to fomesafen, showing low injury. Cluster 1 consisted of plants with injury under 10% and was comprised only of T_1 plants (Figure 5). Plants in cluster 2 had injury ranging from 30 to 60% and included T_1 and WT individuals.

In general, T_0 plants were hemizygous for the inserted gene. Therefore, T_1 plants are expected to be hemizygous, homozygous positive, or null (homozygous negative) for the

transgene after segregation (Christou et al. 1989; Low et al. 2018; Passricha et al. 2016). Out of the 28 T_1 plants sprayed, four did not have the transgene (Figure 6). The plants without the transgene were among the individuals with high injury. All individuals with low injury harbored the transgene. Despite the correlation between transgene presence and low injury, five transgene-positive plants showed high injury. The absence of the expected tolerance level may be the result of gene silencing. Gene copy number and gene expression were investigated in T_2 generation.

It was observed a proportion of seven transgene positive plants to one transgene negative plant implying non-Mendelian transgene transmission patterns. Both Mendelian and non-Mendelian genetic segregation may occur in the offspring of transgenic T_0 plants produced via particle bombardment (Register III et al. 1994; Toki et al. 1992; Tomes et al. 1990; Vain et al. 2002).

Sensitivity of Wild Type Rice to Fomesafen. As observed in the foliar assay, no WT plant was completely controlled with foliar applications of 2X the maximum labeled dose of fomesafen (780 g ha^{-1}). To determine the inherent tolerance level in WT individuals, dose-response assays with foliar- and soil- applied fomesafen were conducted. WT was not controlled 100% with 8X the maximum dose (3120 g ha^{-1}) when fomesafen was sprayed postemergence (Figure 7). However, when fomesafen was applied preemergence, WT was completely controlled at 1X the maximum dose (390 g ha^{-1}) (Figure 8). The estimated fomesafen dose that would result in 50% injury of WT plants was 109 g ha^{-1} and 1020 g ha^{-1} in soil and foliar applications, respectively (Figure 9).

This intrinsic tolerance to PPO-inhibitor herbicides applied postemergence has been observed before. Experiments conducted with two other diphenylether herbicides

(chlomethoxyfen also known as chlomethoxynil and oxyfluorfen) applied on rice indicated that rice was tolerant to the recommended rates of these herbicides. In fact, rice did not exhibit any phytotoxic symptoms when treated with chlomethoxyfen. Fast absorption and metabolic degradation of chlomethoxyfen was detected in rice seedlings (Ishizuka et al. 1988; Niki et al. 1975). Matsumoto et al. (1994) detected higher antioxidant activity and less herbicide absorption in rice compared to other species.

Fomesafen has strong soil activity, consequently, it is also used as a preemergence and preplant herbicide. The half-life of fomesafen in soil varies from 8.5 to 100 days depending on the physical, chemical, and biological features of the soil, and the environmental conditions (Costa et al. 2015; Mueller et al. 2014; Rauch et al. 2007; Shaner 2014; Ying et al. 2012). Organic matter, clay content and soil pH are some of the factors that highly impact the sorption rate of herbicides and their persistence in a specific soil. An increased persistence will likely result in injury to the next crop (Bresnahan et al. 2000; Silva et al. 2013). For this reason, the label for fomesafen does not allow rice planting in a 10-month interval; otherwise the crop may be severely injured (Anonymous 2020). Evaluating the effect of residual fomesafen in soil on rotational crops, Cobucci et al. (1998) found that rice should not be planted for at least 95 days after fomesafen application. Besides fomesafen, various reports have shown that rice seeds are quite vulnerable to the residual activity of other herbicides as well (Lawrence et al. 2018; Marchesan et al. 2010; Zhang et al. 2000).

Response of T_2-*ΔG210* Seed to Soil-Applied Fomesafen. Overall, fomesafen delayed germination for at least 5 days (up to one week) compared to nontreated controls. Means for the germination reduction between WT and T_2 were statistically different. Overall, the germination

of WT seeds was reduced 96% by fomesafen and that of T2 seeds was reduced 27% (Figure 10; Figure 11). This result shows that the presence of Palmer amaranth *ppo2* containing *ΔG210* confers resistance to soil-applied fomesafen in rice. Residual herbicides are recommended preemergence to suppress weeds before the crop emerges. Fomesafen applied preemergence reduced the germination of PPO-resistant Palmer to 64% (Umphres et al. 2018). Since all T_2 survivors from both runs carried the transgene (Figure 12), this increase in tolerance can be explained by the PPO-herbicide-resistant transgene. As shown in Figure 1, the transgene construct used to transform WT with *ppo2 ΔG210* had a ubiquitin promoter. Ubiquitin promoter directs high transgene expression in many young rice tissues (Capell et al. 2004; Toki et al. 1992). Cornejo et al. (1993) analyzed GUS gene expression in transgenic rice tissues transformed with ubiquitin promoter showing that this promoter was more active in young roots and leaves. However, the transgene activity oscillated across developmental stages of the transformed rice plants. For example, leaves collected during flowering stage displayed drastically reduced levels of gene expression when compared to young leaves.

Injury levels among T_2 individuals showed varied widely (from 30 to 95%). After cluster analysis, survivors with 30-50%, 51-70% and 71-95% injury were classified as highly tolerant, moderately tolerant, and slightly tolerant, respectively (Table 3). Although it was confirmed that the presence of the transgene endowed resistance to soil-applied fomesafen, it is necessary to study additional generations, using seeds from plants that exhibited low injury, to determine if high tolerance will be expressed consistently in the offspring.

The transgenic T2 plants were grouped into distinct clusters based on injury level. The phenotypic data of plants across clusters were subjected to analysis of variance. Phenotypic measurements of nontreated WT were also included in the analysis as a comparison factor. The

plants in cluster 1 (50% or less injury) were phenotypically different from those in cluster 3 (more than 80% injury) (Figure 13). Overall, cluster 1 comprised of taller plants with higher number of tillers and panicles. Plants comprising cluster 3 incurred substantial injury to delay their development. Despite initial injury, plants in cluster 1 had significantly more panicles than the WT plants. Similar recovery and higher number of panicles were also in other transgenic rice lines resistant to PPO herbicides (Ha et al. 2003a; Jung et al. 2010).

To determine the frequency of transgene presence (%) in the T_2 generation, the presence of the transgene was analyzed in all nontreated control plants from both runs (33 plants total). Out of 33 plants, 82% harbored the transgene (Figure 14). Comparing this value with that of T_2 survivors of soil-applied fomesafen (73% germination), we can correlate the presence of transgene to increased tolerance to soil-applied fomesafen. The degree of homozygosity and hemizygosity was not examined in this generation, but it can be assumed that the remaining non-germinated T_2 seeds from the soil assay may be hemizygous or null for the transgene.

Several authors have inserted herbicide-resistant mutations into foreign genomes to evaluate their contribution towards resistance and effect over plant characteristics such as yield (Achary et al. 2020; Chen et al. 2019; Fang et al. 2020; Lee et al. 1988; Sathasivan et al. 1991). Sathasivan et al. (1991) isolated a herbicide-resistant mutation from the acetolactate synthase (*ALS*) gene of *A. thaliana* and cloned it into *Nicotiana tabacum* cv Xanthi. This specific mutation conferred resistance to imidazolinone herbicides which inhibit ALS. The authors observed a 100-fold increase in *N. tabacum* resistance to the imidazolinone herbicide, imazapyr, compared to wild type. Chen et al. (2020) produced transgenic rice lines resistant to the herbicide trifluralin, an inhibitor of microtubule formation. The authors obtained these lines by isolating and cloning a specific mutation from the herbicide-resistant *Lolium rigidum* population. Thus far, there is no

published research evaluating the effects of the insertion of *ppo2* harboring *ΔG210* mutation from *Amaranthus* genus into a foreign plant genome. However, the *ppo* mutation Arg-128-Gly which also confers resistance to PPO-herbicides was isolated from PPO-resistant *Amaranthus retroflexus* L. in China and cloned into *A. thaliana.* The presence of this mutation induced high resistance to the PPO-herbicides tested in the study (fomesafen, lactofen and carfentrazone-ethyl) (Huang et al. 2020).

Agar-Based Germination Assay with T_2 Seeds. Seeds have inherent food reserves that sustain the germination until the seedlings start photosynthesis. In monocot seeds, these reserves consist of starch stored in the endocarp (Taiz and Zeiger 2006). Due to these food reserves, all T_2 and WT seeds germinated in agar in the presence of fomesafen. However, the root growth of WT seedlings was inhibited even at the lowest concentration (5 μM) of fomesafen (Figure 15). Except for one plant in the 10 μM treatment, the root growth of T_2 seedlings was not inhibited by up to 10 μM fomesafen (Figure 16). Significant reduction in root growth occurred starting at 20 μM. Although reduced, roots were growing from five T_2 individuals at 20 to 60 μM fomesafen. Only one seedling had root growth at 80 and 100 μM treatment. The estimated fomesafen concentration that would result in 50% growth reduction in T_2 was 45 μM.

In agreement with the results obtained in this experiment, Lee et al. (2004) showed that transgenic rice line overexpressing human *ppo2* in wild type rice (cv. 'Dongjin') was tolerant to up to 5 μM of oxyfluorfen in agar-based seed germination assay, while the wild type was sensitive to the lowest concentration of 2 μM. Also, this transgenic line was able to germinate in the 20 μM treatment, but the seedlings exhibited severe injury compared to nontreated ones. Like fomesafen, oxyfluorfen is also a diphenylether PPO-herbicide. A different transgenic rice line

expressing *Myxococcus xanthus* PPO in chloroplasts and mitochondria showed high tolerance to oxyfluorfen up to 100 μM with germination in up to 500 μM, whereas the wild type did not germinate at the lowest concentration (Jung et al. 2004; Jung et al. 2006). Both studies used the wild type rice line *cv.* 'Dongjin' which is apparently more sensitive compared to the one used in the current study, *cv.* 'Nipponbare'.

With one exception, root growth was correlated to the presence of Palmer amaranth *ppo2 ΔG210* transgene. Since the majority of nontreated T_2 seedlings from the soil assay were transgene positive (Figure 14), it is likely that some of the T_2 seeds, without root growth in agar with higher concentrations of fomesafen, also carried the construct.

Gene Expression Analysis of Transgene Carrying Palmer amaranth *ppo2* gene in T_2 survivors from Soil-Applied Fomesafen. All T_2 plants carrying the transgene had 155- to 1144-fold increase in gene expression when compared to WT and T_2 plants, which were negative for the transgene. The majority of T_2 plants (10 out of 15) showed transgene expression <400-fold when calculated against native *PPO2* from *O. sativa* (Table 4). With one exception, individuals showing low injury assayed by qPCR had values < 400-fold (Figure 17). However, some individuals with higher injury were also part of this range of gene expression. All T_2 carrying the mutation showed expression when calculated against the ΔΔCt obtained with ubiquitin (Table 5 and Figure 18). Transgene expression calculated against eukaryotic elongation factor1-alpha showed no correlation with T_2 injury was detected when transgene expression was calculated against the ΔΔCt values obtained (Table 6 and Figure 19). Contrary to these results, the level of resistance to oxyfluorfen in transgenic rice overexpressing plastidic *A. thaliana ppo* increased with the level of PPO protein expression (Ha et al. 2003a). However, other studies

overexpressing plastidic *Arabidopsis thaliana ppo* or *Bacillus subtilis* PPO gene in tobacco plants demonstrated that the overall overexpression and, consequently, overproduction of the enzyme was enough to inhibit the toxicity caused by diphenylether herbicides. Therefore, the level of expression was not the main factor in conferring herbicide tolerance (Choi et al. 1998; Lermontova and Grimm 2000). In a different study, 50% of transgenic *A. thaliana* plants resistant to hygromycin had transgenic positive progenies susceptible to the antibiotic. A reduced level of transcript was detected in the sensitive transformants (Scheid et al. 1991). This contradicts the results obtained in this current gene expression assay wherein the plants with the highest tolerance attended to have lower *ppo2* gene expression.

Different levels of transgene expression occur frequently among transgenic individuals, and full or partial levels of gene expression may be heritable. Thus, transgene expression was expected to vary among T_2 plants. Gene silencing factors such as DNA cytosine methylation, variation in gene copy number (too low or too high), transgene integration in the chromosome, and low transcription, appear to affect in the transgene expression (Day et al. 2000; Matzke and Matzke 1998; McCabe et al. 1999). Gene segregation of a transgene across generations may impact its expression as well. Vain et al. (2002) analyzed the behavior of transgene expression across two generations where transgenic rice lines were generated through particle bombardment. It was observed that the transmission of partial transgenes to some or all progenies was around 14% of transgenic lines. Also, around 10% of the lines positively expressing the gene of interest in T_0 produced at least one T_1 plant that harbored the gene without expressing it. Therefore, the presence of transgene and its expression do not always result in the expected phenotypic response. Similarly, Ha et al. (2003b) observed that transgenic rice lines overexpressing *B. subtilis ppo* were not tolerant enough to germinate in the presence of

considerably low doses of oxyfluorfen in agar-based assay. Although the presence and expression of the transgene trigger phenotypic responses in plants treated with fomesafen, thus far, there is no clear correlation between transgene expression and injury, which is the indicator for tolerance to herbicide among T_2 survivors.

Gene Copy Number Verification in T_2 survivors from Soil-applied Fomesafen. Copy number ranged from less than one copy to eight copies, independent of the injury level (Figure 20). No correlation was observed between transgene copy number and injury. T_2 survivors showing less than 50% injury had transgene copy numbers that also ranged from 1 to 7 copies. Like in this study, Van der Krol et al. (1990), evaluating Petunia plants transformed to increase the expression of genes involved in flower pigmentation, found no correlation between gene expression or gene copy number with the phenotypic response.

When analyzing gene expression and transgene copy number together, the majority of T_2 plants showed inverse relation of copy number and *ppo2* transgene expression (Figure 21). In other words, when copy number was low, gene expression was high. Thus far, there are studies showing that gene copy number can be either positively (Hobbs et al. 1993; Kohli et al. 1999; McCabe et al. 1999) or negatively (Gendloff et al. 1990; Hobbs et al. 1993; Hobbs et al. 1990; Linn et al. 1990) correlated with transgene expression, or not at all (Bauer et al. 1998). For instance, Kohli et al. (1999) reported that an increase in transgene copy number did not necessarily mean a decline in gene expression or gene silencing induction. McCabe et al. (1999) obtained high expression in lines with multiple gene copies. In both studies, the multiple gene copy lines maintained a stable high gene expression up to at least the third generation. However, Hobbs et al. (1990) observed an overall decrease in gene expression in progenies with multiple copies. These

progenies with multiple copies were also prone to increased methylation of the transgenic DNA. Other researchers also did not find evidence that transgene expression depended on the number of integrated genes (Moravčíková et al. 2004).

Detection of Presence of Transgene Among Selected T_3 Lines. All T_2 survivors after soil application were grown as separate lines to collect T_3 seeds. As mentioned previously, only five plants were selected to detect the presence of the transgene among T_3 lines. Although the injury level varied from 30 to 90% among the selected T_2 plants, all T_3 progenies were confirmed to carry Palmer amaranth ppo2 transgene (Figure 22). As mentioned above, the DNA tested was from only 9-10 seedlings which is not enough to positively affirm 100% homozygosity. However, it would be expected according to Mendelian law that T_3 progeny would have shown a one transgene negative to three transgene positive pattern if T_2 survivors were hemizygous. As described previously, a total of 82% of T2 plants harbored the Palmer amaranth ppo2 transgene, but only 73% were able to germinate in the soil assay. Correlating these previous results with the ones obtained here, it can be assumed that only individuals carrying the mutation in both alleles were able to germinate in the soil assay.

Conclusions

The presence of Palmer amaranth *ppo2* containing *ΔG210* confers resistance to fomesafen in rice. Based on the lack of individuals null for the transgene in the T_3 generation, it can be assumed that the mutation needs to be present in both alleles to attain full resistance to soil-applied fomesafen. Thus far, the connection between gene expression level and gene copy number with injury level among survivors is not clear. The majority of transgenic plants are able to fully recover from preemergence treatment with fomesafen, whereas WT plants are killed.

This research also establishes the foundation that herbicide-resistant genes from weeds can be used as transgene to develop genomically unrelated herbicide-resistant crops.

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Tables

Table 1. Primer sequences used in the gene expression analysis by qPCR.

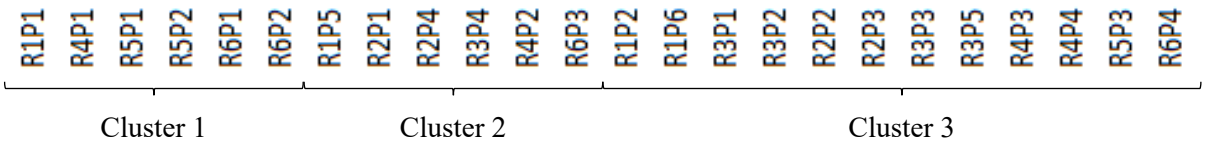
Targeted Gene	Primer Sequence	Primer Name
A. palmeri ppo2 containing *ΔG210* mutation	5'-AGGAAAAGGGTGGAGGAGAA-3'	qPPO2F4
	5'-GGACAGCACCTCACACTGG-3'	qPPO2R4
O. sativa native *PPO2*	5'-TGGTAACGTGAAGCTTGGTACA-3'	OsPPO2F2
	5'-CAGAAATTGACCAACCACCA-3'	OsPPO2R2
Eukaryotic elongation factor1-alpha (Jain et al. 2018)	5'-TTTCACTCTTGGTGTGAAGCAGAT-3'	eEF-1αF
	5'-GACTTCCTTCACGATTTCATCGTAA-3'	eEF-1αR
Ubiquitin	5'-CGCAAGTACAACCAGGACAA-3'	ubiQF
	5'-GCTGTGACCACACTTCTTCTT-3'	ubiQR

Table 2. Hierarchical clustering of injury data from T_1 and WT 'Nipponbare' plants at 2 weeks after postemergence treatment with 780 g ha^{-1} of fomesafen, University of Arkansas, Fayetteville, USA 2018.

Cluster	Category	No. of individuals	T_1	WT	Injury (%), 2 wks after treatment		
					Mean	Min	Max
1	Highly tolerant	19	19	0	6.9	0	10
2	Minimally tolerant	18	9	9	40.3	30	60

Table 3. Hierarchal clustering of 'Nipponbare' T2 survivors based on injury levels (%) at 2 weeks after preemergence treatment with fomesafen at 390 g ha^{-1}, University of Arkansas, Fayetteville, USA 2019.

Cluster	No. of individuals	Injury (%)		
		Mean	Min	Max
1[a]	6	40	30	50
2[b]	6	65	60	70
3[c]	12	88	80	95



[a] Cluster 1 = highly tolerant to fomesafen with injury <50% (6 individuals).
[b] Cluster 2= moderately tolerant to fomesafen with injury ranging from 51 to 70%.
[c] Cluster 3= slightly tolerant to fomesafen with injury >71%.

Table 4. The relative transgene *ppo2* (*A. palmeri*) expression in T_2 and wild type 'Nipponbare' rice calculated against native *PPO2* (*O. sativa*).

Samples	Injury (%)	AVG Ct *ppo2* transgene	AVG Ct *PPO2* native	ΔCt[a]	ΔΔCt[b]	Gene Expression Fold $(2^{-\Delta\Delta Ct})$[c]
R6P1	30	22.04	27.26	-5.22	-7.27	155
R6P2	30	23.44	30.53	-7.09	-9.14	565
R1P1	40	22.08	28.44	-6.36	-8.42	342
R4P1	40	21.23	27.19	-5.96	-8.02	259
R5P2	50	23.52	30.06	-6.54	-8.59	386
R6P3	50	23.36	28.66	-5.30	-7.36	164
R1P5	60	22.19	28.12	-5.93	-7.99	254
R3P4	60	21.90	29.50	-7.60	-9.66	809
R2P1	70	21.15	27.44	-6.29	-8.35	326
R1P2	80	23.28	29.24	-5.96	-8.02	259
R1P6	80	23.03	30.26	-7.23	-9.28	623
R3P1	80	23.59	29.10	-5.51	-7.57	190
R2P2	90	20.59	28.07	-7.48	-9.54	745
R2P3	90	24.30	30.73	-6.43	-8.48	358
R5P3	95	23.56	30.84	-7.28	-9.34	648
T2 (-tg)	-	33.89	31.05	2.84	0.79	0.58
WT1	-	31.61	29.55	2.06	0.00	1.00

[a] ΔCt = average Ct transgene – average Ct native.
[b] ΔΔCt = ΔCt targeted sample – ΔCt wild type.
[c] Gene expression = $2^{-\Delta\Delta Ct}$.

