Characterization of Fenoxaprop-P-Ethyl–Resistant Junglerice (*Echinochloa colona*) from Mississippi

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A population of junglerice from Sunflower County, MS, exhibited resistance to fenoxaprop-P-ethyl. An 11-fold difference in ED_{50} (the effective dose needed to reduce growth by 50%) values was observed when comparing the resistant population (249 g ae ha^{-1}) with susceptible plants (20 g ae ha^{-1}) collected from a different field. The resistant population was controlled by clethodim and sethoxydim at the field rate. Sequencing of the acetyl coenzyme A carboxylase, which encodes the enzyme targeted by fenoxaprop-P-ethyl, did not reveal the presence of any known resistance-conferring point mutations. An enzyme assay confirmed that the acetyl coenzyme A carboxylase in the resistant population is herbicide sensitive. Further investigations with two cytochrome P450 inhibitors, malathion and piperonyl butoxide, and a glutathione-*S*-transferase inhibitor, 4-chloro-7-nitrobenzofurazan, did not indicate involvement of any metabolic enzymes inhibited by these compounds. The absence of a known target-site point mutation and the sensitivity of the ACCase enzyme to herbicide show that fenoxaprop-P-ethyl resistance in this population is due to a non–target-site mechanism or mechanisms.
Nomenclature: Clethodim; fenoxaprop-P-ethyl; sethoxydim; junglerice, *Echinochloa colona* Link. ECHCO
Key words: Acetyl coenzyme A carboxylase, *Echinochloa colona*, fenoxaprop-P-ethyl, herbicide metabolism.

Acetyl coenzyme A carboxylase (ACCase) is the enzyme responsible for the conversion of acetyl CoA to malonyl CoA in fatty acid biosynthesis pathway (Burton et al. 1987; Rendina et al. 1988). ACCase inhibitors, a class of herbicides used to control grass weeds, are composed of three groups: aryloxyphe-noxypropionates (APPs, "fops"), cyclohexanediones (CHDs, "dims"), and phenylpyrazoline (PPZs, "dens"). The selectivity of these herbicides arises from a key difference in graminaceous plants. The difference is that in graminaceous plants, both the plastidic and cytosolic forms of the enzyme are of the sensitive eukaryotic form of ACCase, whereas other plants possess both the eukaryotic (cytosolic) and prokaryotic (plastidic) forms of the enzyme, the latter being insensitive to these inhibitors (Egli et al. 1993; Konishi and Sasaki 1994; Konishi et al. 1996). Thus, insensitivity of the prokaryotic form of the enzyme allows dicots to survive exposure to ACCase-inhibiting herbicides.

Herbicide resistance in weeds is a growing problem that threatens crop yields. Both target-site and non–target-site resistance mechanisms have been reported for ACCase inhibitors. Target-site resistance can result from a point mutation in the carboxyltransferase domain of the eukaryotic ACCase gene; eight point mutations have been documented to date (Powles and Yu 2010). Some, but not all, mutations confer resistance to both APPs and CHDs and have been documented in populations of wild oat (*Avena fatua* L.; Christoffers et al. 2002), American sloughgrass (*Beckmannia syzigachne* Steud.; Li et al. 2013; Pan et al. 2015), goosegrass (*Eleusine indica* L. Gaertn.; Cha et al. 2014; Leach et al. 1995), and annual ryegrass (*Lolium rigidum* Gaudin; Kaundun 2010; Scarabel et al. 2011; Zhang and Powles 2006). More difficult to assess are the non–target-site resistance mechanisms, which can involve metabolism, sequestration, or reduced translocation of the herbicide (Powles and Yu 2010). Increased herbicide metabolism has been reported for blackgrass (*Alopecurus myosuroides* Huds) and annual ryegrass. For some resistant

populations in both species, resistance has been associated with a glutathione-*S*-transferase (Cummins et al. 2013).

