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Resistance to aryloxyphenoxypropionate herbicides in Amazon sprangletop: Confirmation, control, and molecular basis of resistance

Parsa Tehranchian [a,*], Jason K. Norsworthy [a], Nicholas E. Korres [a], Scott McElroy [b], Shu Chen [b], Robert C. Scott [a]

[a] Department of Crop, Soil and Environmental Sciences, University of Arkansas, Fayetteville, AR, USA
[b] Department of Crop, Soil, and Environmental Sciences, Auburn University, 201 Funchess Hall, Auburn, AL, USA

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ABSTRACT

Amazon sprangletop is problematic weed of rice in the midsouthern USA. Two biotypes of this species from rice fields approximately 100 km apart in Louisiana were unaffected when sprayed with the labeled field rate of cyhalofop-butyl (314 g ai ha^{-1}) in 2008. Dose response studies were conducted to confirm the level of resistance to cyhalofop-butyl over a range of doses. Cross-resistance to acetyl-CoA carboxylase (ACCase)-inhibiting herbicides from two different chemical families and multiple herbicide resistance to other mechanisms of action were evaluated. Sequencing using the Illumina Hiseq platform and ACCase gene sequencing revealed two different amino acid substitutions, Trp_{2027}-to-Cys in the first resistant biotype and Asp_{2078}-to-Gly in the second resistant biotype, within the CT domain of the ACCase gene. Two known amino acid substitutions confirmed resistance to cyhalofop-butyl and fenoxaprop-P-ethyl in resistant Amazon sprangletop biotypes. Asp_{2078}-to-Gly amino acid substitution that was detected in one of the resistant biotypes did not result in cross-resistance to clethodim, an ACCase-inhibiting cyclohexandione herbicide which has endowed clethodim resistance in other weed species. Based on this research, both resistant Amazon sprangletop biotypes have evolved target-site resistance to the APP herbicides; yet, alternative herbicides are still active on these plants.

1. Introduction

Amazon sprangletop [*Leptochloa panicoides* (J. Presl) Hitchc.], also known as tighthead sprangletop is an annual species of the Poaceae family [1]. It is native of Brazil and an increasingly problematic weed of rice (*Oryza sativa* L.) in the southern United States [2]. Among the sprangletop species in the midsouthern USA, bearded sprangletop [*Leptochloa fascicularis* (Lam.) Gray] is the most common [3], Amazon sprangletop is becoming increasingly persistent in rice crops in this region. Both bearded and Amazon sprangletop are highly competitive with rice and can cause yield reductions of up to 36% [4].

Management of grass weeds in large-scale rice production systems is efficient with the use of chemical control methods [5]. The aryloxyphenoxypropionate [APP (fops)], cyclohexanedione [CHD (dims)], and phenylpyrazoline [PPZ (dens)] herbicides are the most popular and important class, of herbicides used to control grass weeds in major crops such as soybean [*Glycine max* (L.) Merr.], wheat (*Triticum aestivum* L.), cotton (*Gossypium hirsutum* L.), and rice worldwide. This herbicide class acts by blocking the activity of acetyl-coenzyme A carboxylase (ACCase; E.C. 6.4.1.2), thereby inhibiting lipid biosynthesis in grass species [6,7,8,9]. Both heteromeric prokaryotic and homomeric eukaryotic forms of this enzyme are found in nature [10]. The isoforms of ACCase are prokaryotic and localized in the soluble phase of chloroplast and cytosol of the epidermal and mesophyll tissues [11]. The plastidic form of the enzyme, with more than 80% total activity of ACCase [12], is essential for de novo biosynthesis of primary fatty acid [13,14]. The cytosolic isoform is involved in the synthesis of long-chain fatty acids that are then utilized in cuticular wax production in epidermal tissues [15,16]. The heteromeric plastidic ACCase and cytosolic isoforms in the majority of plant species are insensitive to APPs and CHDs [17]. These classes of herbicides selectively bind to the carboxyl transferase (CT) domain of homomeric plastidic-localized ACCase of Poaceae [7,8]. Nevertheless, there are a few grass weeds, such as red fescue (*Festuca rubra* L.), that are intrinsically tolerant to ACCase inhibitors due to an insensitive homomeric plastidic ACCase isoform [18].