Table 5. The relative transgene *ppo2* (*A. palmeri*) expression in T_2 and wild type Nipponbare calculated against ubiquitin.

Samples	Injury (%)	AVG Ct *ppo2* transgene	AVG Ct ubiQ	ΔCt[a]	ΔΔCt[b]	Gene Expression ($2^{-\Delta\Delta Ct}$) [c]
R6P1	30	22.04	23.02	-0.98	-8.65	403.05
R6P2	30	23.44	24.83	-1.38	-9.06	534.75
R1P1	40	22.08	22.20	-0.12	-7.80	222.27
R4P1	40	21.23	22.65	-1.42	-9.10	549.23
R5P2	50	23.52	25.09	-1.56	-9.24	604.11
R6P3	50	23.36	24.42	-1.06	-8.74	427.46
R1P5	60	22.19	23.52	-1.33	-9.01	514.19
R3P4	60	21.90	23.89	-1.99	-9.67	813.55
R2P1	70	21.15	23.63	-2.48	-10.16	1144.57
R1P2	80	23.28	24.04	-0.76	-8.44	346.52
R1P6	80	23.03	24.04	-1.00	-8.68	410.03
R3P1	80	23.59	24.12	-0.53	-8.21	295.12
R2P2	90	20.59	22.57	-1.99	-9.66	811.68
R2P3	90	24.30	24.84	-0.53	-8.21	295.72
R5P3	95	23.56	25.03	-1.48	-9.15	569.37
T2 (-tg)	-	33.89	25.97	7.92	0.24	0.84
WT1	-	31.61	23.93	7.68	0.00	1.00

[a] ΔCt = average Ct transgene – average Ct ubiquitin.
[b] ΔΔCt = ΔCt targeted sample – ΔCt wild type.
[c] Gene expression = $2^{-\Delta\Delta Ct}$.

Table 6. The relative transgene *ppo2* (*A. palmeri*) expression in T_2 and wild type 'Nipponbare' calculated against native eukaryotic elongation factor1-alpha.

Samples	Injury (%)	AVG Ct *ppo2* transgene	AVG Ct eEF-1α native	ΔCt[a]	ΔΔCt[b]	Gene Expression Fold ($2^{-\Delta\Delta Ct}$)[c]
R6P1	30	22.04	19.46	2.59	-8.31	316.70
R6P2	30	23.44	21.99	1.46	-9.44	693.50
R1P1	40	22.08	19.40	2.68	-8.21	296.96
R4P1	40	21.23	18.92	2.31	-8.58	383.17
R5P2	50	23.52	22.19	1.34	-9.56	752.31
R6P3	50	23.36	22.23	1.13	-9.76	867.39
R1P5	60	22.19	21.25	0.94	-9.96	995.00
R3P4	60	21.90	21.13	0.77	-10.13	1118.28
R2P1	70	21.15	20.18	0.96	-9.93	976.44
R1P2	80	23.28	21.40	1.88	-9.02	517.90
R1P6	80	23.03	21.53	1.51	-9.39	670.62
R3P1	80	23.59	21.34	2.25	-8.64	400.13
R2P2	90	20.59	19.58	1.01	-9.89	948.41
R2P3	90	24.30	21.80	2.50	-8.39	335.79
R5P3	95	23.56	20.51	3.05	-7.84	229.76
T2 (-tg)	-	33.89	23.13	10.77	-0.13	1.09
WT1	-	31.61	20.71	10.89	0.00	1.00

[a] ΔCt = average Ct transgene – average Ct native.
[b] ΔΔCt = ΔCt targeted sample – ΔCt wild type.
[c] Gene expression = $2^{-\Delta\Delta Ct}$

Figures

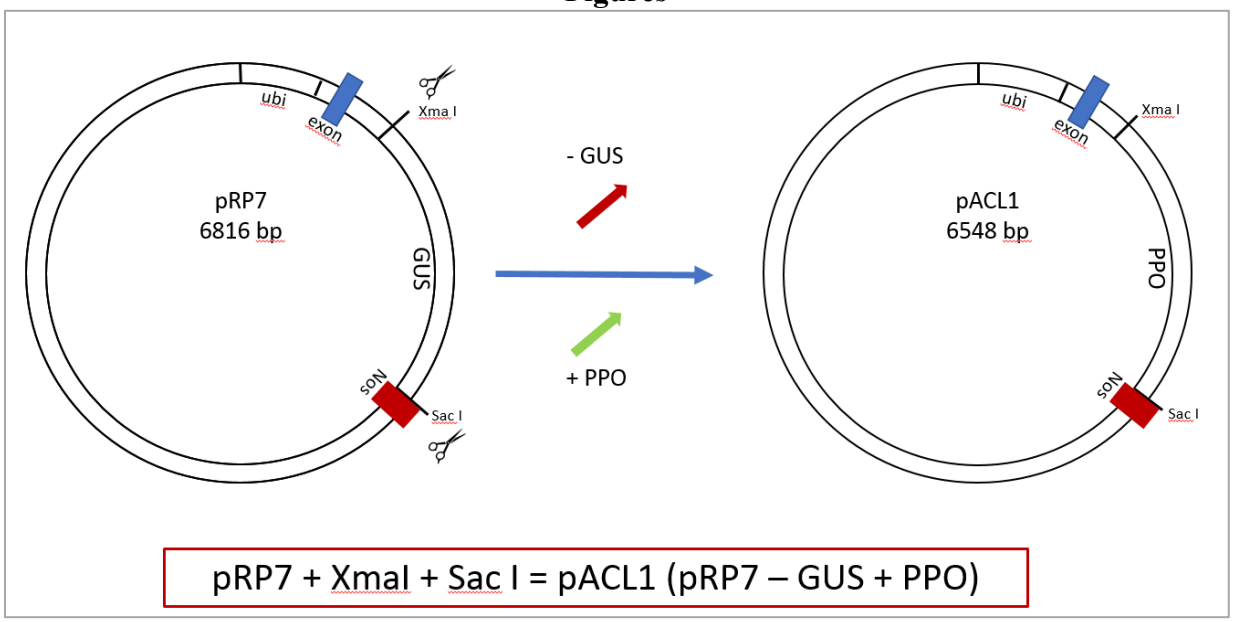


Figure 1. Construct used to transform the wild type 'Nipponbare' rice plants.

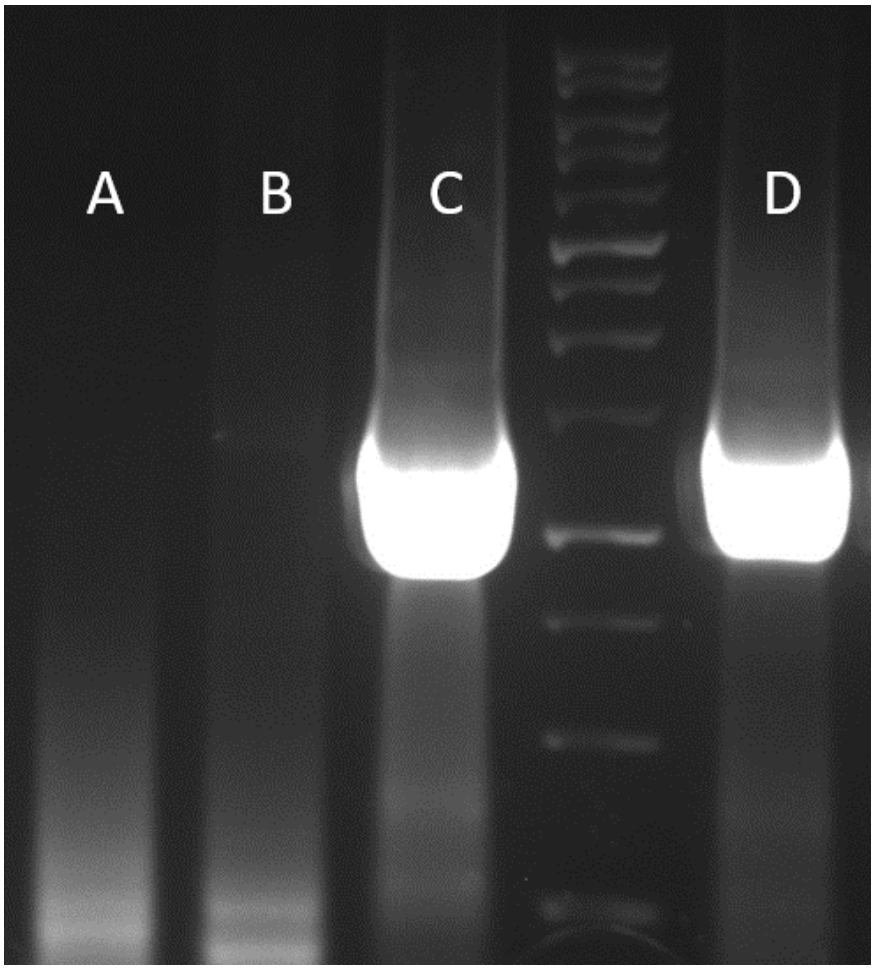


Figure 2. Detection of the *ppo2* (*ΔG210*) transgene in genomic DNA by PCR amplification. The bands were generated using Palmer amaranth *ppo2* primer pair (KpnF x SphR) flanking a 1.6kb region encoding Palmer *ppo2*. Lanes A and B: wild type (negative control); C: plasmid containing the transgene (positive control); D: T0, transformed rice plant regenerated from callus.

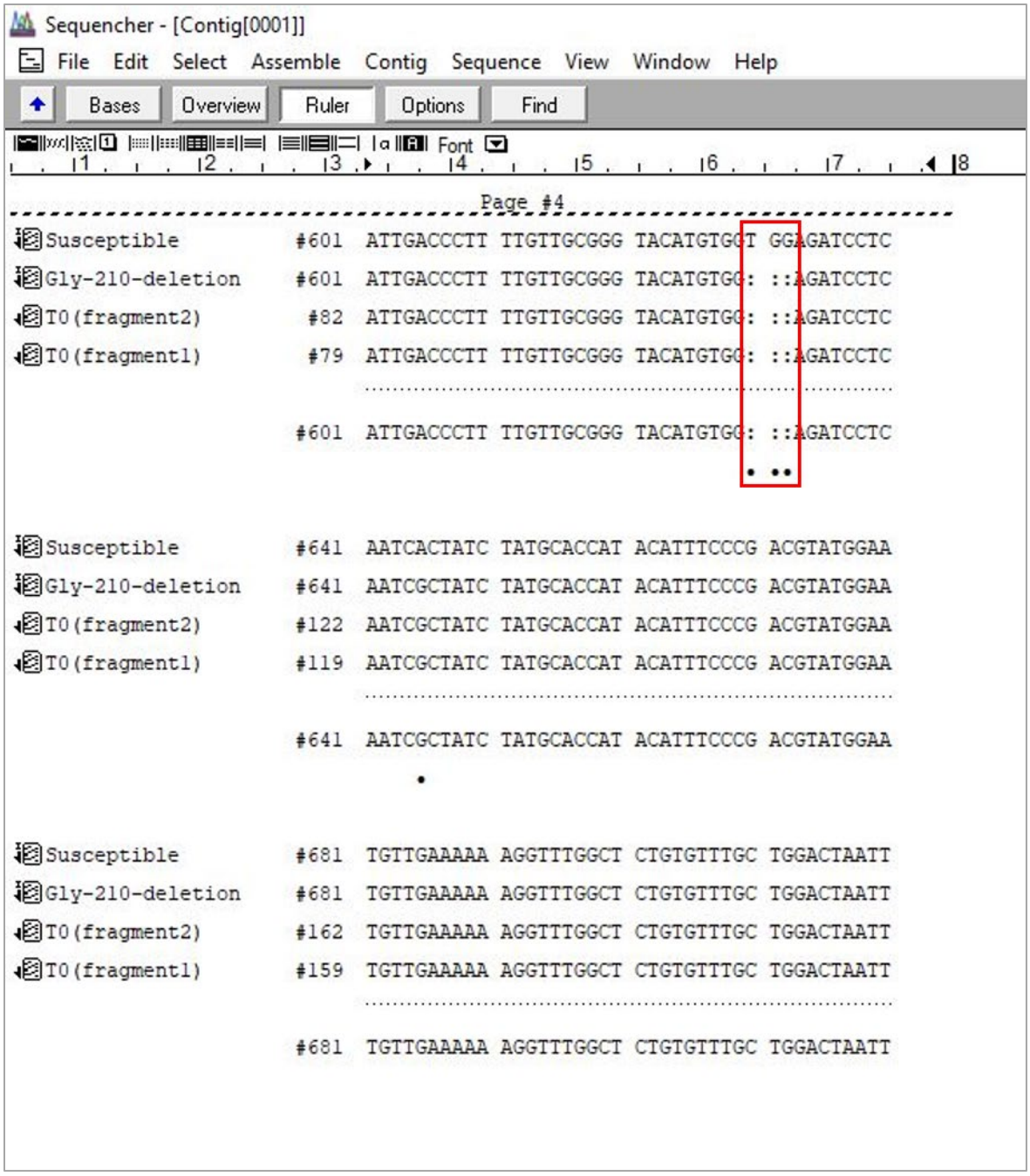


Figure 3. Nucleotide sequence alignment of plastidic protoporphyrinogen IX oxidase (*PPO2*) in sensitive (Susceptible), resistant (*ΔG210*) and transformed survivor (T_0 fragment 1 and 2). Transgenic plant fragments harbored *ΔG210*. *ΔG210* position is marked by red box in the picture.

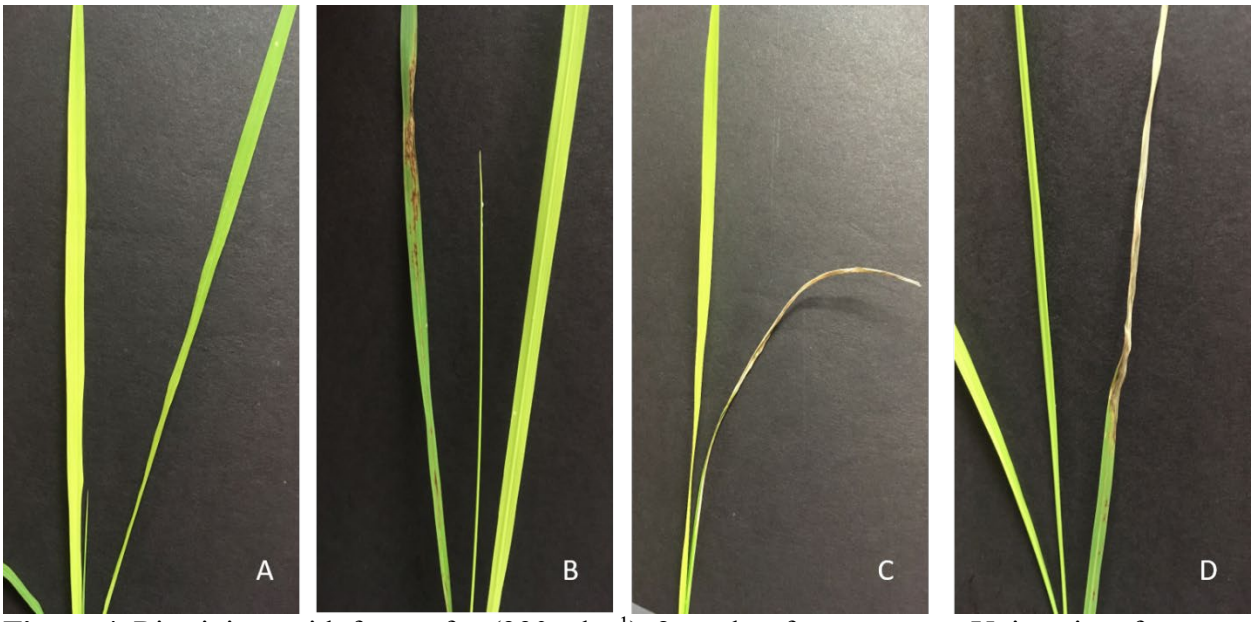


Figure 4. Rice injury with fomesafen (390 g ha^{-1}), 2 weeks after treatment, University of Arkansas, Fayetteville, USA 2018. Shown are T_1-*ΔG210* plants with low (A) and high (B) level of injury. Wild type plants (C and D) had high injury.

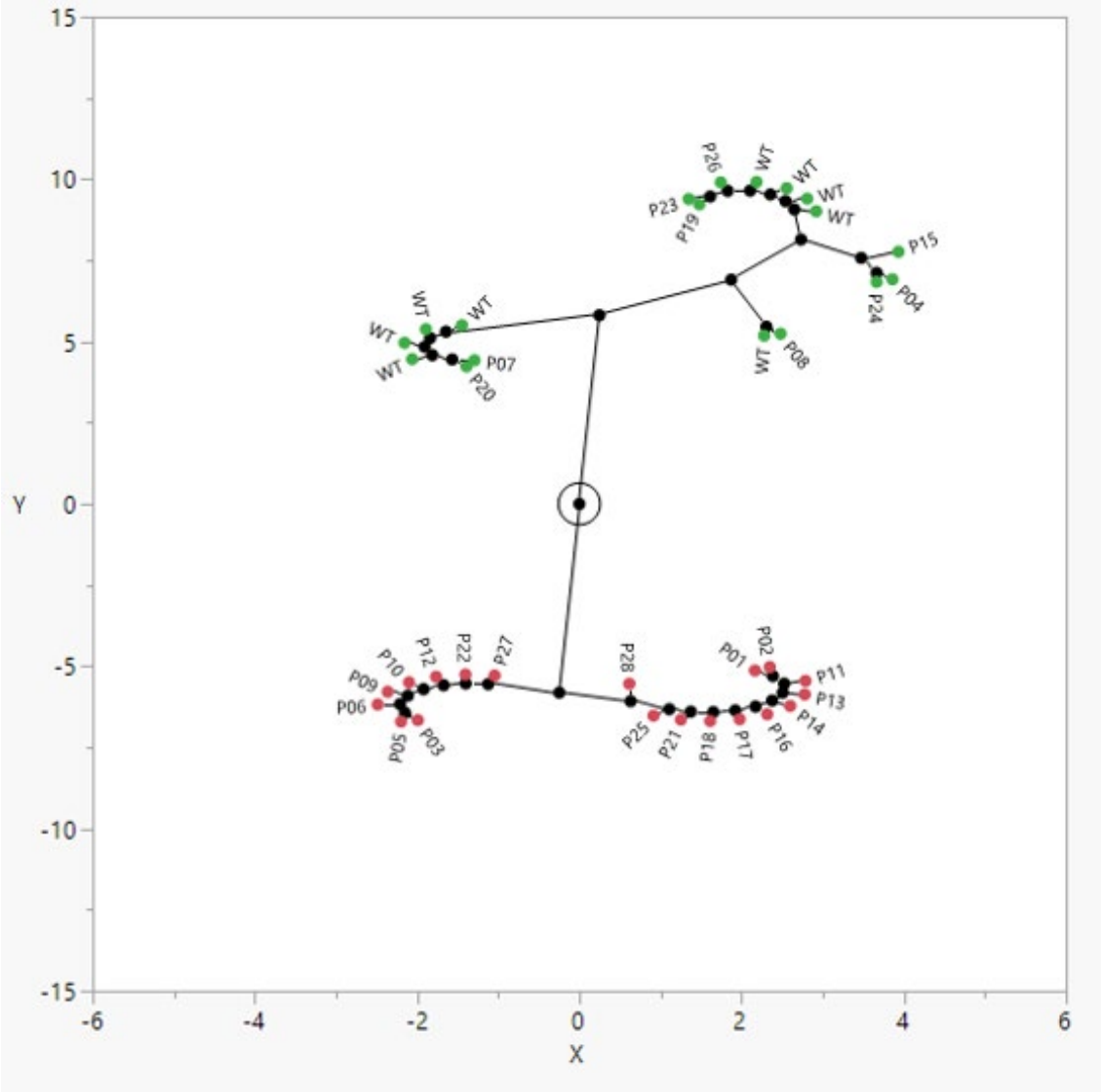


Figure 5. Constellation plot from the hierarchical clustering of T_1 and Wild type injury data collected 2 weeks after treatment with fomesafen (790 g ha^{-1}), University of Arkansas, Fayetteville, USA 2018. Cluster 1 (red) is composed of all transgenic plants. Cluster 2 (green) is composed of WT plants and transformed plants not expressing the transgene.