ACCase inhibitor resistance has also been reported in the *Echinochloa* genus, having been documented in late watergrass [*Echinochloa phyllopogon* (Stapf.) Koso-Pol.; Fischer et al. 2000], early watergrass [*Echinochloa oryzoides* (Ard.) Fritsch; Altop et al. 2014], barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.; Heap 2015] and junglerice (Heap 2015). Of these species, late watergrass has been investigated further, and evidence suggested a non–target-site mechanism that is likely metabolic (Bakkali et al. 2007, Yun et al. 2005). This was supported by two studies. In the first, fenoxaprop-P-ethyl pretreatment of seedlings was found to induce cytochrome P450 activity in microsomes. Further testing revealed that these induced cytochrome P450s had activity specific to fenoxaprop-P-ethyl (Yun et al. 2005). In the second, an increase in metabolism in the resistant biotype compared to the susceptible was detected and, in particular, an increase in glutathione conjugates, suggesting involvement of glutathione-*S*-transferases in the resistance mechanism (Bakkali et al. 2007). Both classes of enzymes, cytochrome P450s and glutathione-*S*-transferases, are known to be involved in metabolic mechanisms of resistance (Van Eerd et al. 2003).

A population of junglerice was reported from Sunflower County, MS, as being difficult to control with currently available herbicide options. Preliminary screening results revealed that population was resistant to fenoxaprop-P-ethyl and three other herbicides: imazamox, quinclorac, and propanil. Imazamox resistance in this population was determined to be a non–target-site mechanism (Riar et al. 2012); however, the mechanisms for fenoxprop-P-ethyl, quinclorac, and propanil resistance are unknown. The goal of this study was to characterize the fenoxaprop-P-ethyl resistance in this population.

Materials and Methods

Dose Responses. In all experiments, resistant plants (R) of a population from a rice field in Sunflower County were compared with a susceptible plants (S) from a population that had no known history of herbicide exposure. These were maintained and allowed to self-fertilize in the greenhouse. Both resistant and susceptible plants were grown in Metromix 360® potting soil (Sun Gro Horticulture,

Bellevue, WA) and grown under a 12-h photoperiod with day and night temperatures of 24 C and 21 C, respectively. Seedlings were transplanted to individual pots and grown to the two- to three-leaf and three- to four-leaf stage, at which point they were treated. All dose responses were conducted with the use of a spray chamber equipped with a 8002E nozzle (Spraying Systems Co, Wheaton, IL). Herbicide treatments were sprayed at a pressure of 221 kPa and at a volume of 187 L ha^{-1}. Ricestar® (fenoxaprop-P-ethyl, Bayer CropScience, Research Triangle Park, NC) was applied with the adjuvant Agridex® (Bayer CropScience) at a concentration of 1% v/v. Rates of fenoxaprop-P-ethyl were applied to both populations at 30.5, 61, 122 (field rate), 244, 488, and 976 g ae ha^{-1}. Following spraying, plants were returned to the greenhouse. At 3 wk after treatment, injury was assessed for each plant on a scale of 0 to 100%. A rating of 0% indicated no injury and 100% indicated plant death, with values in between assessing the degree of injury and growth inhibition (i.e., a rating of 50 would indicate a 50% reduction in growth compared to untreated). The dose responses were performed three times with four to six plants per treatment group. Data were analyzed in SAS® 9.4 (Cary, NC) with the use of PROC GLM. Means and standard error were plotted in SigmaPlot® 12.5 (Systat Software Inc., San Jose, CA) to calculate GR$_{50}$ values with the use of nonlinear regression analysis of the sigmoidal three-parameter form:

$$y = a \big/ \left(1 + \exp\left(-(x - x_0)/b \right) \right).$$

In this equation y represents the percent control, a is the upper asymptote, x the herbicide concentration, x_0 the ED$_{50}$, and b the slope. Six plants of each population were also treated with Poast® (sethoxydim; BASF, Research Triangle Park, NC) and SelectMax® (clethodim; Valent, Walnut Creek, CA), with the crop oil concentrate Agridex (1% v/v), at the field rates of 314 g ai ha^{-1} and 140.2 g ai ha^{-1}, respectively. This was done twice.