Among ACCase-inhibitors, there are four herbicides i.e. fenoxaprop-P-ethyl, cyhalofop-butyl, quizalofop-P-ethyl, and profoxydim used to control sprangletops in direct-seeded rice worldwide [19]. Cyhalofop-butyl (314 g ai ha^{-1}) and fenoxaprop-P-ethyl (123 g ai ha^{-1}) are the preferred herbicides for the postemergence (POST) control of sprangletops in midsouthern USA rice [20,21]. These herbicides, when applied at the four-leaf to two tiller, also effectively control Amazon sprangletop [22].

Widespread application of ACCase-inhibiting herbicides, which have been used since 1977, led to the evolution of a resistant annual ryegrass (*Lolium rigidum* L.) biotype in Australia in 1982 [23]. To date, 47 weed

* Corresponding author at: Department of Crop, Soil and Environmental Sciences, University of Arkansas, 1366 West Altheimer Drive, Fayetteville, AR 72704, USA.
E-mail address: ptehranc@uark.edu (P. Tehranchian).

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species have evolved resistance to ACCase-inhibitors worldwide [24]. In 2002, the first report of ACCase-resistant red sprangletop (*Leptochloa chinensis* L.) was documented in Thailand in a field sprayed with fenoxaprop-P-ethyl and cyhalofop-butyl [19]. In the United States, there have been recent reports of a bearded sprangletop (*Leptochloa fusca ssp. fascicularis*) in California with resistance to cyhalofop-butyl and quizalofop-P-ethyl [25]. Additionally, two putative cyhalofop-butyl-resistant Amazon sprangletop biotypes were found in Louisiana in 2007 and 2008. These biotypes were detected in fields under continuous rice production with one to two applications of cyhalofop-butyl. Additional use history of herbicides in these fields is unknown; however, rapid evolution of herbicide resistance after three to four exposures to ACCase inhibitors has been reported in annual ryegrass populations in South Australia [26,27].

The mechanisms of resistance to ACCase inhibitors have been extensively studied in grass weeds including non-target-site (NTSR) and target-site based resistance (TSR) [17]. The latter was detected in many weed species and so far, seven ACCase codon positions have been identified within homomeric plastidic ACCase results in different levels of herbicide resistance [10]. Among these amino acid substitutions, residue 1781 is the most common found in plant species [28].

This study reports the first case of herbicide resistance in Amazon sprangletop worldwide. The objectives of this research were to (1) verify the level of resistance to cyhalofop-butyl, (2) test for cross resistance across two distinct chemical groups, (3) characterize the mechanism of resistance through transcriptome analysis using RNA sequencing and ACCase gene amplification, and (4) assess the effectiveness of additional herbicide options.

2. Materials and methods

2.1. Plant propagation

Seeds of two putative resistant Amazon sprangletop biotypes were collected in Rapides Parish near Bunkie (12–08, hereafter referred to *Res1*) and St. Landry Parish near Whiteville (14–08, hereafter referred to *Res2*), Louisiana, respectively. Seeds of a susceptible biotype (*Sus*) were purchased from a commercial seed supplier (Azlin Seed Company, Leland, MS). All experiments were conducted in the greenhouse at the Agricultural Research and Extension Center, Fayetteville, AR. Initially both *Res1* and *Res2* biotypes were confirmed resistant to a labeled field rate of cyhalofop-butyl in comparison to the susceptible biotype in a greenhouse study (data not shown). Plants of each biotype were self-pollinated in a Conviron growth chamber (CMP 6050) at 30/20 °C (day and night) under 14-h photoperiod. Seeds of both resistant and susceptible biotypes were sown individually in plastic trays (52.5 × 25.5 × 5.5 cm) filled with commercial potting mix (LC1, Sun Gro Horticulture, AB, Canada) in the greenhouse under 14-h photoperiod provided by high pressure sodium lights (400 μmol/m^2/s) at 35/25 °C day/night temperature. Seedlings at two-leaf growth stage were transplanted into plastic pots (8 cm height × 10 cm diameter). Plants were watered on a daily basis. All herbicide treatments were applied to three- to four-leaf plants using an automated research track sprayer equipped with a boom containing two flat-fan nozzles with 80,067 tips (Teejet® Technologies, Springfield, IL) delivering 187 L ha^{-1} at a pressure of 276 kPa.