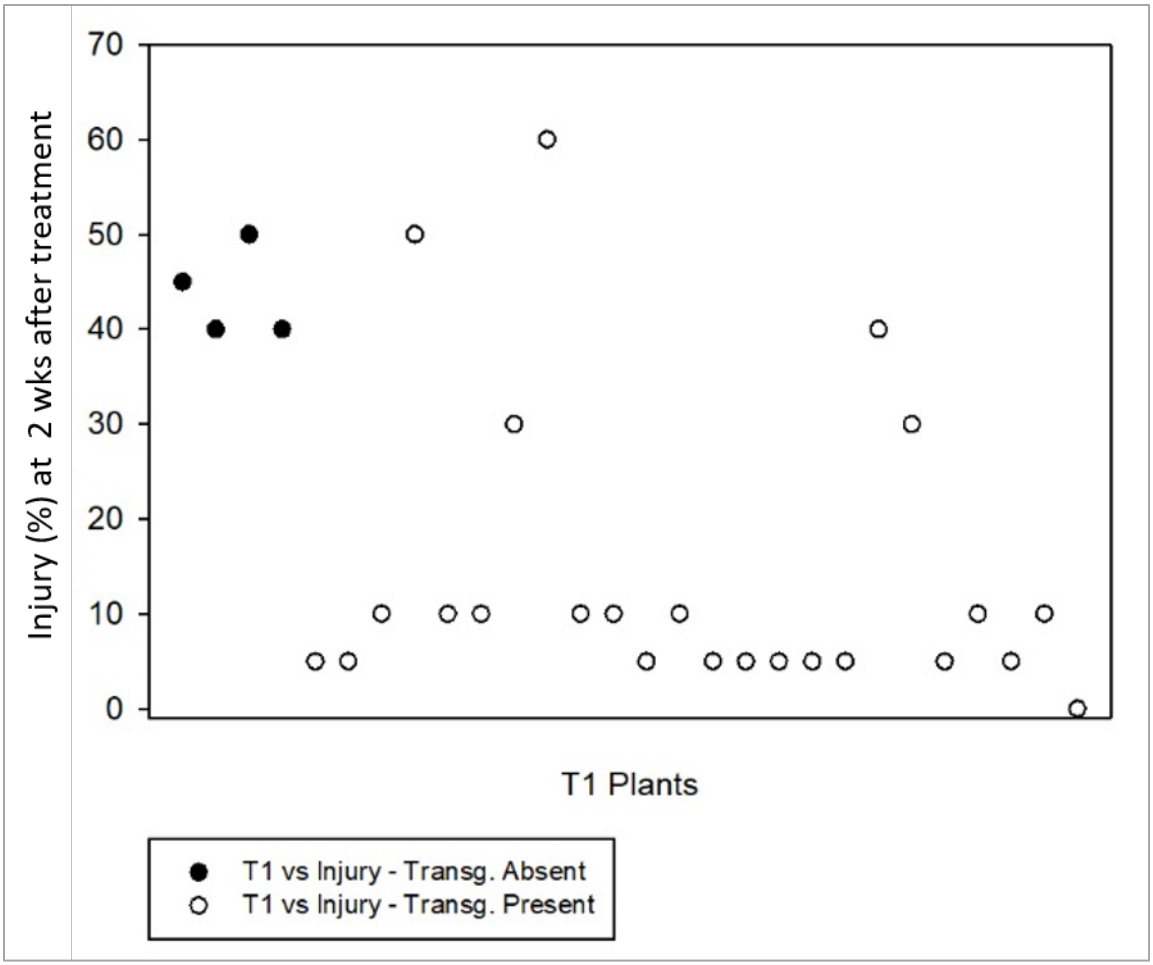


Figure 6. Scatter plot of foliar injury levels of T1 plants with (white circles) or without (black circles) the Palmer amaranth *ppo2 ΔG210* transgene, University of Arkansas, Fayetteville, USA 2018.

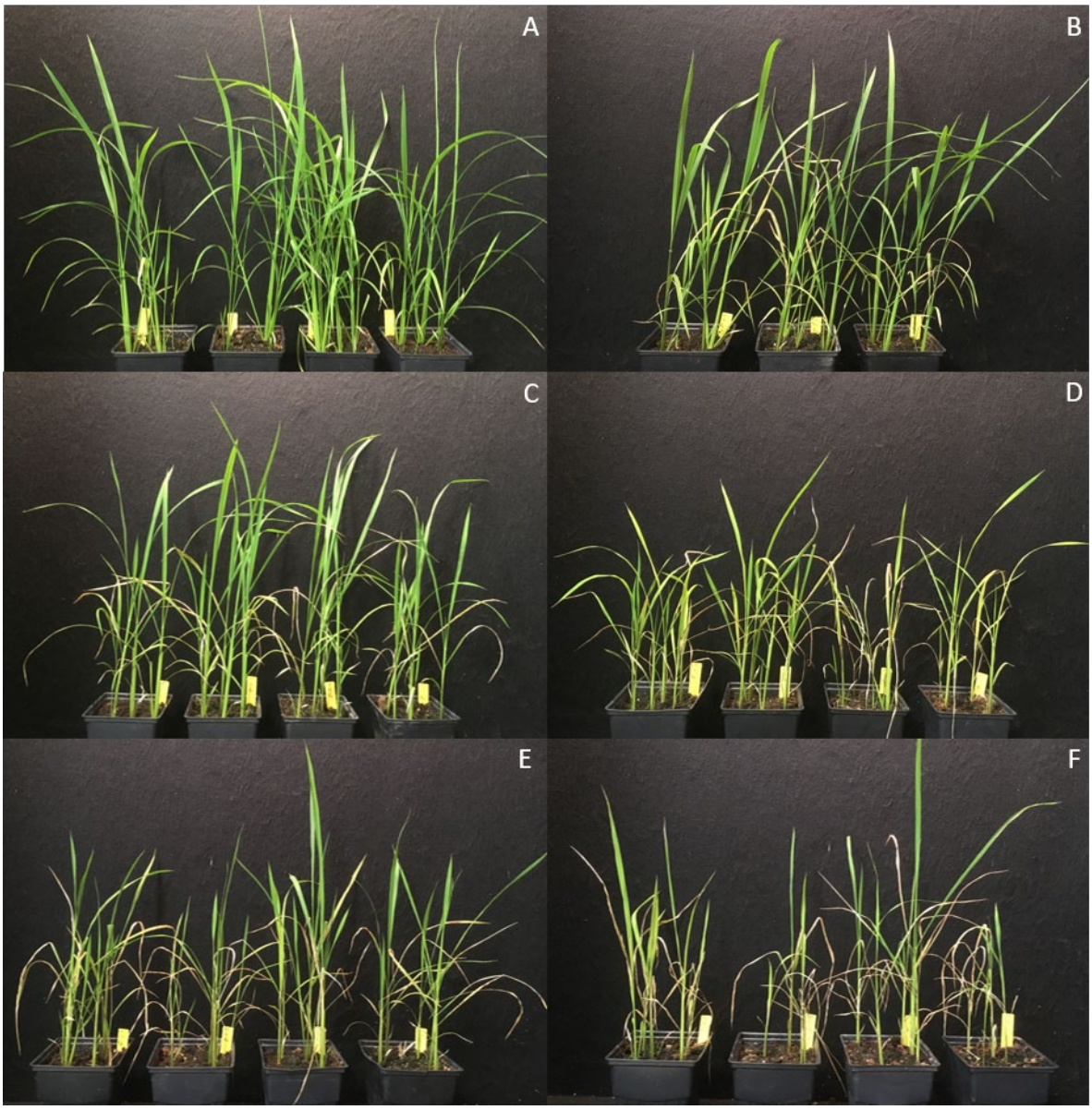


Figure 7. Rice injury (%) resulting from postemergence application of fomesafen on wild type 'Niponbare'. Picture contains all replications. A: nontreated check, B: 1x (390 g ha^{-1}), C: 2x (780 g ha^{-1}), D: 3x (1170 g ha^{-1}), E: 4x (1560 g ha^{-1}), and F: 8x (3120 g ha^{-1}), University of Arkansas, Fayetteville, USA 2019.

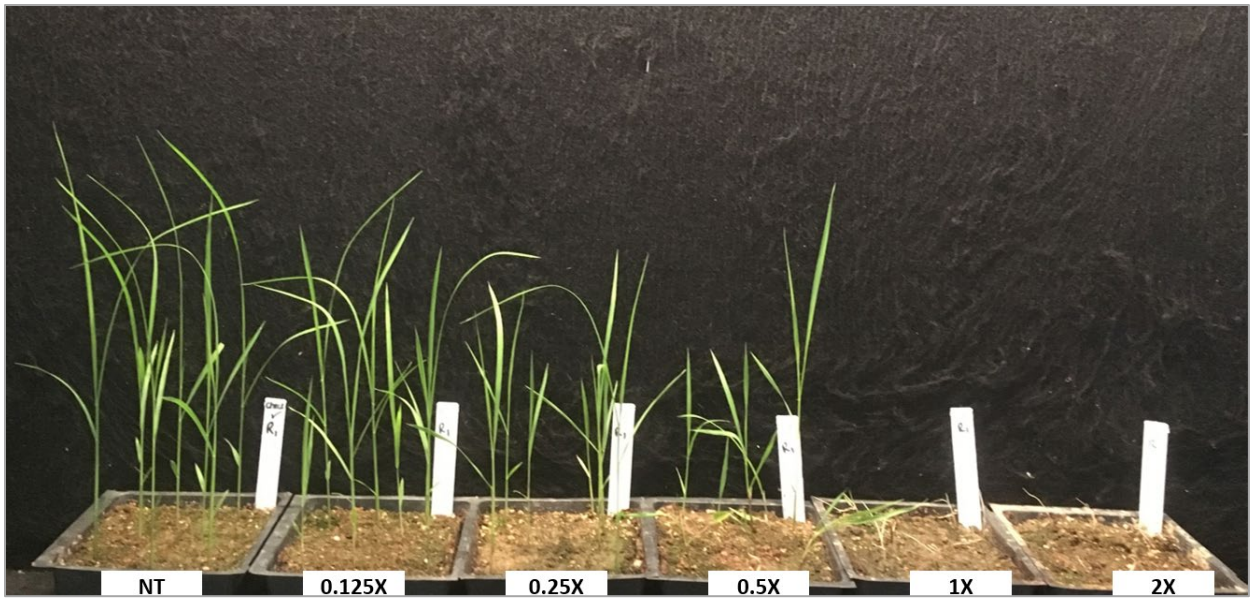


Figure 8. Wild type 'Niponbare' rice injury (%) from soil-applied fomesafen. NT: nontreated check, 0.125x (48.75 g ha^{-1}), 0.25x (97.5 g ha^{-1}), 0.5x (195 g ha^{-1}), 1x (390 g ha^{-1}) and 2x (780 g ha^{-1}), University of Arkansas, Fayetteville, USA 2019.

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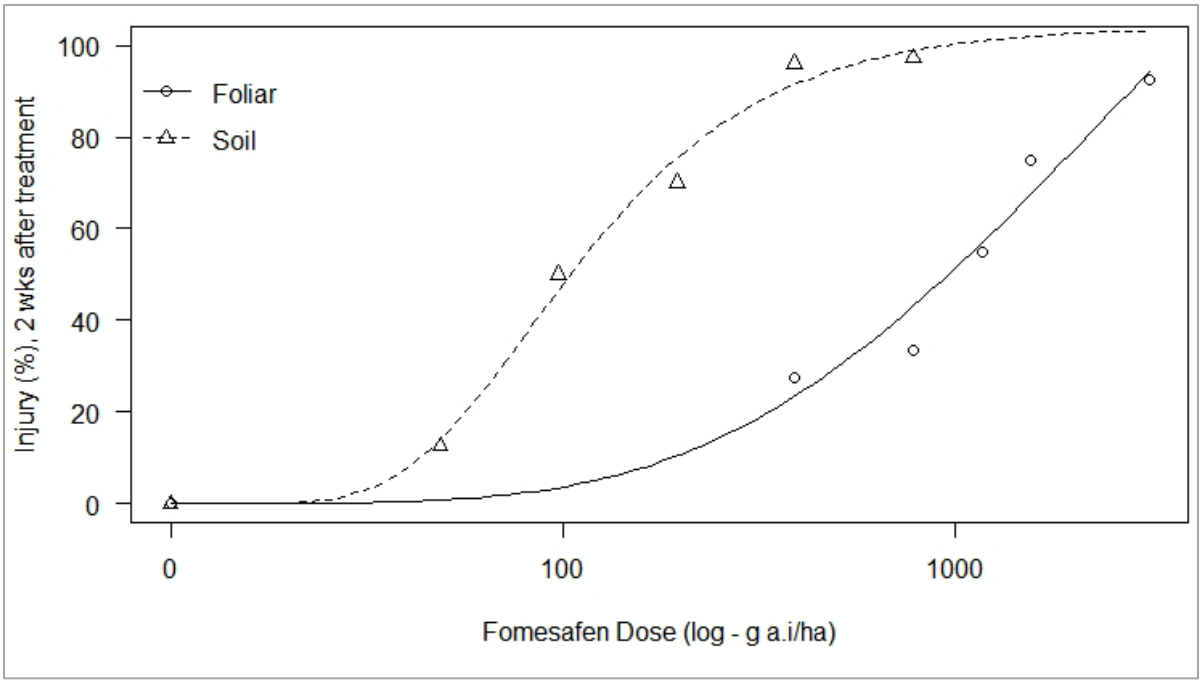

Figure 9. Dose response curve generated with the visible injury data (%) of wild type 'Niponbare' plants treated with fomesafen preemergence or postemergence, University of Arkansas, Fayetteville, USA 2019. Circles and triangles represent values from foliar and soil applications of fomesafen, respectively. Data were fitted to a non-linear, three-parameter Weibull I regression function $Y=c+(d-c)exp\{-exp[b(log(dose)-log(ED50)]\}$.

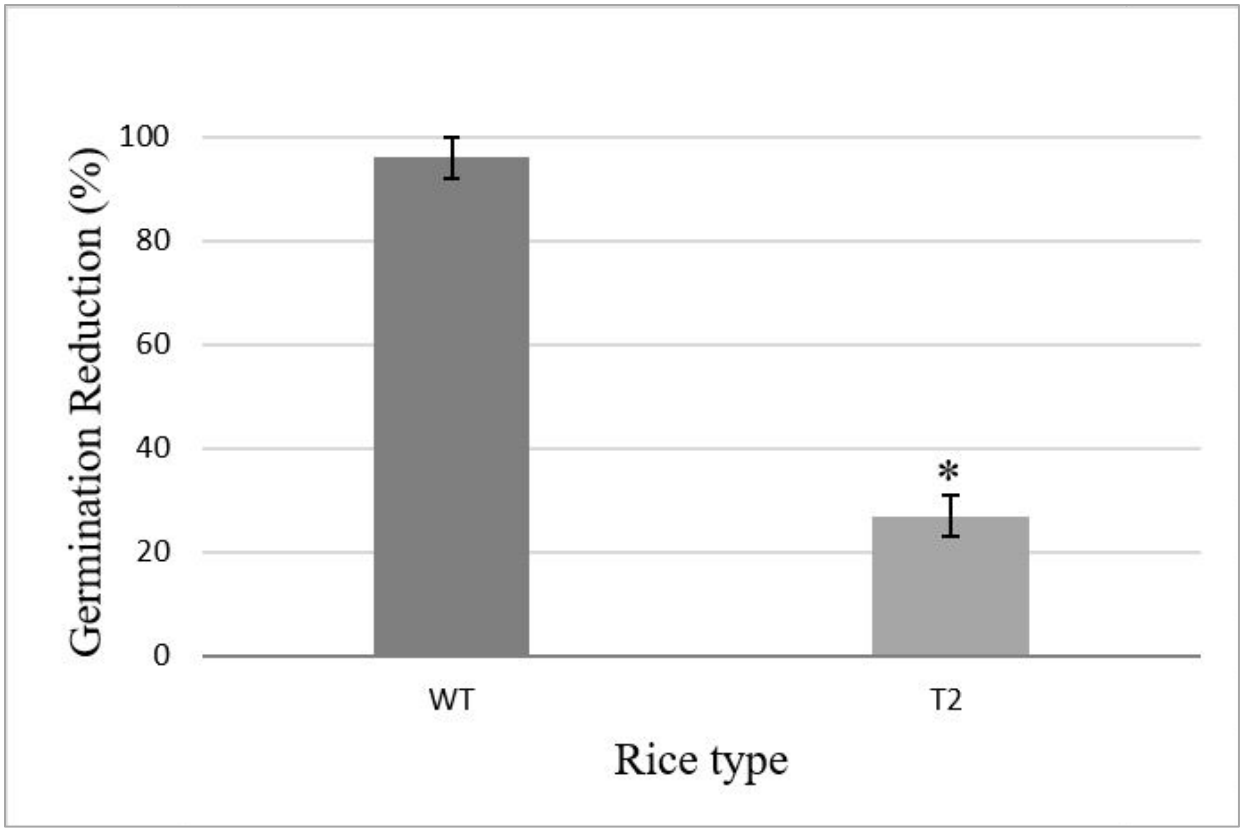

Figure 10. Wild type and T_2 germination as affected by soil-applied fomesafen (390 g ha-1), University of Arkansas, Fayetteville, USA 2020. Means were derived from combined analysis of two runs since plant response to treatments did not vary across runs. *Significant difference ($p <$ 0.05).

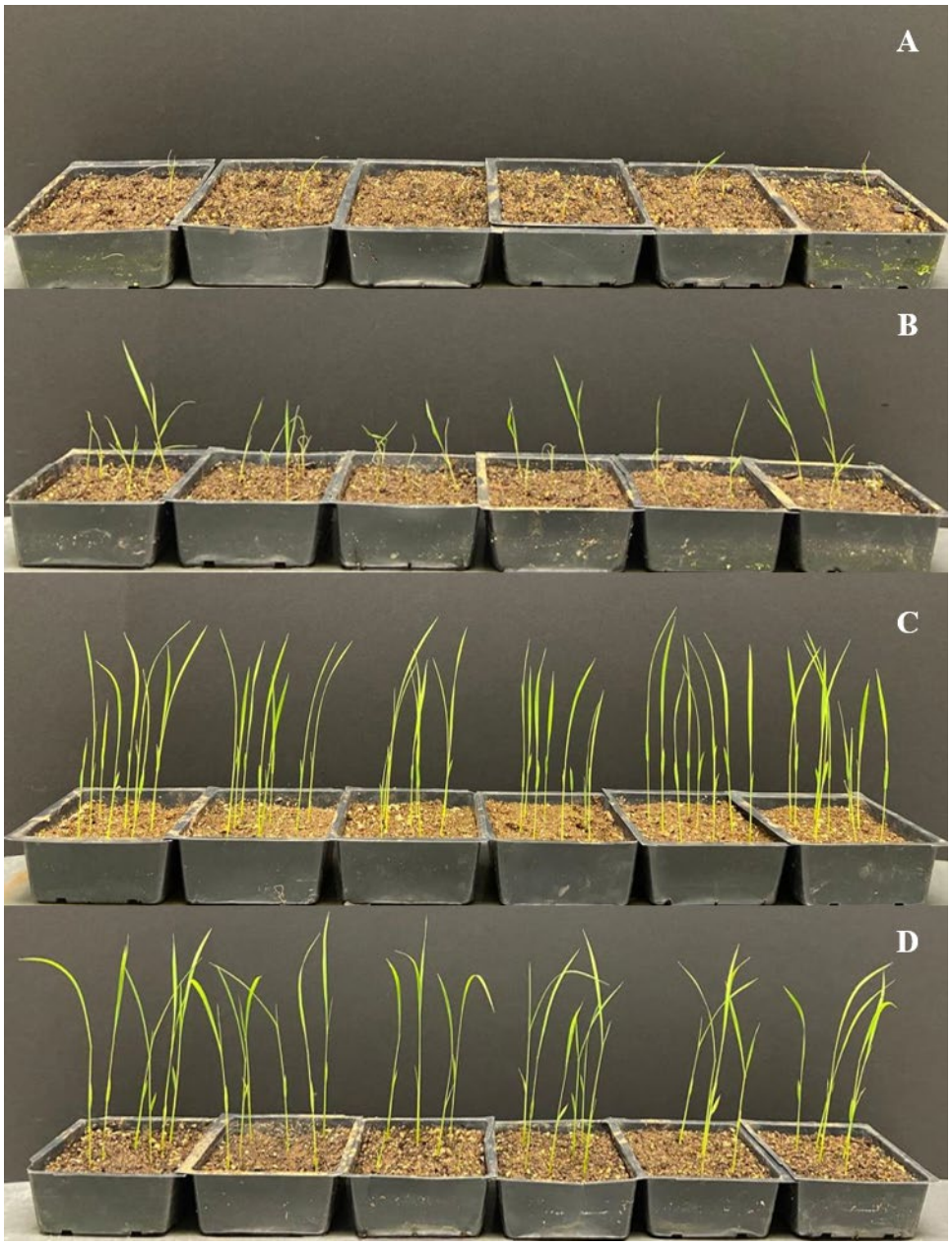

Figure 11. Response of wild type and T_2 'Nipponbare' rice to soil-applied fomesafen (390 g ha^{-1}) 3 weeks after treatment, University of Arkansas, Fayetteville, USA 2020. The photos show two runs of the experiment, three replications per run. Shown are WT treated (A) and nontreated (C), and T_2 treated (B) and nontreated (D).