ACCase Sequencing. The ACCase gene was sequenced as part of a larger transcriptomics project to investigate non–target-site acetolactate synthase (ALS) inhibitor resistance (yet to be published). Plants for R and S were divided into two groups: untreated and those treated with imazamox (Beyond®, BASF) at 52.7 g ai ha^{-1}. Tissue was harvested from the second leaf of four to six plants per population per treatment 1 h after imazamox exposure. RNA was extracted with the use of an

RNEasy plant mini kit (Qiagen, Valencia, CA) and DNase treated to remove DNA. Samples were submitted to the Institute for Genomics, Biocomputing & Biotechnology at Mississippi State University (Mississippi State, MS) for library construction and sequencing on Illumina MiSeq and HiSeq platforms. Transcriptomes were assembled for both the S and R populations. Contigs containing the ACCase transcripts were extracted from each of these transcriptomes and aligned in Geneious 7.1.4 (Kearse et al. 2012) to search for point mutations. The consensus sequences for the R and S populations have been submitted to GenBank (accession numbers KX236329 and KX236328, respectively).

ACCase Enzyme Assay. ACCase was extracted from the combined meristematic tissue of 24 plants for each of the S and R populations. The tissue was frozen in liquid nitrogen and ground into a powder, 5 g of which was added to an extraction buffer (50 mM Tris-HCl pH 8.0, 5 mM DTT, 10% glycerol, 2.5 w/v polyvinylpolypyrolidone, and one complete protease inhibitor tablet [Sigma Aldrich, St. Louis, MO] 10 ml^{-1}). The dissolved tissue was ground with a polytron homogenizer for 30 s and then filtered through Miracloth. Cellular debris was collected by centrifugation at 30,000 × g for 20 min at 4 C. Ammonium sulfate was added to the supernatant to produce a 20% w/v solution and the mixture was incubated on ice for 15 min. The supernatant was collected after centrifugation, as before, and saturated to 50% with ammonium sulfate. After a third centrifugation the supernatant was discarded and the pellet dried. The pellet was resuspended in 700 μl of chilled resuspension buffer (50 mM Tris-HCl, 1 mM DTT, 50 μM EDTA, 10% v/v glycerol, and one complete mini-EDTA protease inhibitor tablet (Sigma Aldrich)/10 ml). Any undissolved pellet was removed by centrifugation at 30,000 × g for 20 min at 4 C. Protein concentration was determined by a Bradford assay (Bradford 1976).

For the enzyme assay, the protein solution was diluted in 50 mM Tris HCl (pH 8.0) with 10% v/v glycerol. Each reaction contained 10 μg of protein added to the reaction mix (50 mM Tris HCl pH 8.0, 1 mM DTT, 50 mM KCl, 2.5 mM MgCl$_2$, 1 mM ATP, 10 mM NaHCO$_3$, and 5 μCi mL^{-1} NaC^{14}HCO$_3$). Reactions were performed in a 96-well microtiter plate. Fenoxaprop-P-ethyl was added to each reaction to produce final concentrations of 0.1, 0.5, 1, 5, and 10 μM. Reactions were initiated with acetyl CoA at a final concentration of 1 mM

(control reactions omitted the acetyl CoA). Plates were incubated at room temperature for 60 min and reactions were stopped by addition of 50 μl of 5 N HCl. Two hundred microliters of the assay were spotted onto a Whatman filter in a scintillation vial. After being dried overnight, the filter was dissolved in 500 μl of 50% v/v methanol and 10 ml EcoLite(+)™ liquid scintillation cocktail (MP Biomedicals LLC, Solon, OH) for 2 h. The disintegrations per minute (DPMs) were counted for each sample for five minutes. Two technical replicates were performed per experiment and the experiment was performed twice. Data were plotted in SigmaPlot® 12.5. No curve was calculated for the enzyme data, as none were available that fit the data in SigmaPlot® and there was no significant difference between the two populations.