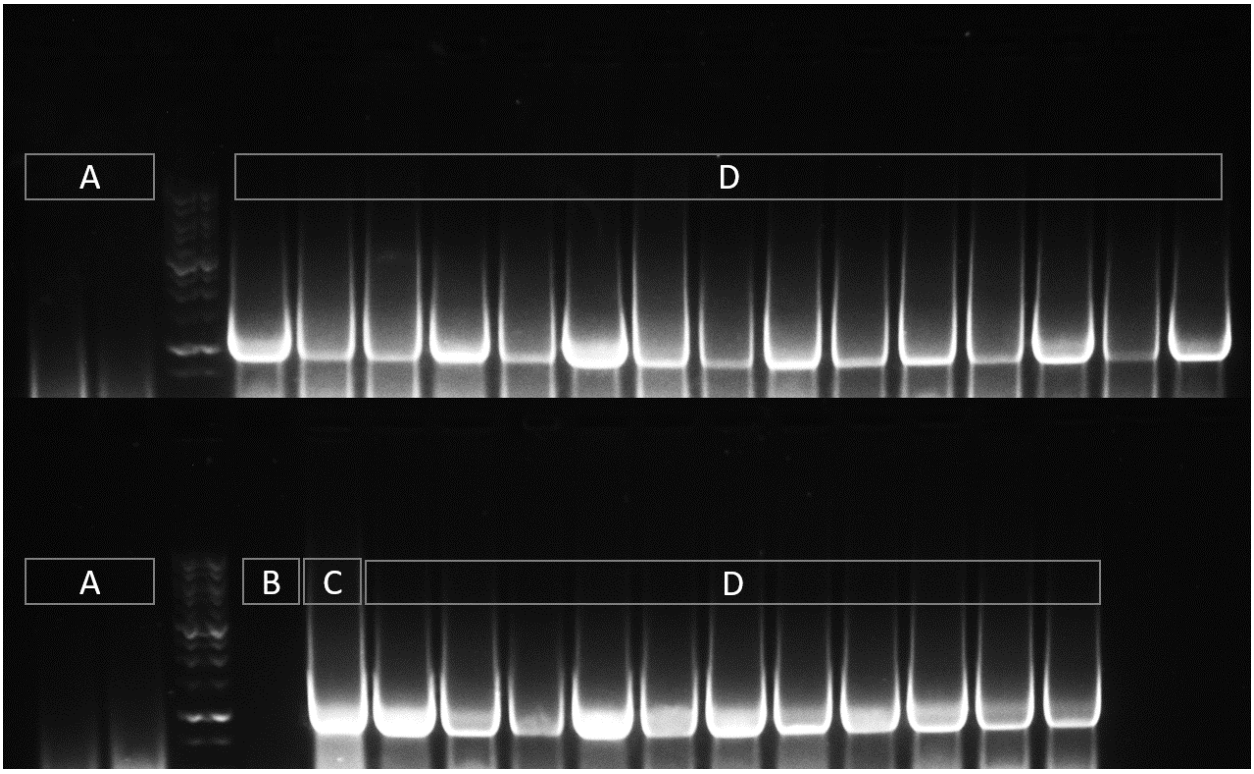

Figure 12. Detection of the *ppo2* (*ΔG210*) transgene in genomic DNA of T_2 survivors of soil-applied fomesafen by PCR amplification. The bands were generated using Palmer amaranth *ppo2* primer pair (KpnF x SphR) flanking a 1.6kb region encoding Palmer *ppo2*. Lanes A: wild type (negative control); B: water; C: T_0 containing the transgene (positive control); D: T_2, soil-based assay survivors.

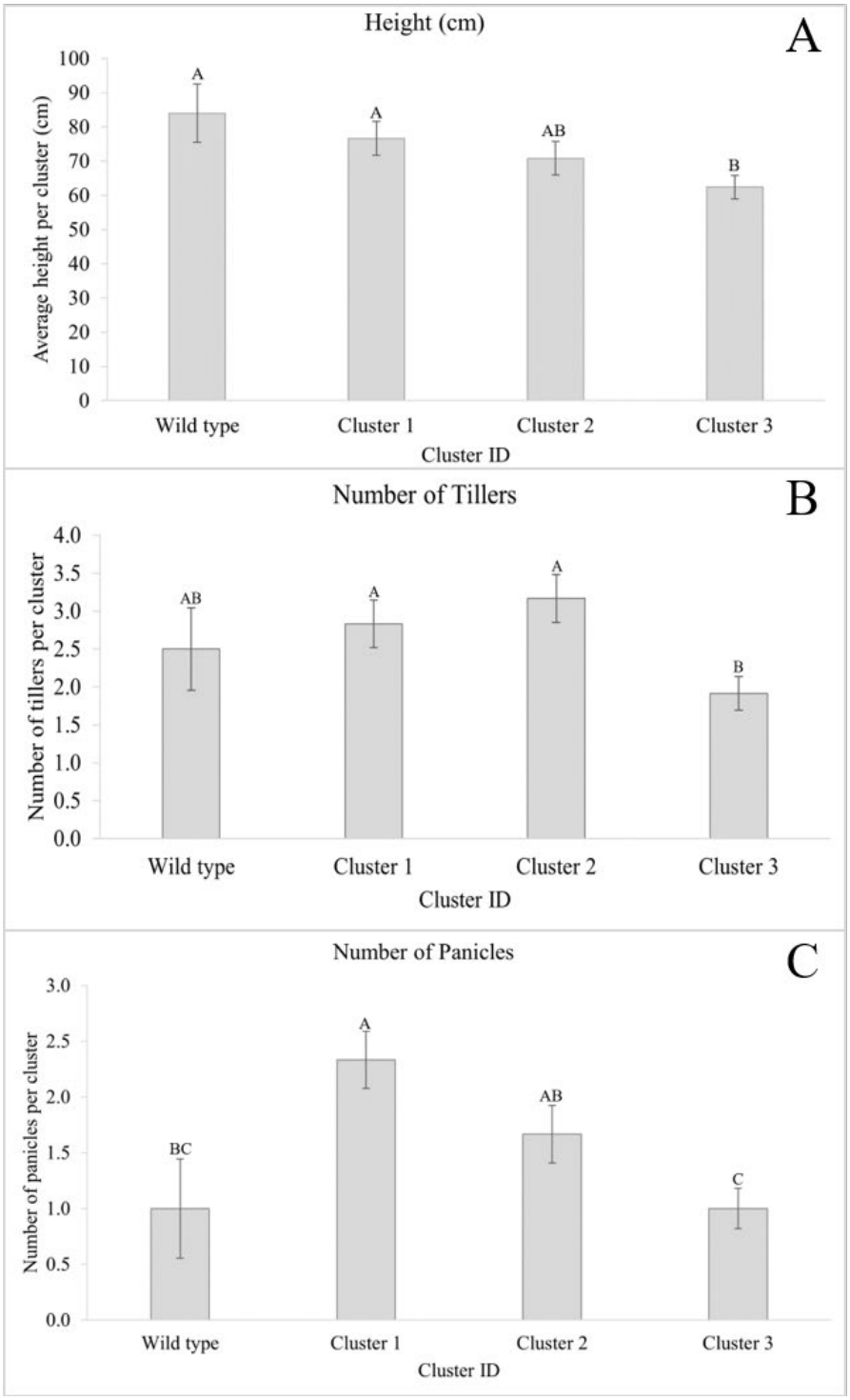

Figure 13. Height (cm) (A), number of tillers (B), and number of panicles (C) of T_2 survivors from soil-based assay, by phenotypic trait cluster. Data were collected when the majority of survivors transitioned to reproductive stage. University of Arkansas, Fayetteville, USA 2020. Significant means were separated using Fisher's protected LSD ($p < 0.05$).

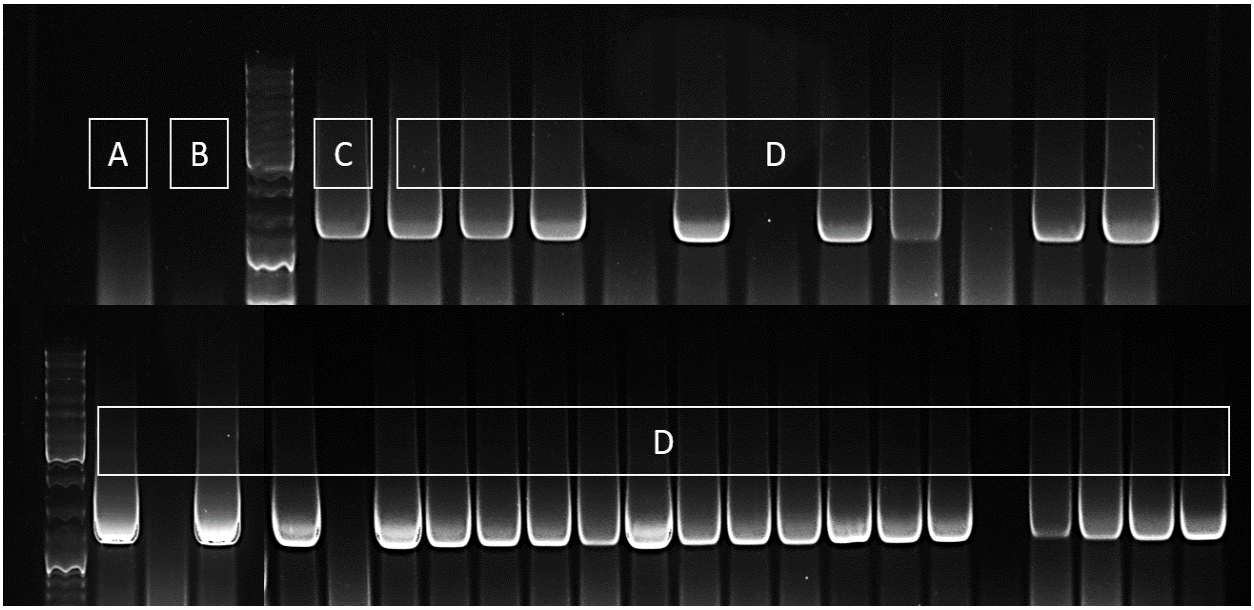

Figure 14. Detection of the *ppo2* (*ΔG210*) transgene in genomic DNA from T_2 nontreated plants by PCR amplification. The bands were generated using Palmer amaranth *ppo2* primer pair (KpnF x SphR) flanking a 1.6kb region encoding Palmer *ppo2*. Lanes A: wild type (negative control); B: water; C: T_0, containing the transgene (positive control); D: T_2, nontreated plants.

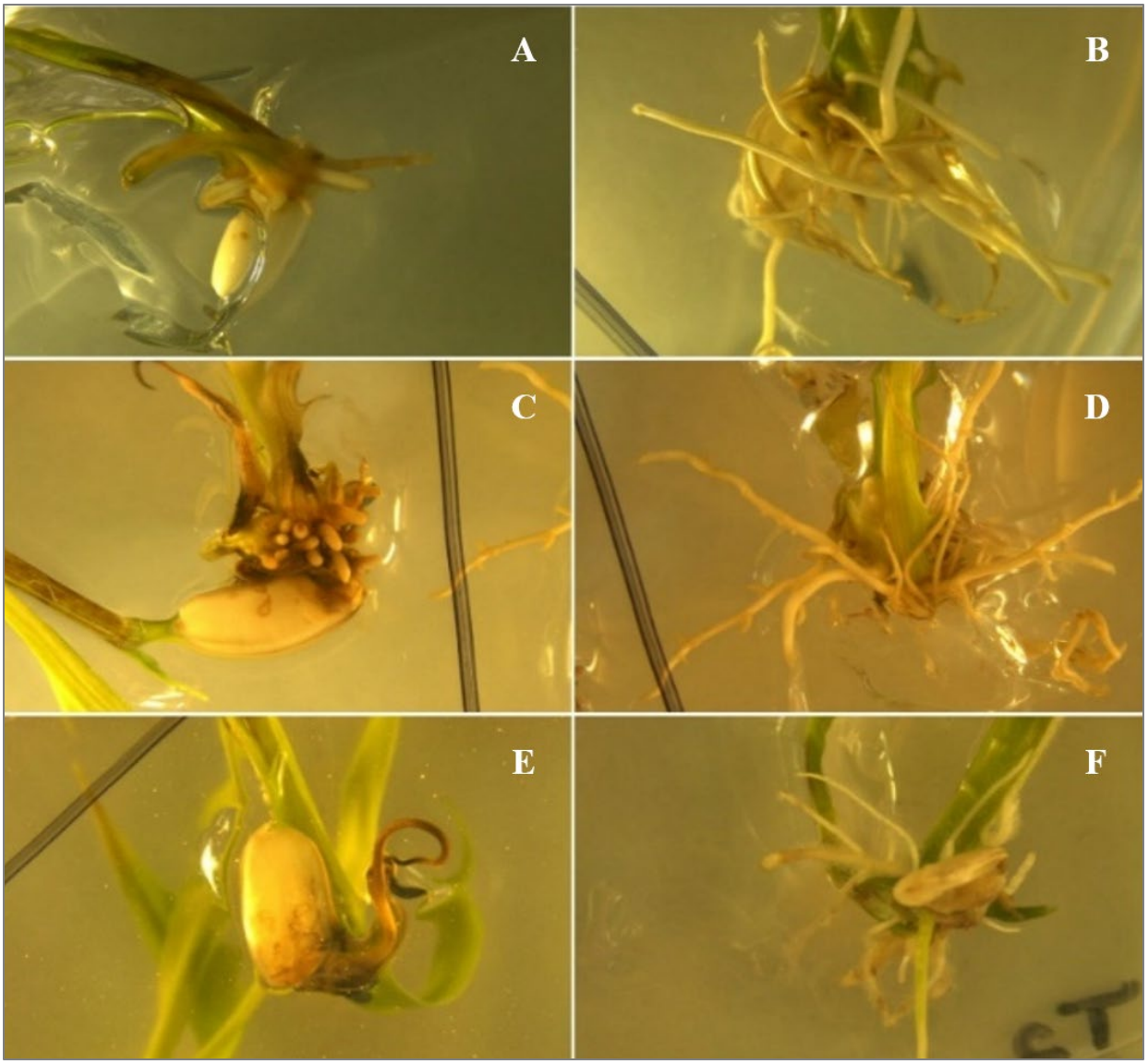

Figure 15. Root growth in different concentrations of fomesafen, University of Arkansas, Fayetteville, USA 2019. Root growth of wild type 'Nipponbare' at 5 µM (A), 40 µM (C) and 100 µM (E). Root growth of T_2 plants 5 µM (B), 40 µM (D) and 100 µM (F).

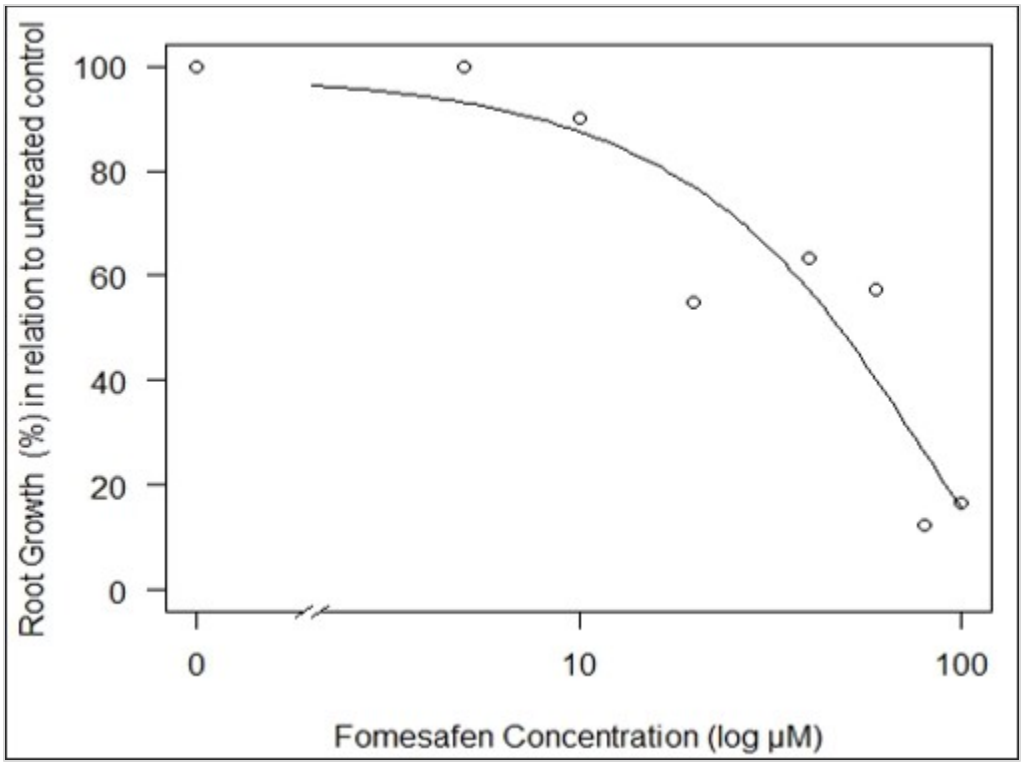

Figure 16. Dose response curve generated with the root growth (%) data collected from T_2 seeds in different fomesafen concentrations, University of Arkansas, Fayetteville, USA 2019. Data were fitted to a non-linear, three-parameter log-logistic regression function $Y=d/1+\exp\{[\log(x) - \log(ED50)]\}$.

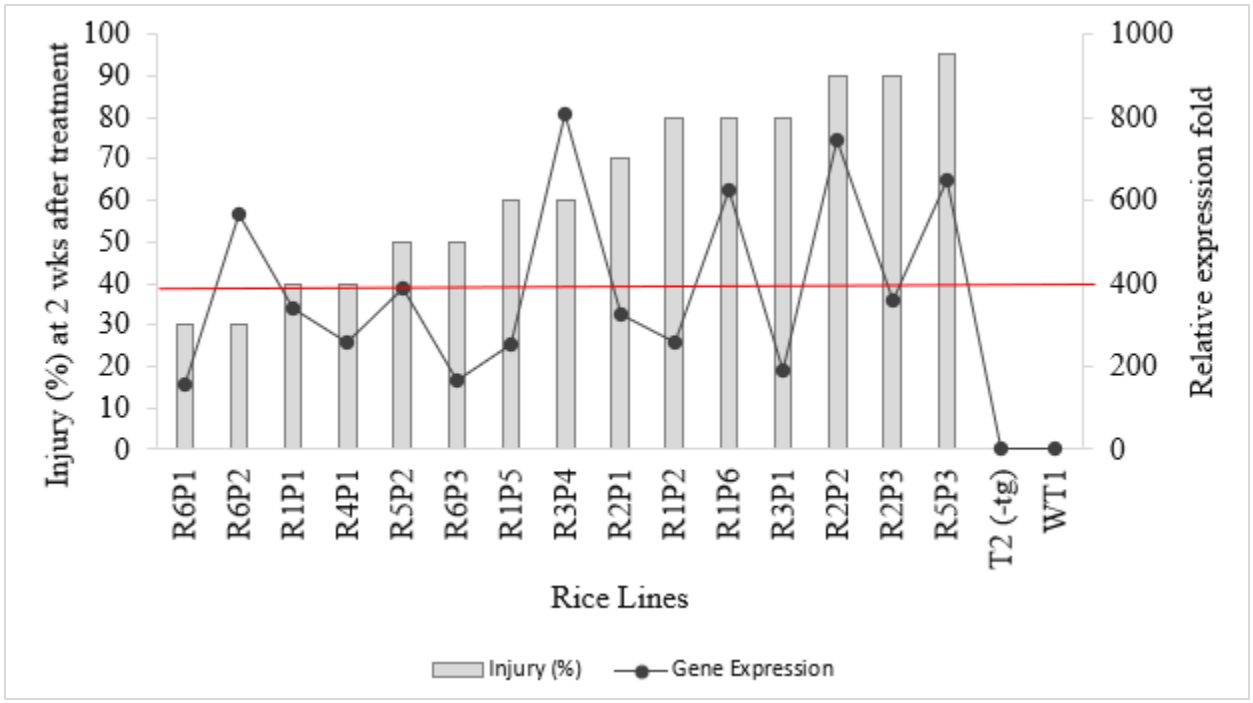

Figure 17. Visible injury (%) and transgene expression calculated relative to the native *PPO2* from *O. sativa*. Samples are organized from lowest to highest values. Red line delimits values above 400-fold.

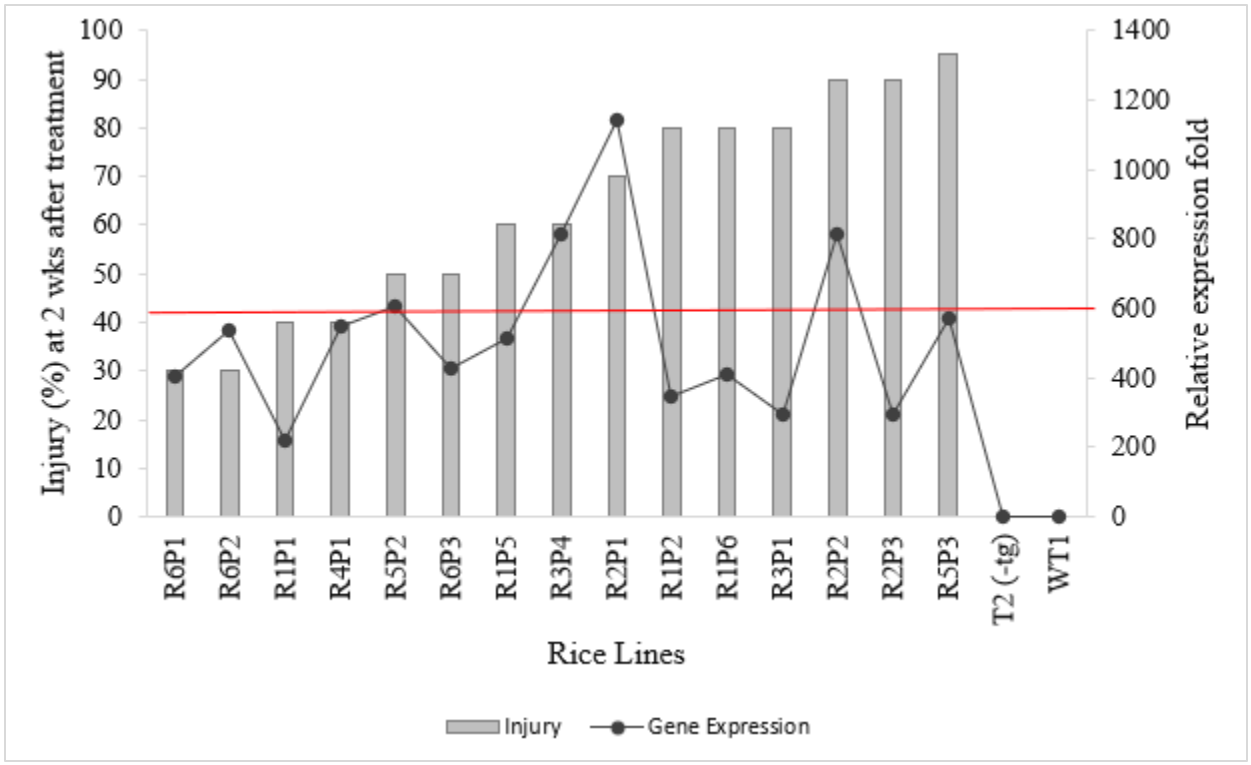

Figure 18. Visible injury (%) and transgene expression calculated relative to the ubiquitin. Samples are organized from lowest to highest values. Red line delimits values above 600-fold.

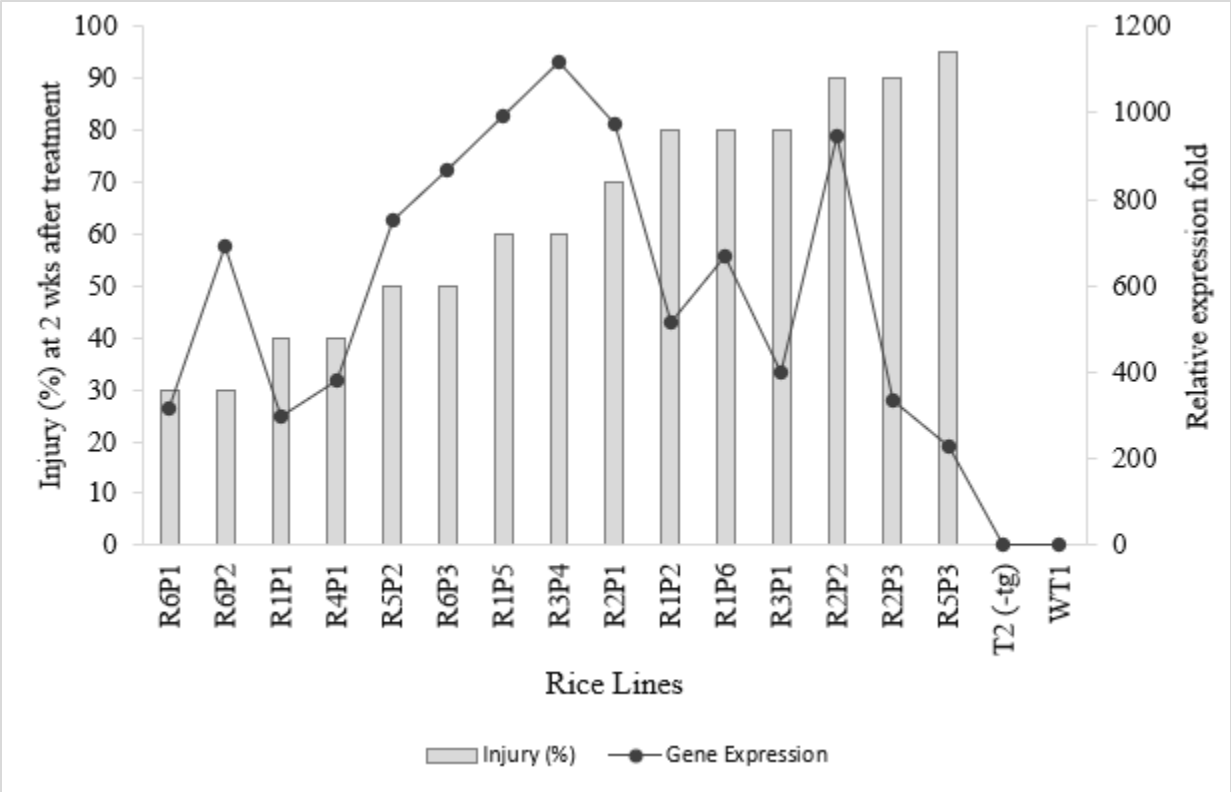

Figure 19. Visible injury (%) and transgene expression calculated relative to the native eukaryotic elongation factor1-alpha. Samples are organized from lowest to highest values.

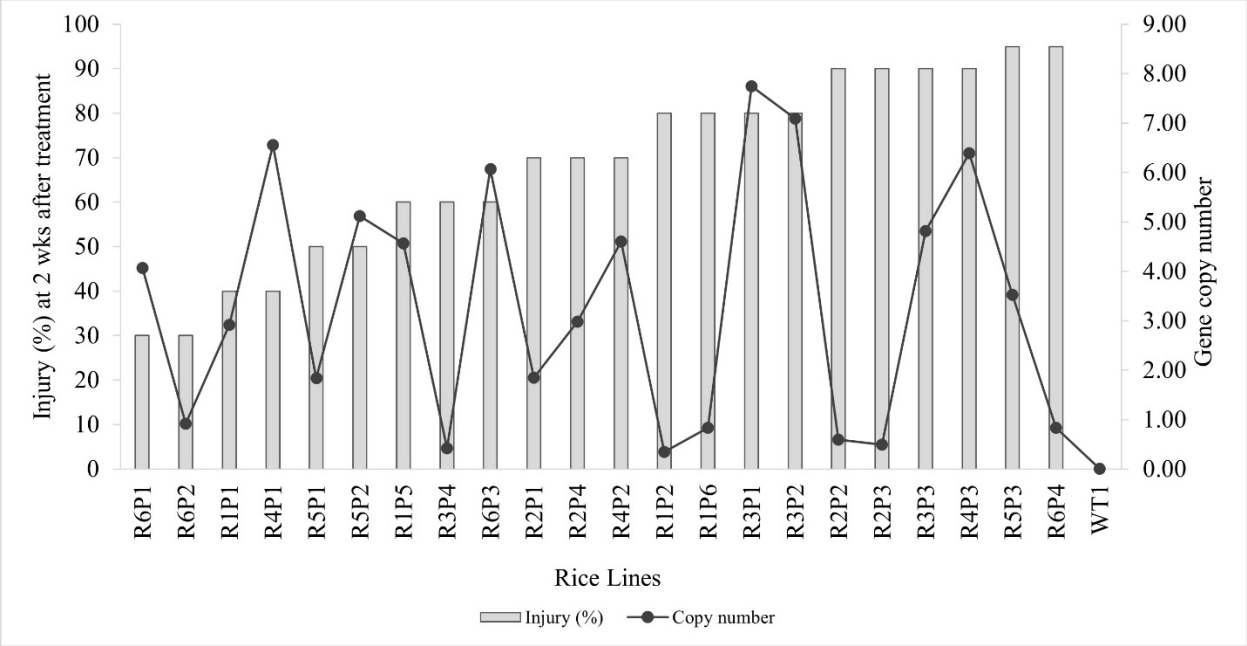

Figure 20. Visible injury (%) and gene copy number relative to the native rice *PPO2*. Samples are organized from lowest to highest values.

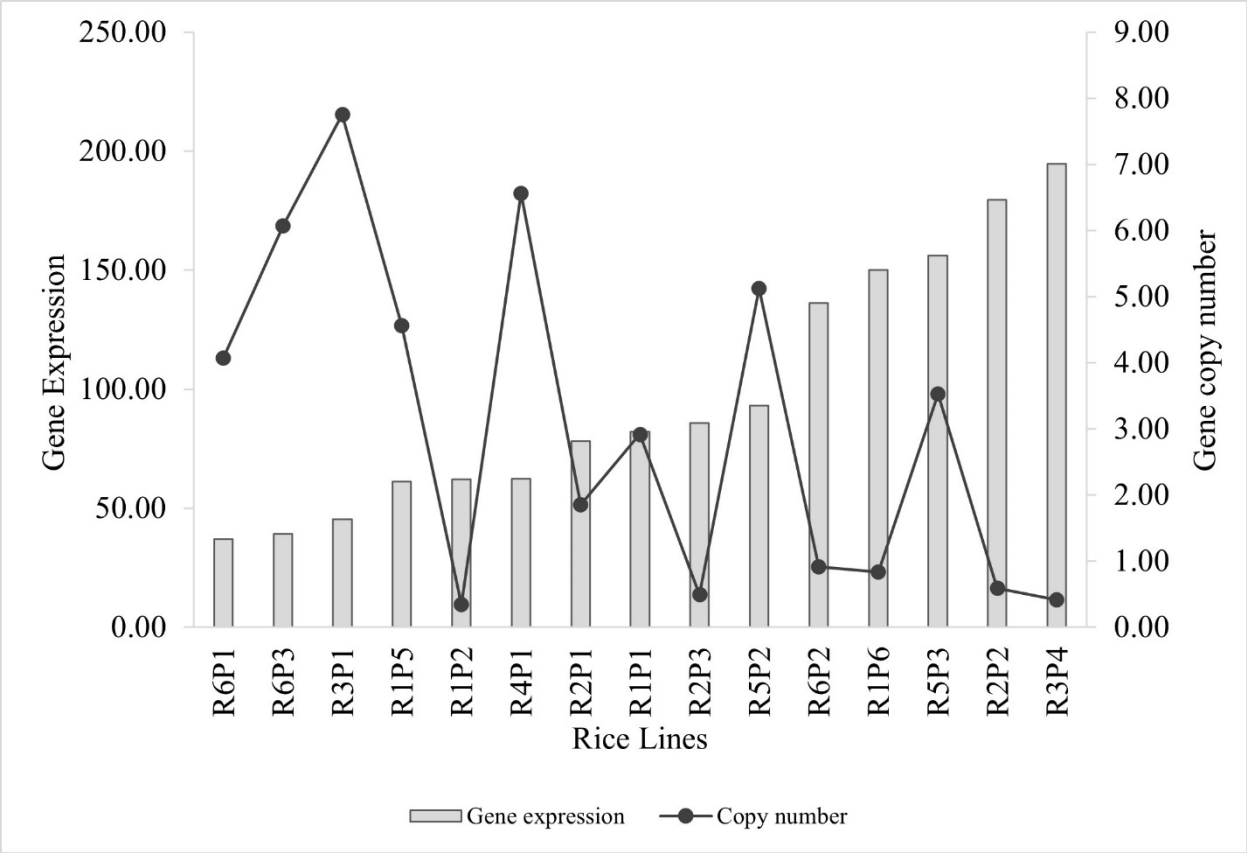

Figure 21. Transgene expression and gene copy number calculated against native rice *PPO2.* Samples are organized from the lowest to the highest gene expression values.

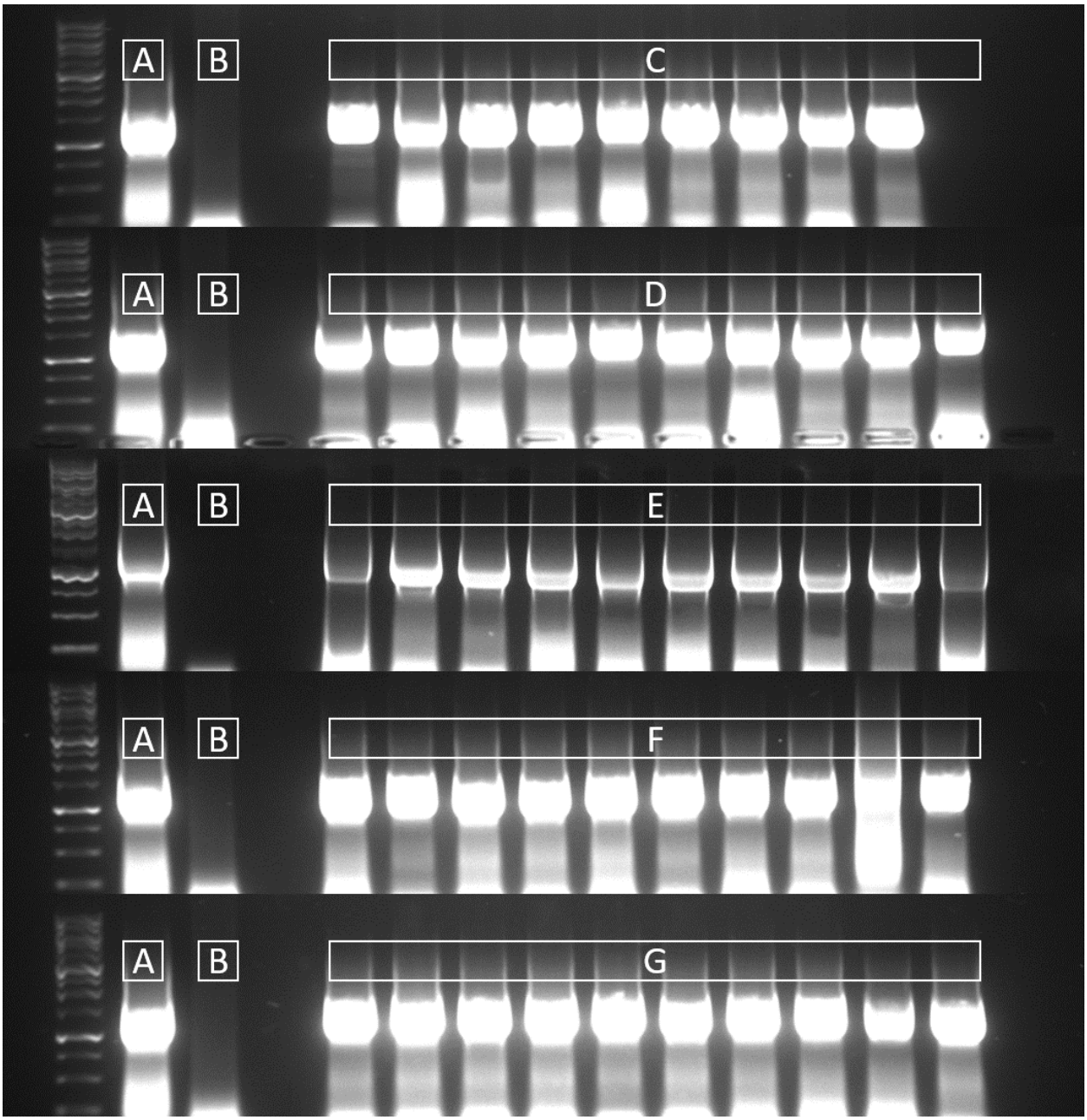

Figure 22. Detection of the *ppo2* (*ΔG210*) transgene in genomic DNA of T_3 seedlings from T_2 soil survivors by PCR amplification. The bands were generated using Palmer amaranth *ppo2* primer pair (KpnF x SphR) flanking a 1.6kb region encoding Palmer *ppo2*. Lanes A: T_0 containing the transgene (positive control); B: wild type (negative control); C and D: T_3, high injury progeny; E, F and G: T_3, low injury progeny.

Appendix

Appendix A – Murashige and Skoog medium (Murashige and Skoog 1962) for plant growth.

- 800 ml of autoclaved distilled water
- 4.6 g of MS plant salt mixture (Caisson Labs, Smithfield, UT)
- 1 ml of 1000x MS-vitamin (Caisson Labs, Smithfield, UT)
- 0.1 g of myo-inositol (Sigma Life Science, Saint Louis, MO)
- 30 g of sucrose (PhytoTechnology Laboratories, Lenexa, KS)
- Mix and dissolve the ingredients
- Adjust the pH to 5.8
- 3 g of phytagel (Sigma Life Science, Saint Louis, MO)
- Adjust the last volume to 1 liter with autoclaved distilled water.
- Close the containing leaving the lid loose.
- Autoclave
- Let the medium cool to about 50°C
- Pool the medium over Petri dishes inside sterilized laminar flow hood.
- Let it solidify and dry before use it

CHAPTER IV

RESISTANCE LEVEL TO FOMESAFEN IN *Amaranthus palmeri* ACCESSIONS CARRYING DIFFERENT PROTOPORPHYRINOGEN IX OXIDASE MUTATIONS

Abstract

Protoporphyrinogen IX oxidase (PPO) inhibiting herbicides were heavily used to control Palmer amaranth (*Amaranthus palmeri* S. Wats.) populations resistant to glyphosate and acetolactate synthase-inhibitor herbicides. The continuous use of herbicides with the same mode of action imposed high selection pressure towards PPO-herbicide-resistant genotypes. Palmer amaranth PPO-mutations are well studied, but information is lacking regarding the resistance level and cross-resistance pattern that each mutant *ppo* endows to weed populations. Therefore, this study was conducted to evaluate the level of fomesafen resistance conferred by the *ppo2* mutations *ΔG210* and *G399* in Palmer amaranth PPO-resistant field accessions, separately or combined in the same plant. The response to other PPO inhibitors saflufenacil and trifludimoxazin as well as to the alternative herbicide, dicamba (auxin mimic), was also investigated in the same populations. One susceptible and six resistant accessions were subjected to a dose response assay with the PPO-herbicides and dicamba. Some survivors were genotyped to characterize the mutation profile. The predicted dose to control 50% of the population (ED_{50}) ranged from 55 to 171 g ha^{-1} among the resistant populations. The increase in resistance relative to that of the susceptible accession ranged from 2- to 7-fold. Palmer amaranth control with other foliar-applied herbicides tested was as follows: saflufenacil < trifludimoxazin < dicamba. High frequency of homozygous *ΔG210* confers high population-level resistance to fomesafen. The accession with a higher frequency of *ΔG210*-homozygous survivors showed the higher predicted ED_{50} for fomesafen. The accessions with high frequency of homozygous *ΔG210* and with individuals accumulating *ΔG210+G399A* showed higher potential for cross-resistance to the other PPO-herbicides tested, which is highly informative with respect to the proper stewardship of the new and improved PPO-herbicide formulations soon to be commercialized. Survivors that

are homozygous for *ΔG210* or accumulating *ΔG210+G399A* are less injured compared to heterozygous individuals at the highest fomesafen rate tested. Survivors from one resistant accession were mostly wild type for both mutations, but were not genotyped for other mutations, nor was the *PPO* gene sequenced. This accession may harbor other mutations or may harbor non-target-site-resistance mechanism. This same accession was less sensitive to dicamba.