Treatment with Metabolic Inhibitors. Plants from the R and S populations were each treated with one of three metabolic inhibitors, malathion, piperonyl butoxide (PBO; Chem Service, West Chester, PA), and 4-chloro-7-nitrobenzofurazan (NBD-Cl, Sigma Aldrich) to test for a metabolic herbicide resistance mechanism. Plants were grown as described above. At the two- to three-leaf and three- to four-leaf stage, plants were divided into four treatment groups: no treatment, the inhibitor alone, fenoxaprop-P-ethyl alone, or the inhibitor in combination with fenoxaprop-P-ethyl. All treatments were performed in the spray chamber. Fenoxaprop-P-ethyl was applied at a rate of 122 g ae ha^{-1}. PBO and NBD-Cl were prepared in methanol and acetone, respectively, and applied at rates of 1,400 g ha^{-1} and 270 g ha^{-1}, respectively. Malathion was applied at a rate of 1,000 g ha^{-1}. NBD-Cl was applied 2 d before, PBO 1 d before, and malathion at the time of herbicide treatment. Following herbicide treatment, plants were returned to the greenhouse. At 3 wk, plants were rated for injury on a scale of 0 to 100, with 0 being no injury and 100 being plant death. The inhibitor treatments were performed twice.

Results and Discussion

Herbicide Treatments. Dose-response assays were performed to determine the level of resistance in the R population. Plants from both the R population and the S population were treated with rates ranging from a quarter (1/4×) of the field rate to eight (8×) times the field rate of fenoxaprop-P-ethyl (with the field rate being 122 g ae ha^{-1}). The S population
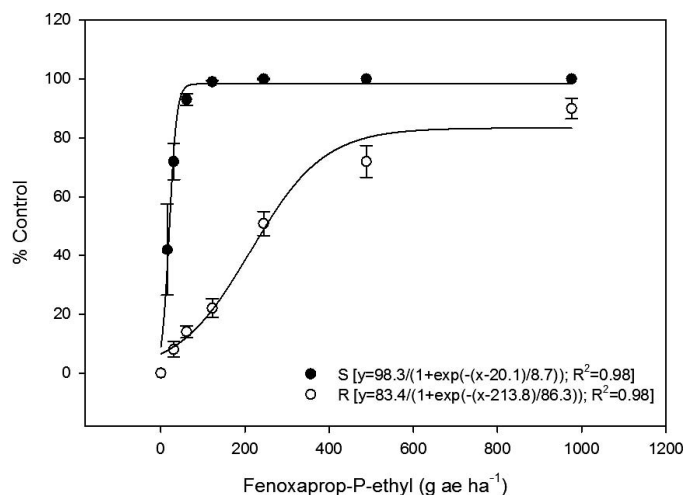
Figure 1. Dose-response curve for susceptible (closed circles) and resistant (open circles) populations treated with fenoxaprop-P-ethyl. Error bars represent standard error.

The plot legend reads:
S [y=98.3/(1+exp(-(x-20.1)/8.7)); R^2=0.98]
R [y=83.4/(1+exp(-(x-213.8)/86.3)); R^2=0.98]

Figure 2. Control of resistant population with (A) clethodim at 314 g ai ha^{-1} and (B) sethoxydim at 140.2 g ai ha^{-1}. In both images, the plant on the left is untreated and the plant on the right is treated. (Color for this figure is available in the on-line version of this article.)

exhibited sensitivity to the herbicide at the lowest dose and was completely controlled at the field rate (Figure 1, closed circles). The resistant biotype exhibited little injury at the field rate and was mostly controlled at the highest dose (Figure 1, open circles). ED$_{50}$ (the effective dose at which a 50% reduction in growth is achieved) values for the resistant and susceptible biotypes were 249 ± 51 and 20 ± 3 g ae ha^{-1}, respectively. The R/S ratio was 10.7, indicating an 11-fold resistance to fenoxaprop-P-ethyl in the R population compared to the S population. R/S values of 27.2 and 3.9 have been reported for blackgrass biotypes exhibiting non–target-site resistance (Hall et al. 1997) and an R/S value of 10 was reported for an late watergrass biotype with suspected metabolic resistance to fenoxaprop-P-ethyl (Bakkali et al. 2007). Populations of American sloughgrass and annual ryegrass exhibiting target site resistance to fenoxaprop-P-ethyl had R/S values of 21.73 and 6.3 (Li et al. 2013; Tal and Rubin 2004). The variation of R/S values for fenoxaprop-P-ethyl resistance demonstrates that this value is not a good predictor of resistance mechanism, necessitating the investigation of both target-site and non–target-site mechanisms.