Introduction

The release of genetically modified crops resistant to highly effective and non-selective herbicides has greatly impacted weed management. Even though chemical control was the main weed control method prior to the release of transgenic crops, herbicide-resistant crops allowed farmers to rely on a single herbicide site of action to control weeds, which reduced the diversity of weed management practices and chemistries used in a crop season (Bonny 2016; Duke 2005; Mazur and Falco 1989; Owen 2016; Vencill et al. 2012). Herbicide resistance in weeds is a consequence of evolution through herbicide selection pressure. By relying on a single herbicide, diverse chemistries that could control a resistant individual will be excluded. Therefore, resistant plants will survive, reproduce, and, consequently, increase the frequency of resistant plants in a population. Also, resistant alleles may be introduced in a population through gene flow from other fields (Christoffers 1999; Duke 2005; Gaines et al. 2020; Jasieniuk et al. 1996).

Palmer amaranth (*Amaranthus palmeri* S. Wats.) is a highly competitive weed with dioecious habit and is genetically compatible with other species in the Amaranthaceae family (Franssen et al. 2001; Molin et al. 2016; Steckel 2007). Thus far, this species has evolved resistance to herbicides pertaining to eight different site of actions which, combined with its competitiveness, makes this weed a serious problem in several crops (Bensch et al. 2003; Morgan et al. 2001; Heap 2020; Massinga et al. 2001; McGowen et al. 2018; Meyers et al. 2010). This species is highly resistant to acetolactate synthase (ALS)- and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-inhibiting herbicides. In fact, there are at least ten reports where a single Palmer amaranth population carries multiple resistance to both herbicides (Chaudhari et al. 2020; Kohrt et al. 2017; Küpper et al. 2017; Salas-Perez et al. 2017; Schwartz-Lazaro et al. 2017; Sosnoskie et al. 2011; Spaunhorst et al. 2019).

The herbicides inhibiting the enzyme protoporphyrinogen oxidase (PPO, EC 1.3.3.4) were extensively used to control ALS- and EPSPS-resistant Palmer amaranth populations. By inhibiting this enzyme, PPO-inhibitor herbicides stop the oxidation of protoporphyrinogen IX into protoporphyrin IX which leads to accumulation of protoporphyrinogen IX. The excess protoporphyrinogen IX is exported into the cytoplasm where oxidative reactions with the free oxygen will occur, producing a photosensitive protoporphyrin IX. Upon exposure to light, the photosensitive protoporphyrin IX will generate singlet oxygen molecules, ultimately, leading to cellular death in susceptible plants (Jacobs et al. 1991; Lee et al. 1993; Matringe et al. 1989; Orr and Hess 1982; Poulson and Polglase 1975).

Palmer amaranth is resistant to PPO-inhibitor herbicides due to target-site (TSR) and non-target-site resistance (NTSR) mechanisms. However, the majority of the PPO-resistant populations tested had target-site mutations. The PPO enzyme is nuclear-encoded and exists in two forms in plants; *PPO1* is located in the chloroplast and *PPO2* is located in the mitochondria and, in a few plants, also in the chloroplast (Lermontova et al. 1997; Watanabe et al. 2001). Alterations in the *PPO2* have been found in PPO-resistant Palmer amaranth. The first modification was the deletion of a glycine at the 210th position (ΔG210), previously reported in tall waterhemp (*Amaranthus tuberculatus*) (Copeland et al. 2018; Patzoldt et al. 2006; Salas et al. 2016; Spaunhorst et al. 2019). The second was a substitution of arginine with glycine or methionine at the 128th position (*R128G* or *R128M*) (Giacomini et al. 2017; Salas-Perez et al. 2017; Varanasi et al. 2018a). This mutation was previously identified in common ragweed (*Ambrosia artemiisifolia*) (Rousonelos et al. 2012). The substitution of glycine at the 399th position with alanine was the latest identified herbicide resistance-conferring mutation in *PPO2* (Rangani et al. 2019).

Until recently, there were no reports regarding the accumulation of PPO-mutations in the same plant. This changed when Wu et al. (2020) identified few fomesafen survivors carrying *ΔG210+R128G*. The authors did not provide further details regarding the level of resistance in these plants or if the mutations co-occurred in the same allele. Also, working with several PPO-resistant Palmer amaranth accessions from four USA states, Noguera et al. (2020) reported that accessions with more than one *ppo* mutation grouped in one cluster, which collectively exhibited stronger resistance. Further evaluation revealed a few plants in these accessions accumulating the mutations *ΔG210+G399A* and *G399A+R128G* (in the same allele), and plants carrying *ΔG210+R128G* (may or may not occurred in the same allele). How these double mutations might affect the resistance level per plant is yet to be investigated. Therefore, this study was conducted to identify the level of resistance to fomesafen conferred by the *ppo2* mutations *ΔG210* and *G399* in Palmer amaranth PPO-resistant field accessions, separately or co-occurring in the same plant or allele. It was also investigated the response of the same populations to three foliar-applied herbicides: dicamba (synthetic auxin), saflufenacil (PPO-inhibitor), and, a new PPO-inhibitor active ingredient, trifludimoxazin.

Materials and Methods

Fomesafen Dose-Response Bioassay. Palmer amaranth accessions collected in 2017 and 2018 were screened for fomesafen resistance and genotyped for the presence of *ΔG210* and *G399A* mutations (Noguera et al. 2020). From this initial screening, six populations collected in 2017 from fields located in Arkansas and Missouri were selected with distinct mutation profiles (Table 1). The six PPO-resistant accessions, and the susceptible (SS), were used in whole-plant bioassays to determine the resistance level to fomesafen. The accessions were expected to

contain *ΔG210* (PHI-C and LAW-E), *G399A* (PHI-I and SC-C), and both mutations (NM-J and PEM-F). Seedlings were grown in 11 x 11 cm pots filled with commercial potting soil (Sunshine® Premix No. 1; Sun Gro Horticulture, Bellevue, WA) and thinned to 4 plants pot^{-1}. The experiment was conducted twice and had four replications. Each replication was one pot. Seedlings, 8- to 10-cm tall, were sprayed with 6 doses of fomesafen (Flexstar®, Syngenta Crop Protection, Greensboro, NC 27419) from 70 to 1120 g ai ha^{-1} for resistant populations, corresponding to 1/4 to 4x the recommended field dose. The dose range for SS was from 17.5 to 280 g ha^{-1}, corresponding to 1/16 to 1x the recommended dose. A nontreated check was used as reference for evaluation. Following the label requirement, the herbicide was applied with 0.5% non-ionic surfactant (Induce, Helena Chemical, Collierville, TN). Replications were sprayed separately in an air propelled, motorized spray chamber with 1100067 nozzles (Teejet, Wheaton, IL) calibrated to deliver 187 L ha^{-1}. At 3 weeks after treatment (WAT), plants were evaluated for injury. Visible injury (%) was rated on a scale of 0 to 100%, where 0 represented no effect, and 100% was dead (Burgos et al. 2013; Frans et al. 1986). The data were analyzed by regression using the "drc" package in R 3.5.1 (Ritz et al. 2015). A three-parameter log-logistic model was fitted to the data using the equation:

$$Y = \frac{d}{1 + \exp\{b[\log(x) - \log(ED50)]\}} b$$

where *Y* is visible injury relative to the nontreated check (%), d is the upper horizontal asymptote, x is the herbicide dose, and *b* is the slope around ED_{50} which is the herbicide dose causing 50% injury (Ritz 2010). The resistance index was the ratio of the ED_{50} values of the R accession and SS accession. Injury of survivors (%) was also recorded to select plants for genotyping.

Response to Other Foliar-Applied Herbicides. The seven accessions (including SS) used in the dose-response assay were also tested for the response to saflufenacil, trifludimoxazin, and dicamba (Table 2). Saflufenacil (pyrimidinedione) and trifludimoxazin (triazinone) are also PPO-inhibiting herbicides from different chemical families than fomesafen (diphenyl ether). These two other PPO herbicides were chosen because these are the newest PPO-inhibitor herbicides and are being commercialized as a pre-mix to control a broad spectrum of herbicide-resistant weeds in Australia (Wang et al. 2019; Armel et al. 2017). With the recent launching of soybean varieties resistant to dicamba, dicamba became a useful postemergence option for the control of PPO-resistant Palmer amaranth in soybeans. Fomesafen was also included in this assay. The experiment was conducted twice with four replications per treatment. A square pot, 11 x 11 cm, filled with commercial potting soil (Sunshine® Premix No. 1; Sun Gro Horticulture, Bellevue, WA) with four (in the first run) or five (in the second run) plants consisted one replication. Therefore, the total number of plants per accession was 16 and 20 for the first and the second run, respectively. In the greenhouse, plants were grouped by herbicide and dose, and the accessions were completely randomized within each herbicide group. Nontreated check was used as control. Treatments were sprayed when seedlings were 8- to 10-cm tall in the first run and 6- to 9-cm tall in the second run. Herbicide application was conducted as described earlier. Saflufenacil treatments included 1% methylated seed oil (v/v) and 1% ammonium sulfate (w/v). Trifludimoxazin treatments were sprayed with 1% methylated seed oil (v/v). At 3 WAT, injury per survivor and mortality (%) were assessed. The data were analyzed by herbicide by accession using JMP Pro v. 15 (SAS Institute, Cary, NC). Student's t test ($p < 0.05$) was used for comparison. Mortality (%) was calculated using the formula:

$$\text{Mortality (\%)} = \frac{Number\ of\ dead\ plants}{Initial\ number\ of\ plants} x\ 100$$

Detection of Mutations by TaqMan Assay. DNA was extracted from leaf tissues collected from selected survivors (less than 85% injury) from both runs of the fomesafen dose-response assay. Individuals of each accession were genotyped individually for the presence of the target-site mutations (*ΔG210* and/or *G399*). Leaf tissues were collected from survivors at 1x, 2x, and 4x the recommended dose of fomesafen ha^{-1} for the accessions LAW-E, NM-J, PEM-F, and PHI-I; at 1x and 2x of fomesafen ha^{-1} for the accession SC-C; and at 1x of fomesafen ha^{-1} for the accession PHI-C. Following the protocol previously used by Noguera et al. (2020), the tissues were placed separately in 1.5-mL Eppendorf tubes (VWR International LLC, Radnor, PA) with two 2.4-mm metal beads (VWR International LLC). The tubes were stored in -80 °C until processed. The tubes with leaf tissues and beads were placed in a laboratory mixer mill (MM400, Retsch GmbH, Haan, Germany) for 15 s at 30 Hz to grind the leaf tissues. Genomic DNA was extracted using a modified CTAB protocol (Doyle and Doyle 1987) and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). The extracted DNA was diluted to about 150 ng uL^{-1}.

The diluted DNA samples were used in the TaqMan® SNP Genotyping Assay. Fluorescent probes were used to discriminate between the resistant and susceptible alleles of the *ppo2* (*ΔG210* and *G399A*) gene. For *ΔG210* detection, the forward (5'-TGATTATGTTATTGACCCTTTTGTTGCG -3') and reverse (5'-GAGGGAGTATAATTTATTTACAACCTCCAGAA -3') primer pair was used (Giacomini et al. 2017). Probes overlapping the *ΔG210* mutation, targeting wild type (5'-TTGAGGATCTCCACCACATG-3') and positive *ΔG210* (5'-CGATTGAGGATCTCCACATG-3'), were used (Wuerffel et al. 2015). For *G399A* detection, the forward (5'-TGTTTATTTGATAAACATATCATAGAATCTAATGCTAGTTTCTT-3') and reverse (5'-

AGCACGATCAGGAAACATCATAGAC-3') primers were used. Probes overlapping the *G399A* mutation, targeting wild type (5'-ACGTCGCAGGTACTTT-3') and positive *ΔG210* (5'-CGTCGCAGCTACTTT-3'), were used (Noguera et al. 2020).

The qPCR reaction mixture (5.5 µl) consisted of 1 µl GoTaq® Flexi buffer (Promega, Madison, WI), 0.6 µl 25 mM $MgCl_2$ (Promega), 0.25 µl 10 mM dNTP mix (Promega), 0.25 µl primer-probe mix (Thermo Fisher Scientific, Waltham, MA), 0.05 µl GoTaq® Flexi DNA polymerase (Promega) and 1 µl of genomic DNA. The qPCR was conducted using a CFX96 Real-Time PCR machine (BioRad, Hercules, CA) using the following conditions: 3 min at 95°C, followed by 40 cycles of 15 s denaturation at 95°C, 1 min at 60°C, followed by a plate read at the end of every cycle. The plates included a known homozygous and heterozygous resistant allele for each mutation, and a homozygous susceptible. Allelic discrimination was performed using the Bio-Rad CFX software based on the relative fluorescence units. This data was used to describe the profile of individual survivors per accession and dose.

Results and Discussion

Fomesafen Dose-Response Bioassay. To determine the resistance level to fomesafen, a dose-response bioassay was conducted with LAW-E, NM-J, PEM-F, PHI-C, PHI-I, SC-C, and SS accessions. Except for SC-C, none of the resistant accessions were completely controlled at 1x dose (280 g ha^{-1}) of fomesafen (Figure 1). Regardless of accession, survivors at 280 g ha^{-1} fomesafen showed a wide range of injury (from no symptoms to severe plant necrosis and stunting). The approximate fomesafen dose that would cause 50% injury (ED_{50}) varied widely among the resistant accessions. The ED_{50} ranged from 55 to 171 g ha^{-1} with the order of resistance level as follows: LAW-E > PEM-F > PHI-I > NM-J > PHI-C). The increase in

resistance (R/S) ranged from 2- to 7-fold (Figure 2). It turned out that SC-C was more sensitive to fomesafen (ED_{50} = 13 g ha^{-1}) than the the SS population (ED_{50} =24 g ha^{-1}). SC-C was included in this test because rare individuals harboring the *G399A* mutation were detected in this population in the general resistance screening and *ppo* mutation survey. This case highlights the fact that resistant individuals would already have been selected in the field several years prior to detection of field-level resistance (Salas et al. 2016).

Similar ED_{50} values were found in other Palmer amaranth field populations resistant to PPO-herbicides. The susceptible population used in this study seemed to have higher tolerance to fomesafen than the other susceptible standards used in previous studies, although direct comparison cannot be made across different studies. To gauge relative susceptibilities of populations, all have to be tested in one experiment. The resistance index values obtained here were slightly lower compared to those reported in other studies (Rangani et al. 2019; Salas et al. 2016; Salas-Perez et al. 2017; Varanasi et al. 2019). Wide ranging ED_{50} values were also reported for PPO-resistant Palmer amaranth populations from other states. Lillie et al. (2019a) estimated higher ED_{50} values (up to 614 g fomesafen ha^{-1}) PPO-resistant populations from Kentucky carrying *ΔG210* mutation. On the other hand, Wu et al. (2020) obtained quite low ED_{50} values (from 12.4 to 28.5 g fomesafen ha^{-1}) for populations from Tennessee. Even with low ED_{50} values, these Tennessee populations had survivors at fomesafen rates of up to 3360 g ha^{-1}.

Response to Other Foliar-Applied Herbicides. The response of the same accessions to the foliar herbicides tested was as follows: fomesafen < saflufenacil < trifludimoxazin < dicamba. Observing the least efficacy with fomesafen was not surprising since fomesafen was the PPO inhibitor most widely used for the longest time. Mortality was different between runs for

fomesafen. Therefore, the data across runs were analyzed separately. Mortality at 280 (1x) and 560 g fomesafen ha^{-1} ranged from 10 to 99 and from 31 to 100, respectively (Table 3). In both runs, the mortality rate of PHI-C and SC-C did not statistically differ from SS. All other accessions had more survivors than SS at 1x dose of fomesafen and still had survivors at the 2x dose.

The mortality across runs for saflufenacil, trifludimoxazin and dicamba were similar. Therefore, the data from both runs were analyzed together by herbicide. Except for LAW-E and PEM-F, the mortality rates with 2x dose of saflufenacil were similar to that of SS (Figure 3; Table 3). At the recommended dose of saflufenacil, LAW-E, NM-J and PEM-F had number of survivors than SS, ranging from 52 to 78% mortality. Saflufenacil is applied pre- and postemergence on field corn, cotton, and soybean, to control susceptible broadleaf weeds, including Palmer amaranth (Anonymous 2020a). Previous results declared saflufenacil as a potent herbicide option for the control of Palmer amaranth (Montgomery et al. 2014; Morichetti et al. 2012). However, whenever saflufenacil was applied to fomesafen-resistant populations, its efficacy declined significantly (Houston et al. 2019; Salas-Perez et al. 2017).

At 1x, three accessions displayed less sensitivity to trifludimoxazin than SS (Figure 4; Table 3). These accessions were LAW-E, NM-J, and PEM-F with 82%, 85% and 81% of mortality, respectively. The injury of survivors, regardless of the accession, was higher than 75% at 21 d after treatment in ideal growing conditions (Figure 6). This indicates that the resistance level to trifludimoxazin low. The accessions LAW-E and PEM-F, which had the highest ED_{50} and lowest mortality with fomesafen, also showed the lowest mortality at both doses of saflufenacil, and at the 1x dose of trifludimoxazin. At 2x trifludimoxazin, the mortality rates of fomesafen-resistant accessions ranged from 91 to 100% and did not differ from the SS standard.

Most of the survivors (17 out of 28) had injury ≥ 90. Survivors showing injury higher than 90% are classified as susceptible due to the small chances of survivability on field conditions.

Various cross-resistance levels to PPO-chemistries were reported previously among highly fomesafen-resistant Palmer amaranth and tall waterhemp populations (Evans et al. 2019; Salas-Perez et al. 2017; Shoup et al. 2003). The common pattern is that the great majority of fomesafen-resistant populations are susceptible to saflufenacil and populations cross-resistant to the newest PPO-inhibitor chemistry, trifludimoxazin, have not yet evolved at the field level. However, individuals with cross-resistance to trifludimoxazin have already been selected in a few field populations. As mentioned previously, a premix formulation of saflufenacil and trifludimoxazin was launched this year in Australia. This formulation is more effective in burndown application than other burndown herbicides and is a promising tool for the control of PPO-herbicide-tolerant or -resistant weeds. Nevertheless, to curtail further resistance evolution, it is crucial to combine this new product with other herbicide groups (APVMA 2020; Anonymous 2020b; Armel et al. 2017; Bi et al. 2020; Lillie et al. 2019b).

Dicamba is one alternative herbicide that is promoted for the control of herbicide-resistant broadleaf weeds. Dicamba obtained the highest mortality ratings (Figure 5; Table 3) compared to the PPO-inhibitor herbicides tested. At 1x (560 g ai ha^{-1}) dicamba, all PPO-resistant accessions were controlled the same as SS, except PHI-I. The mortality rate of PHI-I was significantly lesser than that of SS. Overall, the survivors showed 80 to 99% injury at 21 d after treatment under ideal growing conditions (Figure 6). Apparently, some survivors with >90% injury (15 out of 19) are most likely not going to recover and are not truly resistant. All individuals with injury lower than 90% were from the PHI-I accession. Besides PHI-I, all accessions were susceptible to dicamba with mortality >90% at 1x and >97% at 2x. Similar

efficacy levels of dicamba on PPO-resistant Palmer amaranth and tall waterhemp populations were reported (Evans et al. 2019; Salas-Perez et al. 2017; Sarangi et al. 2019). Thus far, there is only one report of resistance to dicamba in the genus *Amaranthus* worldwide. Smooth pigweed (*Amaranthus hybridus*) populations were identified resistant to the auxinic herbicides 2,4-D and dicamba, possibly due to increased detoxification or metabolism (Dellaferrera et al. 2018). Although there is no confirmed case of *Amaranthus* resistance to dicamba in the USA, Bernards et al. (2012) reported that waterhemp population resistant to 2,4-D was 2.7 times less sensitive to dicamba than the susceptible population. The release of dicamba-resistant crops has increased the selection pressure on *Amaranthus* populations. Reports of dicamba failures have been increasing in the US mid-south, specifically in Tennessee (Steckel 2020). There is an undeniable correlation between the release of herbicide-resistant crops with the increased evolution of weed resistance to the same herbicide used on such crops due to the decrease in number of chemistries used and tremendous selection pressure on weed populations (Bonny 2016; Brookes 2014).

***ppo2* Mutations Among Survivors.** The presence of *ΔG210* and *G399A ppo2* mutations was observed in survivors of the accessions NM-J, PEM-F, and SC-C (Table 4). However, only PEM-F and SC-C had survivors with both mutations in the same plant. Only one individual from SC-C survived the 2x dose of fomesafen, and this plant carried both mutations. This individual was excluded from the other comparisons because it had 85% injury, which is atypical of any other survivor carrying both mutations (injury range from 10 to 40%). Based on the dose response assay SC-C was sensitive to fomesafen, but it contained rare individuals that are resistant to PPO inhibitors. On the other hand, the accession PEM-F which also carried individuals accumulating both *ppo2* mutations was the second most resistant to fomesafen.

However, the high resistance level of PEM-F accession cannot be solely attributed to this accumulation since only 32% of the survivors were carrying both mutations. Both mutations were heterozygous in all accessions. Therefore, Δ*G210* and *G399A* may or may not co-exist in the same allele of the survivors tested. Singh et al. (2019), when working with Palmer amaranth accessions resistant to ALS herbicides, detected the three combinations of two point mutations in *ALS* gene of six resistant populations. The accumulation of ALS-mutations did not impact the resistance level since one specific mutation was the main resistance mechanism for these populations, independently of the combination.

The only *ppo2* mutation present in LAW-E and PHI-C survivors was *ΔG210*. Based on accession-level responses to fomesafen, saflufenacil, and triflodimoxazin, LAW-E had the highest resistance to PPO-inhibitor herbicides. The majority of the genotyped survivors from LAW-E (11 out of 16) had the *ΔG210* mutation in both alleles (Table 4). The high frequency of *ΔG210*-homozygous individuals and the high resistance to PPO inhibitors in this accession indicates that the homozygous state of *ΔG210* confers high resistance level to PPO herbicides (injury under 50%) (Figure 7). Similarly, the homozygous *ΔG210* F1 crosses of Palmer amaranth had the highest ED_{50} for fomesafen compared to heterozygous ones and those harboring both G210 and R128G mutations (Brabham et al. 2018).

Overall, across accessions, the *ΔG210* mutation was present in most survivors up to the highest dose of fomesafen (1120 g ai ha^{-1}) (Table 4; Figure 7). This mutation is predominant among PPO-herbicide-resistant Palmer amaranth and tall waterhemp across the US (Copeland et al. 2018; Noguera et al. 2020; Salas-Perez et al. 2017; Varanasi et al. 2018a; Wu et al. 2020; Wuerffel et al. 2015). Although several researchers studied the resistance level of plants harboring the *ppo2* mutations, these studies were conducted with survivors from 1x doses. Up to

this point, data on the *ppo2* mutation profile of individuals surviving higher doses are not yet available.

At 4x fomesafen, survivors accumulating both mutations (*ΔG210+G399A*) or carrying *ΔG210* in both alleles incurred similar levels of injury (20 to 50%) while the *ΔG210* heterozygous or WT survivors had higher injury from 40 up to 70% (Figure 7). Regardless of accession and fomesafen rate, this pattern was the same across all the genotyped survivors (78 total). This indicates that survivors carrying the *ΔG210* in both alleles and the ones co-existing with *G399A* will recover better than those with *G399A* alone, or those without any of these two mutations. Even in the heterozygous state, the *ΔG210* mutation, which is the most frequent resistant allele, is effective in endowing resistance to PPO-herbicides (Patzoldt et al. 2006). Although there is no previous information regarding the level of resistance provided by the accumulation of two, resistance-conferring *ppo2* mutations in the same plant, there are some studies showing the effects of multiple mutations. The *epsps* point mutations, T102I or P106S, were previously identified as glyphosate-resistant conferring mutations (Arnaud et al. 1998; Kishore et al. 1992; Funke et al. 2009). However, while investigating one goosegrass (*Eleusine indica*) population highly resistant to glyphosate, Yu et al. (2015) identified the combination of these two mutations in 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene in the same allele of survivors. The population with individuals accumulating *epsps* mutations showed 180x more resistance than the susceptible standard. When compared to the resistant-population with plants harboring only the P106S mutation, the double mutant goosegrass was 32x more resistant than the susceptible standard higher resistance. On the downside, a severe fitness cost was observed in the double *epsps* mutant goosegrass plants (Han et al. 2017). The accumulation of mutations in the same plant have been detected before in another herbicide target site in Palmer

amaranth. The accumulation of two *als* mutations in the same plant was detected in Palmer amaranth from Arkansas (Singh et al. 2019).

Unlike ΔG210, homozygous *G399A* was rare among the populations tested. Only three, out of 78 survivors genotyped, harbored this mutation in the homozygous state (Table 4). When *G399A* occurred by itself, the heterozygous survivors incurred 20-80% injury while the homozygous ones had 40-80% injury (Figure 7). Thus, the relationship between the occurrence of *ppo2* mutation(s) in field-selected plants and resistance level is not straightforward. Other resistance mechanism(s) could modify the plant response to herbicides in the field and these combinations of mechanisms can vary across resistant plants in one population (Rangani et al. 2019; Wu et al. 2020). As discovered in other species, the same target-site mutation confers different resistance levels across field-selected plants of the same species (Yu et al. 2012; Zhao et al. 2020; Zheng et al. 2005).

Of the 16 survivors of 4x fomesafen, none harbored the *G399A* mutation by itself, either heterozygous or homozygous (Figure 7). This was also reported previously by Rangani et al. (2019) where a field population of Palmer amaranth field harboring exclusively the *G399A* mutation did not survive the 4x dose (1053 g ha^{-1}) of fomesafen. The study by Montgomery et al. (2020) using lactofen instead fomesafen showed that the ED_{50} of Palmer amaranth populations harboring either *G399A* or *ΔG210* was similar (Montgomery et al. 2020). This higher resistance to lactofen conferred by *G399A* mutation was predicted earlier with *in vitro* studies (Rangani et al. 2019). Thus far, the *G399A* mutation has been reported in a few Palmer amaranth populations in Arkansas, Kansas, Missouri, and Tennessee. Further research is necessary to fully characterize the physiological effect of *G399A* substitution, and if it contributes to increased resistance level with complementary resistance mechanisms.

The accession PHI-I which harbors plants carrying the *ppo2 G399A* mutation was classified as resistant in the dose response assay. However, only one out of sixteen survivors exhibited the *G399A* mutation. The other survivors were wild type for both mutations. Since the *PPO2* gene of these plants were not sequenced nor tested for other PPO mutations, the mechanism of resistance in these plants are not known. Compared to the other accessions studied here, PHI-I was the only accession that showed less mortality (73%) to 1x dicamba compared to the other accessions (Figure 5; Figure 6). Such a case hints at the existence of NTSR mechanism, which is a subject for follow-up research. In Argentina, Dellaferrera et al. (2018) encountered *Amaranthus hybridus* populations resistant to auxinic herbicides, dicamba and 2,4-D. After pre-treatment with cytochrome P450 inhibitors before applying the auxin mimics, the resistant populations were controlled, suggesting metabolic degradation of herbicides by cytochrome P450. Cytochrome P450 monooxygenases play a key role in several biochemical and physiological processes in the plant. Specifically, some members of this enzyme family are responsible for catalyzing hydroxylation and dealkylation reactions of herbicide (Barrett 1995; Powles and Yu 2010). Even though TSR is the prevalent mechanism of resistance among PPO-inhibitor-resistant Palmer amaranth populations, other researchers have reported or suggested the possible existence of NTSR mechanisms based on the absence of target-site mutations in some PPO-herbicide-resistant plants (Copeland et al. 2018; Rangani et al. 2019; Salas et al. 2016; Salas-Perez et al. 2017; Varanasi et al. 2018a). PPO-resistant Palmer amaranth and waterhemp populations harboring NTSR mechanisms of resistance were identified in Arkansas and Illinois, respectively, based on plant response to P450 inhibitor application ahead of the PPO herbicides (Obenland et al. 2019; Varanasi et al. 2018b).

Conclusion

The *ppo2* mutation *ΔG210* is the primary mechanism of resistance to PPO-inhibitor herbicides among Palmer amaranth accessions. High frequency of homozygous *ΔG210* confers high population-level resistance to fomesafen. The accession with a higher frequency of *ΔG210*-homozygous survivors showed the higher predicted ED_{50} for fomesafen. Survivors from treatments with the highest fomesafen rate (1120 g ha^{-1}) carrying *ΔG210* in both alleles or accumulating *ΔG210+G399A* showed less injury compared to heterozygous *ΔG210*. The *G399A* mutation by itself, either heterozygous or homozygous, was not detected among survivors treated with 1120 g ha^{-1} fomesafen. Populations with high frequency of homozygous *ΔG210* and with individuals accumulating *ΔG210+G399A* are less sensitive to the other two PPO-herbicides tested, saflufenacil and trifludimoxazin.

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Tables

Table 1. Expected mutation profile of Palmer amaranth field accessions used in the experiment.

Accession	Origin State	*ΔG210*	*G399A*
LAW-E[a]	Arkansas	Present	Absent
NM-J	Missouri	Present	Present
PEM-F	Missouri	Present	Present
PHI-C	Arkansas	Present	Absent
PHI-I	Arkansas	Absent	Present
SC-C	Missouri	Absent	Present
SS[b]	Arkansas	Absent	Absent

[a] The resistant accessions were harvested in 2017.
[b] The susceptible accession was harvested in 2018.

Table 2. Information about foliar herbicides used.

Site of action	Common name	Trade name	Chemical family	Field rate[a] g ai ha^{-1}	Manufacturer
PPO[b]	Fomesafen	Flexstar® 1.88SL	Diphenyl ether	280	Syngenta Crop Protection
PPO	Saflufenacil	Sharpen® 4F	Pyrimidinedione	25	BASF Corporation
PPO	Trifludimoxazin	Tirexor™ [c]	Triazinone	30	BASF Corporation
Auxin	Dicamba	Engenia	Benzoic acid	560	BASF Corporation

[a] Recommended field dose of the herbicides used in this study.
[b] Protoporphyrinogen IX oxidase inhibitors.
[c] Commercial name used in Australia; not registered in the United States of America.

Table 3. Response of fomesafen-resistant Palmer amaranth accessions to the 1x and 2x new, foliar PPO-inhibitor herbicides and dicamba, Altheimer Laboratory, University of Arkansas, Fayetteville, USA 2020.

Accession	Dose[a]	Mortality			
		Fomesafen[b]	Saflufenacil[d]	Trifludimoxazin[d]	Dicamba[d]
		%			
LAW-E	1x	10(39)[c] *	52 *	82 *	97 [NS]
	2x	31(41) *	72 *	91 [NS]	100 [NS]
NM-J	1x	55(66) *	78 *	85 *	94 [NS]
	2x	50(60) *	97 [NS]	94 [NS]	100 [NS]
PEM-F	1x	25(35) *	74 *	81 *	97 [NS]
	2x	35(58) *	76 *	94 [NS]	100 [NS]
PHI-C	1x	80(83) [NS]	97 [NS]	100 [NS]	90 [NS]
	2x	75(100) [NS]	100 [NS]	100 [NS]	100 [NS]
PHI-I	1x	38(65) *	91 [NS]	91 [NS]	72 *
	2x	65(75) *	92 [NS]	100 [NS]	94 [NS]
SC-C	1x	85(99) [NS]	89 [NS]	97 [NS]	100 [NS]
	2x	88(100) [NS]	97 [NS]	100 [NS]	100 [NS]
SS[e]	1x	100	100	100	100
	2x	100	100	100	100

[a] Recommended field rate (1x) per herbicide in g ai ha^{-1}: fomesafen, 280, with 0.5% v/v nonionic surfactant (NIS); saflufenacil, 25, with 1% v/v methylated seed oil and 1% w/v ammonium sulfate; and trifludimoxazin, 30, with 1% v/v methylated seed oil.
[b] Mortality ratings from fomesafen treatments differed across runs; data were analyzed separately.
[c] Numbers in parenthesis are mortality data from the second run. Seedlings were 8- to 10-cm-tall when treated in run 1 and 6- to 9 cm in run 2.
[d]. Mortality data across two runs were similar with saflufenacil, trifludimoxazin and dicamba treatments. Data were analyzed together.
[e] Susceptible population (SS).
* Significant difference ($p < 0.05$) in comparison to susceptible standard.
[NS] No significant difference with the susceptible standard.

Table 4. Genotype and zygosity of Palmer amaranth survivors from treatments with 280, 560, and 1120 g ha^{-1} fomesafen (Flexstar® 1.88 EC) + 0.5% v/v nonionic surfactant.

Accession	Number of plants genotyped	*ΔG210* only		*G399* only		*ΔG210+ G399A*	WT[c]	WT Injury (%)		
		RR[a]	Rr[b]	RR	Rr			Min	Max	Average
LAW-E[d]	16	11	4	-	-	-	1	50	50	50
NM-J[d]	13	-	2	2	4	-	5	40	70	45
PEM-F[d]	22	2	6	-	6	7	1	50	50	50
PHI-C[e]	4	-	2	-	-	-	2	30	30	30
PHI-I[d]	16	-	-	-	1	-	15	30	60	46
SC-C[f]	5	-	1	1	1	-	1	50	50	50

[a] Homozygous (mutation present in both alleles)
[b] Heterozygous (mutation present in one allele)
[c] Mechanism of resistance was not investigated.
[d] Leaf tissues from 280, 560 and 1120 g fomesafen ha^{-1}.
[e] Leaf tissues from 280 g fomesafen ha^{-1}.
[f] Leaf tissues from 280 and 560 g fomesafen ha^{-1}.

Figures

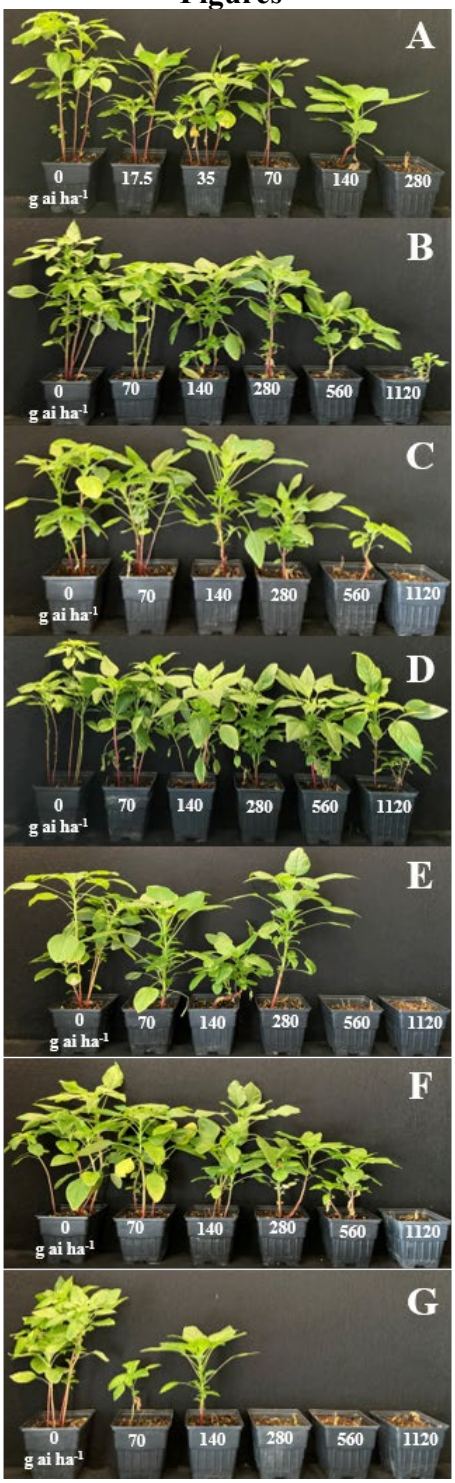
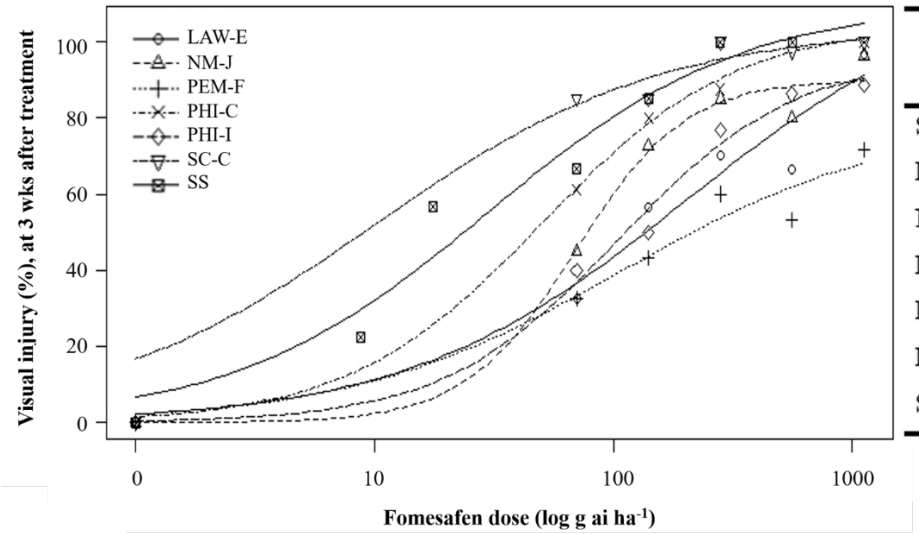


Figure 1. Palmer amaranth accessions susceptible and resistant to fomesafen in greenhouse dose-response experiment. Pictures were taken 3 weeks after treatment with 6 doses of fomesafen, Altheimer Laboratory, University of Arkansas, Fayetteville, USA 2020. Each letter represents one specific accession: **A**, susceptible; **B**, LAW-E; **C**, NM-J; **D**, PEM-F; **E**, PHI-C; **F**, PHI-I; **G**, SC-C. The first pot to the left of each photo was nontreated. Fomesafen doses were in g ai ha^{-1}. The dose range for susceptible and resistant populations differed.



ACC	ED_{50} (g ai ha^{-1})	Fold
SS	24	1
LAW-E	171	7
NM-J	67	3
PEM-F	149	6
PHI-C	55	2
PHI-I	128	5
SC-C	13	0.5

Figure 2. Dose response curve generated and ED_{50} generated with the visual injury (%) data collected from Palmer amaranth accessions after treatment with different fomesafen concentrations, Altheimer Laboratory, University of Arkansas, Fayetteville, USA 2020. Fold increase was calculated by ED_{50} *R*/ED_{50}*SS*. Symbols and lines represent actual and predicted herbicide injury responses. Data were fitted to a non-linear, three-parameter log-logistic regression function $Y=d/1+\exp\{[\log(x) - \log(ED50)]\}$.