Cross-resistance to another class of ACCase inhibitors, the CHDs (dims), in the R population was examined by treating plants with field rates of clethodim and sethoxydim. These two herbicides successfully controlled the R population at their respective field rates (Figure 2). Althou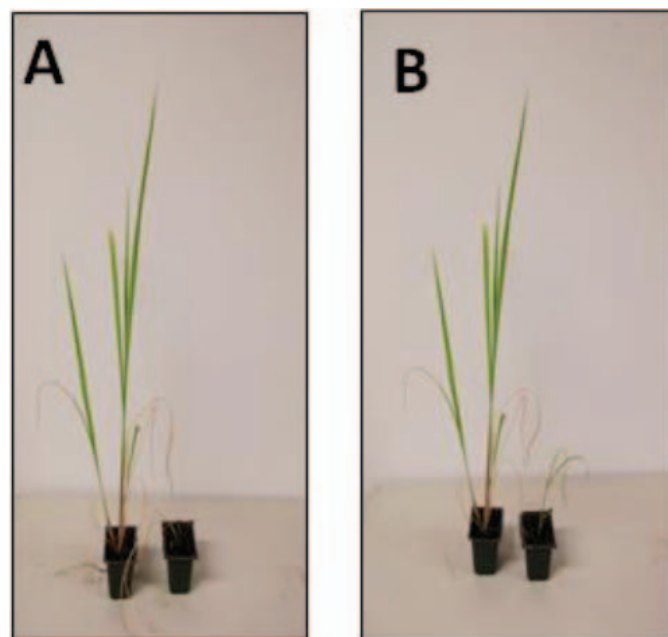gh APPs like fenoxaprop-P-ethyl no longer control the R population, CHDs do, leaving the grower the latter class as an option for controlling junglerice.

ACCase Sequencing. Some of the known resistance-conferring point mutations provide resistance to APPs, but not CHDs (Délye et al. 2003; Powles and Yu 2010). To determine if one of these target-site point mutations is responsible for fenoxaprop-P-ethyl resistance, the ACCase sequence in the R population was examined. The ACCase sequence for the R and S populations was extracted from an RNA-seq data set (unpublished data) in which the transcriptomes of both populations had been sequenced. The amino acid consensus sequences of residues 1763 through to the carboxy terminus were aligned for both populations to the amino acid sequence for blackgrass (accession number AJ310767). None of the known resistance-conferring point mutations were present (Figure 3). Because junglerice is hexaploid, all transcripts were searched for any of the known point mutations; however, none were found (data not shown). Nevertheless, the possibility of a novel point mutation exists. Differences were observed between S and R in the amino acid sequence of the carboxyltransferase domain, including S1789R, Q1976E, and Q2009R. To address this, it was necessary to determine if the enzyme itself was resistant to the herbicide.

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Figure 3. Alignment of susceptible and resistant ACCase sequences with blackgrass (accession number AJ310767). The region containing the carboxyltransferase domain is shown. The known resistance-conferring point mutations are annotated in dark blue, none of which are present in the sequence from the resistant biotype.

ACCase Enzyme Assay. The ACCase enzyme assay is a definitive means of determining if the enzyme in the R population is sensitive to the herbicide. The assay measures malonyl-CoA production in the presence of increasing concentrations of herbicide (0.1, 0.5, 1, 5, and 10 µM) with the use of radiolabeled bicarbonate. There was a precipitous drop in enzyme activity between 0.1 and 1 µM fenoxaprop-P-ethyl, and between 1 and 10 µM enzyme activity ranged from 20–40% of the untreated control (Figure 4). This was true for both the S and R populations. The lack of differential response between the S and R populations indicates that the enzyme from the R population is sensitive to the herbicide. This is not surprising, as no resistance-conferring point mutation was detected in any of the ACCase transcripts. Therefore, the resistance mechanism in the R population is a non–target-site mechanism.