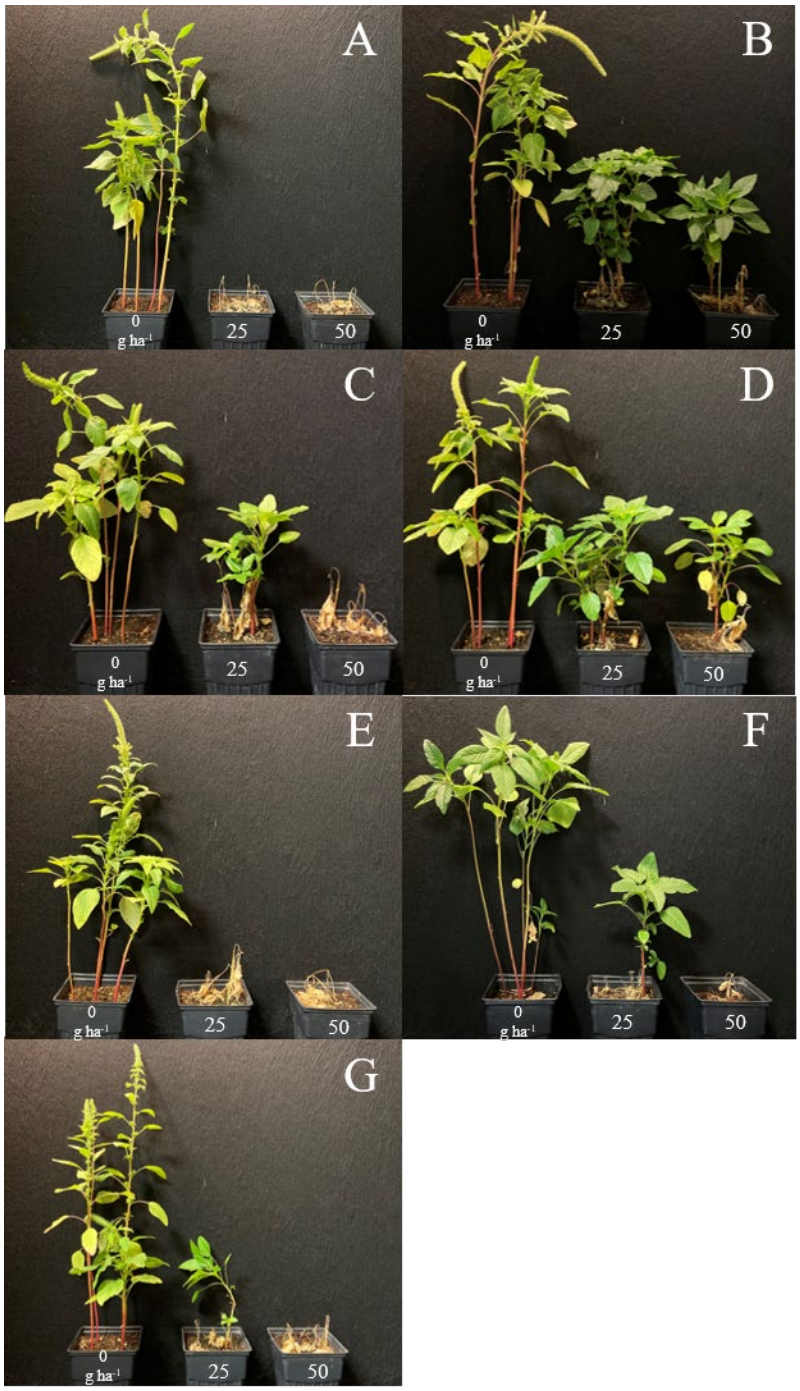


Figure 3. Response of Palmer amaranth accessions, susceptible and resistant to fomesafen, to foliar applications of saflufenacil. Pictures were taken 3 weeks after treatment with 2 doses of saflufenacil, Altheimer Laboratory, University of Arkansas, Fayetteville, USA 2020. Each letter represents one specific accession: **A**, susceptible; **B**, LAW-E; **C**, NM-J; **D**, PEM-F; **E**, PHI-C; **F**, PHI-I; **G**, SC-C. The first pot to the left of each photo was nontreated. Saflufenacil doses were in g ai ha^{-1}.

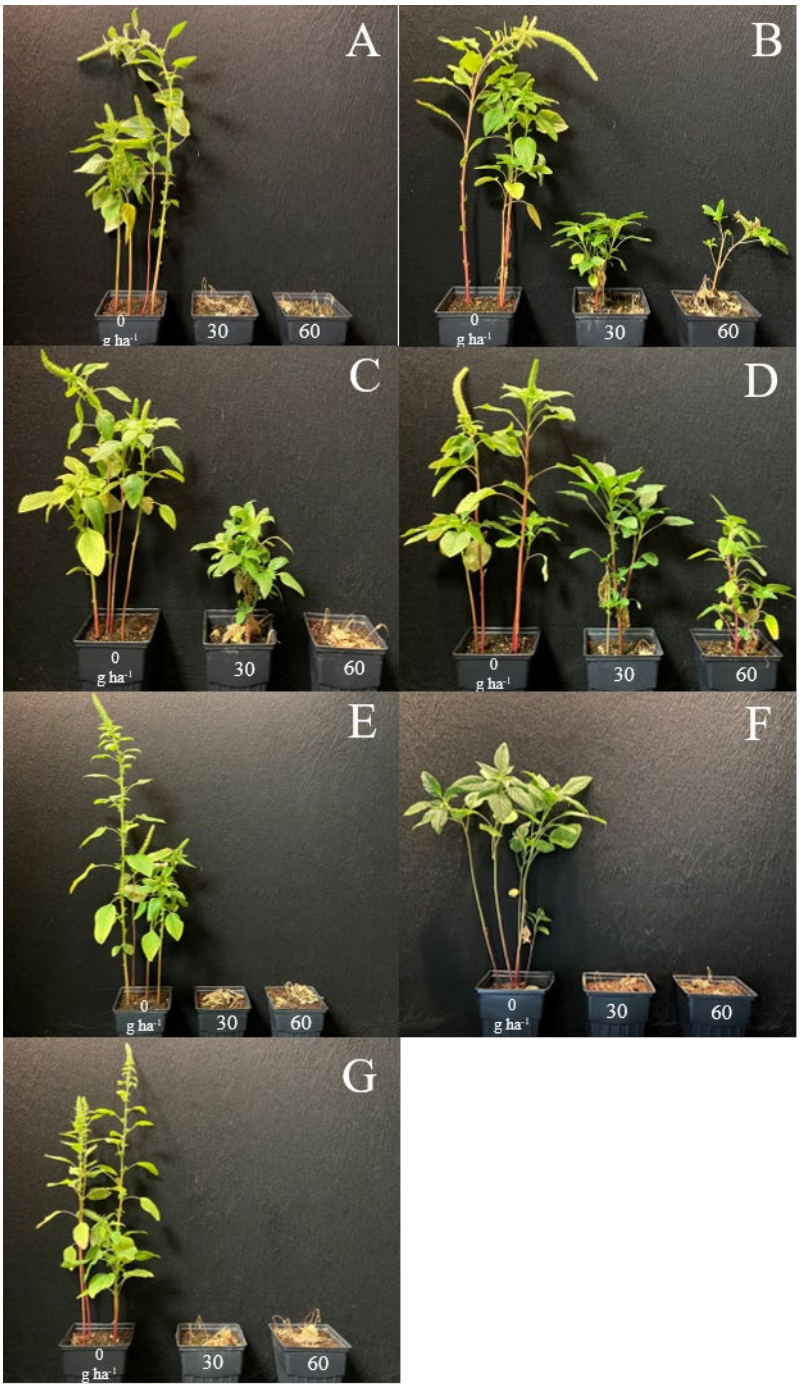


Figure 4. Response of Palmer amaranth accessions, susceptible and resistant to fomesafen, to foliar applications of trifludimoxazin. Pictures were taken 3 weeks after treatment with 2 doses of trifludimoxazin, Altheimer Laboratory, University of Arkansas, Fayetteville, USA 2020. Each letter represents one specific accession: **A**, susceptible; **B**, LAW-E; **C**, NM-J; **D**, PEM-F; **E**, PHI-C; **F**, PHI-I; **G**, SC-C. The first pot to the left of each photo was nontreated. Trifludimoxazin doses were in g ai ha^{-1}.

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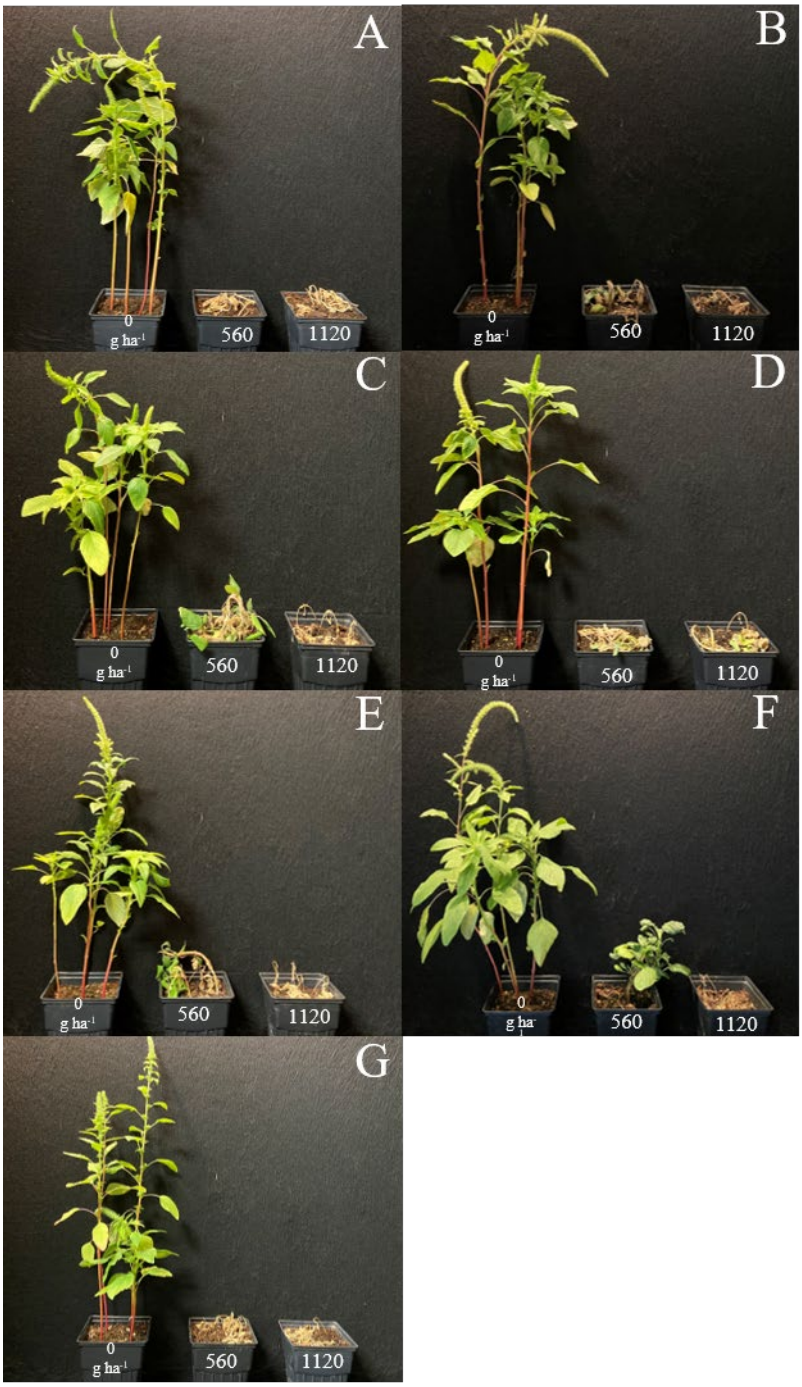

Figure 5. Response of Palmer amaranth accessions, susceptible and resistant to fomesafen, to foliar applications of dicamba. Pictures were taken 3 weeks after treatment with 2 doses of dicamba, Altheimer Laboratory, University of Arkansas, Fayetteville, USA 2020. Each letter represents one specific accession: **A**, susceptible; **B**, LAW-E; **C**, NM-J; **D**, PEM-F; **E**, PHI-C; **F**, PHI-I; **G**, SC-C. The first pot to the left of each photo was nontreated. Dicamba doses were in g ai ha^{-1}.

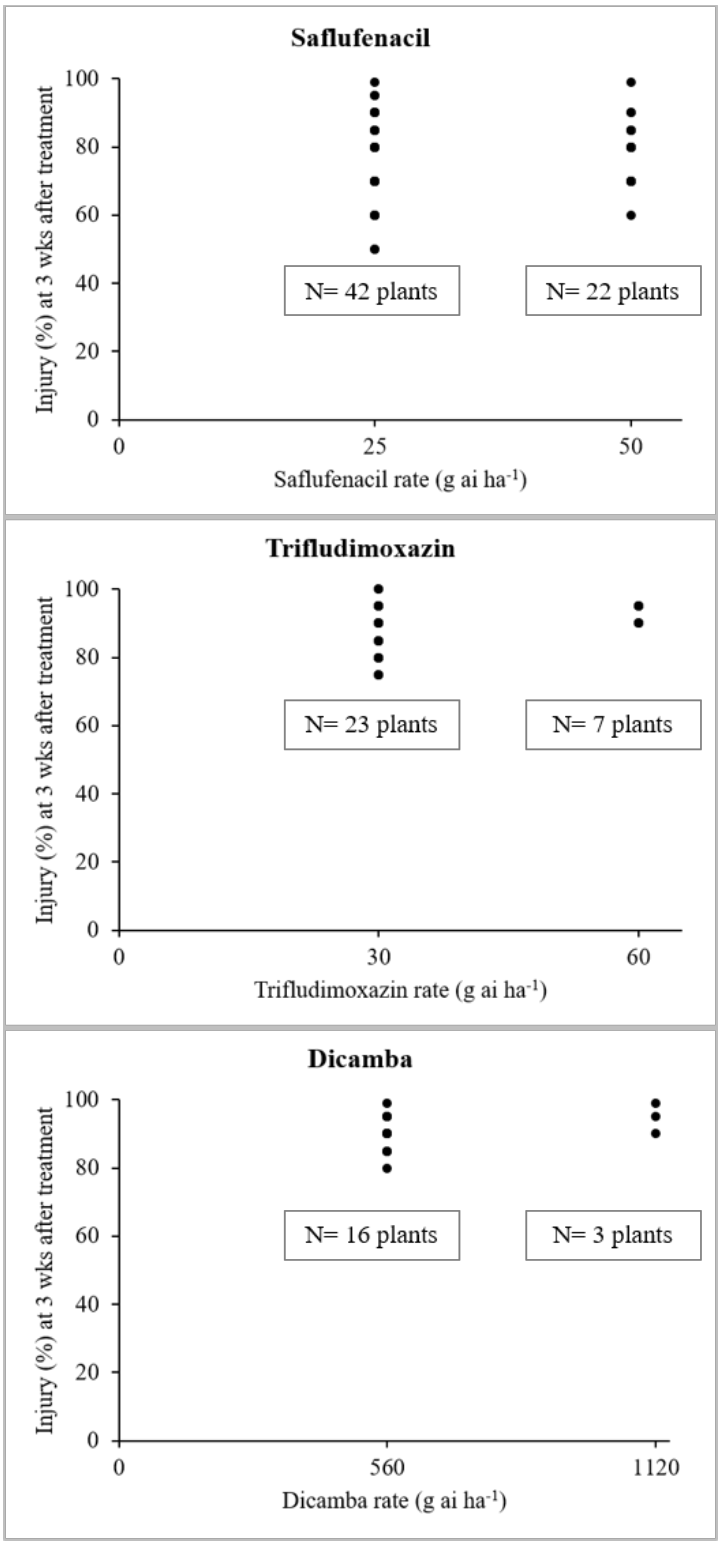

Figure 6. Injury (%) of Palmer amaranth survivors from treatments with saflufenacil (Sharpen® 4F) + 1% v/v methylated seed oil and 1% w/v ammonium sulfate, trifludimoxazin + 1% v/v methylated seed oil, or dicamba (Engenia), Altheimer Laboratory, University of Arkansas, Fayetteville, USA 2020.

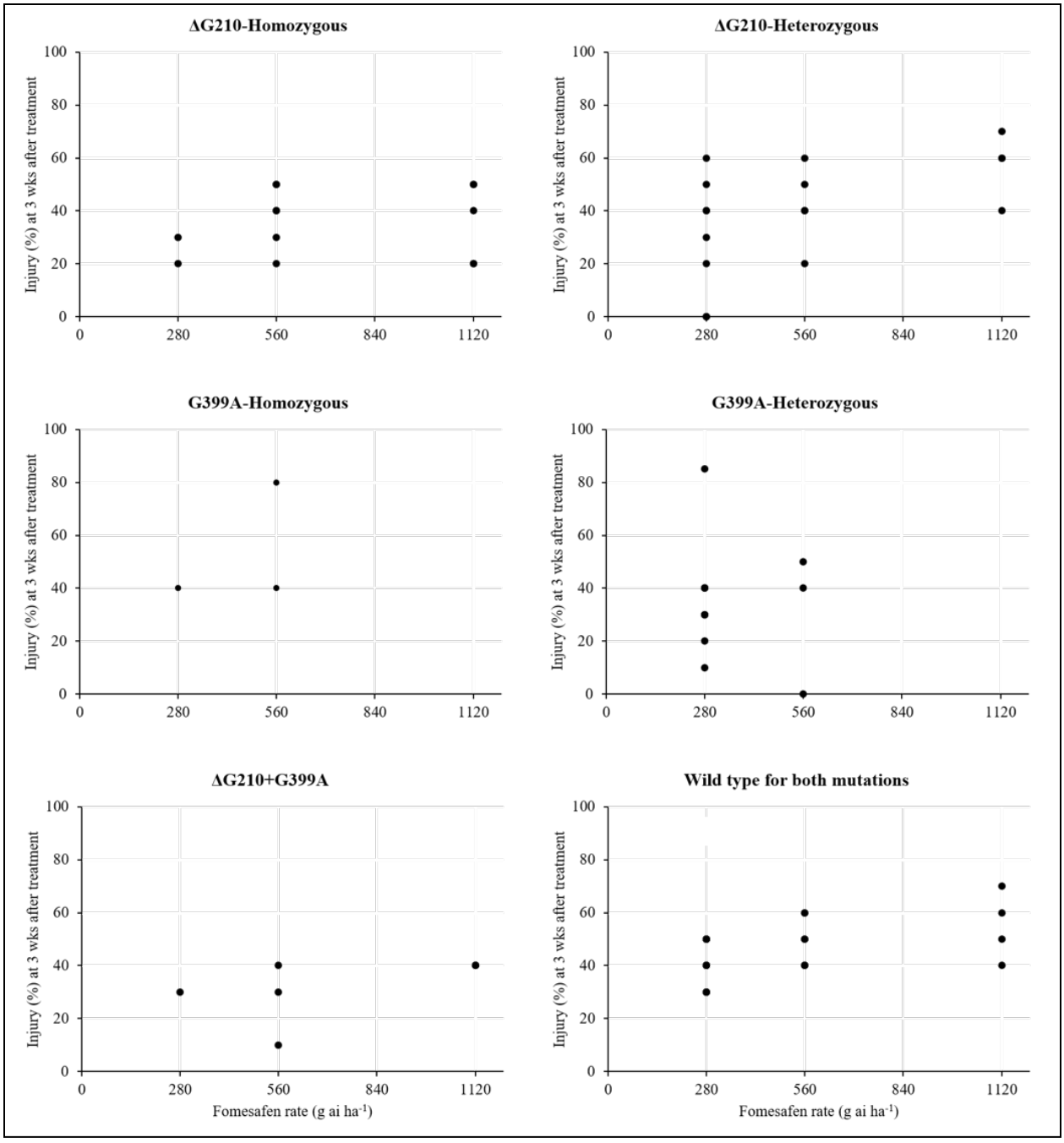

Figure 7. Injury (%) of Palmer amaranth survivors from treatments with 280, 560 and 1120 g ha^{-1} fomesafen (Flexstar® 1.88 EC) + 0.5% v/v nonionic surfactant separated by genotype.

CHAPTER V

CONCLUSIONS

The species Palmer amaranth (*Amaranthus palmeri* S. Wats.) have evolved resistance to several herbicide modes of action, including to protoporphyrinogen IX oxidase (PPO) inhibiting herbicides. Thus far, target-site resistance is the main mechanism of resistance in this species. Among the mutations detected in Palmer amaranth populations, *ΔG210* seems to be the most common one. Palmer amaranth *ppo2 ΔG210* mutation has the greatest herbicide resistance potential when present in both alleles. When higher rates of fomesafen was used, survivors carrying *ΔG210* in both alleles or accumulating *ΔG210+G399A* showed less injury compared to heterozygous *ΔG210*. Homozygosity for *ΔG210* and combination of *ΔG210+G399A* is correlated with rapid cross-resistance evolution. New and effective PPO-formulations are being released in the market as an option to control PPO-resistant populations. However, based on the findings in this study, it is crucial the use of different modes of action in fields where the percentage of individuals carrying *ΔG210* mutation is high. When the transgenic construct Palmer amaranth *ppo2* containing *ΔG210* was transferred into the monocot rice (*Oryza sativa*), it conferred resistance to the PPO-herbicide, fomesafen. This research paves the foundation that herbicide-resistant mutant genes from weeds can be used as transgene to develop genomically unrelated herbicide-resistant crops. This may consist a powerful tool in the future development of transgenic plants.