Metabolic Inhibitors. To investigate the involvement of metabolism in the resistance mechanism, metabolic inhibitors were applied with fenoxaprop-P-ethyl. Two cytochrome P450 inhibitors, malathion and piperonyl butoxide (PBO), and a glutathione-*S*-transferase inhibitor, 4-chloro-7-nitrobenzofurazan (NBD-Cl), were applied with or before application of the herbicide. If these compounds inhibit activity of an enzyme essential to the resistance mechanism, the R population should show a reduction in resistance following treatment with both the herbicide and the inhibitor. Malathion, in the absence of fenoxaprop-P-ethyl, had no effect on plant growth and did not reduce resistance to fenoxaprop-P-ethyl in the R population when applied with the herbicide (Figures 5A and 5B). This indicates that the resistance mechanism does not involve a cyto-

chrome P450 enzyme whose activity would be inhibited by malathion. Similar results were observed when PBO and NBD-Cl were applied, indicating the lack of involvement in resistance by any enzymes, cytochrome P450 or glutathione-*S*-transferase, respectively, inhibited by these two chemicals (Figures 5C–F). This is in contrast to some biotypes of blackgrass and annual ryegrass in which resistance was greatly reduced when the herbicide was applied following NBD-Cl treatment (Cummins et al. 2013). In large crabgrass [*Digitaria sanguinalis* (L.) Scop.], where a metabolic resistance mechanism has been established, neither malathion nor PBO inhibited fluazifop metabolism (Hidayat and Preston 2001). Therefore metabolism is not ruled out as a mechanism, but enzymes
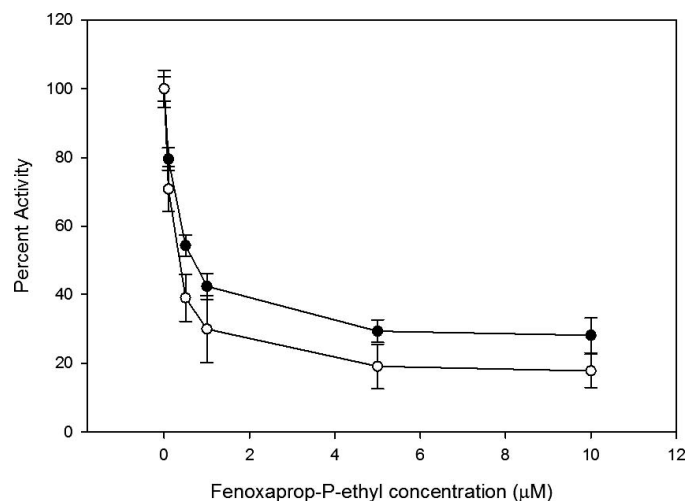

Figure 4. Percent activity of acetyl coenzyme A carboxylase for resistant (open circles) and susceptible (closed circles) populations in the presence of increasing concentrations of fenoxaprop-P-ethyl. Error bars represent standard error.
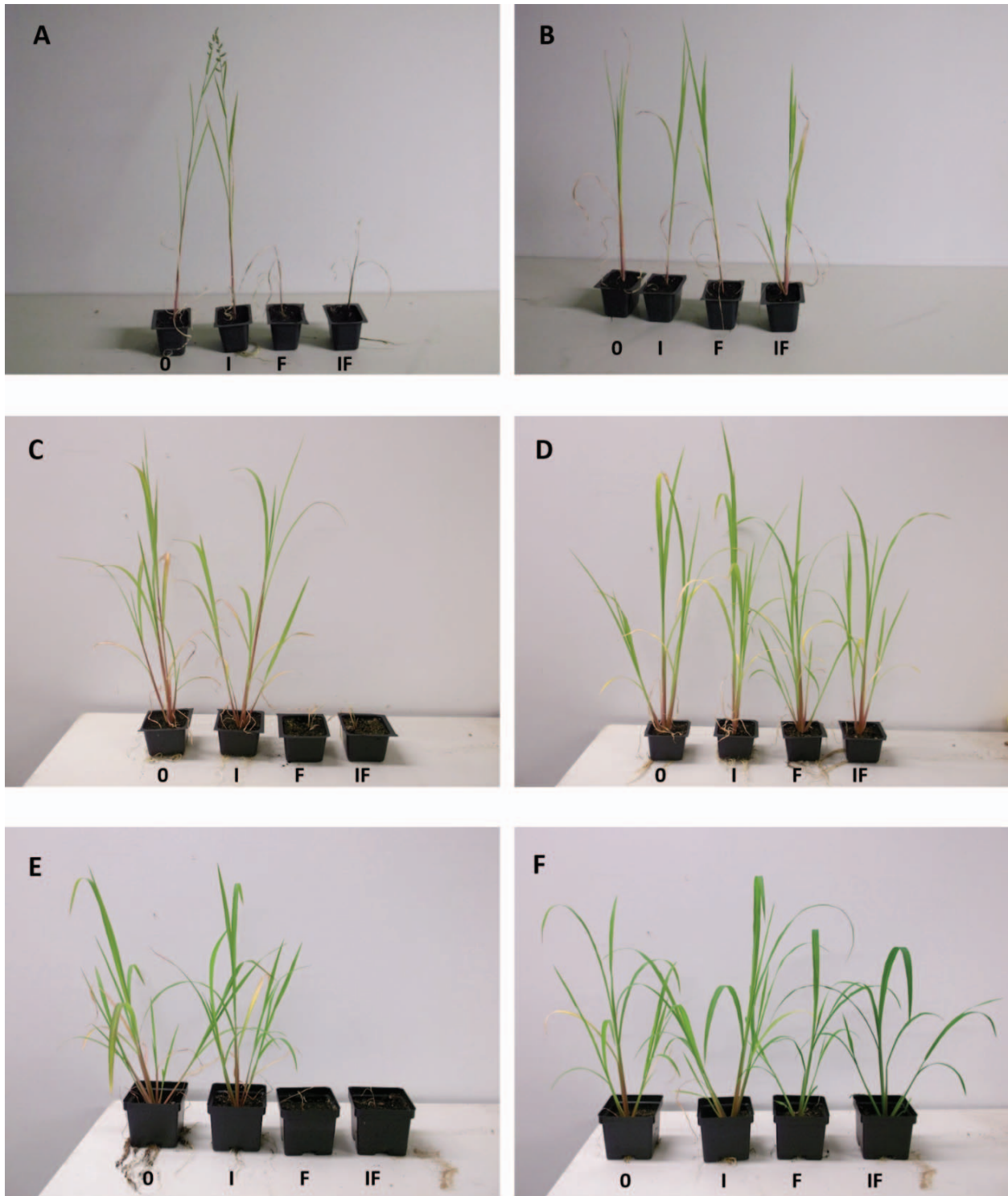
Figure 5. Treatment with metabolic inhibitors malathion (A and B), 4-chloro-7-nitrobenzofurazan (C and D), and piperonyl butoxide (E and F) for susceptible (A, C, and E) and resistant (B, D, and F) populations. From right to left are untreated (0), inhibitor only (I), fenoxaprop-P-ethyl alone (F), and inhibitor with fenoxaprop-P-ethyl (IF). (Color for this figure is available in the on-line version of this article.)

inhibited by these metabolic inhibitors are not involved in the resistance mechanism.

The R population of junglerice identified in Sunflower County is resistant to fenoxaprop-P-ethyl with an R/S value of 10.65. At this level, the population cannot be controlled with fenoxaprop-P-ethyl, necessitating the use of different control options such as other herbicides. CHDs, another

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class of ACCase inhibitors, effectively control this weed. The resistance mechanism has been difficult to determine. No known point mutations responsible for resistance were found in the ACCase sequence and the enzyme assay confirmed sensitivity of the enzyme to fenoxaprop-P-ethyl. None of the tested metabolic inhibitors affected resistance. Additional work will be required to determine what non–target-site mechanism is responsible for resistance. This may include metabolic studies to identify differences between R and S in the types and amount of metabolites produced. Monitoring uptake and translocation of the herbicide in R and S populations could determine if there are any differences in movement of the herbicide into and within the plant. RNA-seq analysis will provide the sequence and gene expression data needed to identify candidate resistance genes for further study. These experiments will aid in elucidating the non–target-site mechanism present in this population.

Acknowledgments

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