2.2. Herbicide response evaluation

2.2.1. Dose response

The treatments were arranged in a Randomized Complete Block Design (RCBD) with 20 replications for each cyhalofop-butyl dose including a non-treated control. The experiment was conducted twice. Plants of both resistant and susceptible biotypes were treated with different doses of cyhalofop-butyl (Clincher SF®, Dow AgroSciences) containing 1% v/v of crop oil concentrate (AGRI-DEX®, Helena Company). The herbicide doses for the *Sus* biotype were 19.5, 39, 78.5, 157, 314, 628, and 1256 g ai ha^{-1}, which equates to 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 times the labeled field rate of cyhalofop-butyl whereas doses for resistant biotypes were 314, 628, 1256, 2512, 5024, 10,048, 20,096, and 40,192 g ai ha^{-1} which equates to 1, 2, 4, 8, 16, 32, 64, and 128 times the labeled field rate of cyhalofop-butyl. Plants were kept in the greenhouse after herbicide applications under the same conditions as mentioned earlier. Plant mortality was recorded 21 days after treatment (DAT) and subsequently the aboveground biomass was harvested and oven dried at 60 °C for 48 h. All data were subjected to analysis of variance (ANOVA) in SAS (Version 9.1.3., SAS Institute Inc., Cary, NC, USA) using PROC MIXED procedure. As the interaction of treatment × run was not statistically significant, data for both runs were pooled for subsequent analysis. Means were separated using Fisher's protected LSD at $\alpha = 0.05$. To calculate the lethal dose of cyhalofop-butyl at 50% (LD_{50}), the mortality data were subjected to probit analysis using PROC PROBIT in SAS. Sensitivity of the resistant biotypes to cyhalofop-butyl dose was assessed by determining 50% plant growth reduction (GR_{50}). Dry weight data were calculated as a percentage of the mean of the non-treated control and a four-parameter log-logistic model (Eq. (1)) was fitted to the data[29] using SigmaPlot (Version 12.5, Systat Software Inc., San Jose, CA).

$$y = y_0 + a/\left(1 + \mathrm{abs}(x/x_0)^b\right) \quad (1)$$

where y is percentage of dry weight reduction caused by cyhalofop-butyl dose x (g ha^{-1}), a is a fixed value, x_0 and y_0 are asymptotes, and b is the relative slope around GR_{50}.

2.2.2. Cross- and multiple-resistance

The experiment conducted in a factorial RCBD with eleven herbicides (Table 1) by three biotypes to assess the efficacy of the ACCase inhibitors and alternative herbicides currently labeled in either rice or soybean. All herbicide treatments were at the recommended field rate [21] as shown in Table 1. Each treatment contained 10 replications and the experiment repeated. Visual assessments of percentage control were taken 21 DAT on a scale of 0 (no injury) to 100 (dead plant), and dry weights were recorded as mentioned earlier. All data were subjected to ANOVA using PROC MIXED in SAS. Data from the repeated experiment pooled because of non-significant treatment by experiment interactions. Means were separated using Fisher's protected LSD at $\alpha = 0.05$.

2.3. Transcriptome construction, ACCase gene assembly, mapping and single nucleotide polymorphism (SNP) detection

Initially, three sets of primers were designed to amplify the CT domain of the ACCase gene of Amazon sprangletop biotypes based on multiple alignments of the coding region sequences of blackgrass [*Alopecurus myosuroides* (Huds.)] (GenBank accession number AJ310767), annual bluegrass (*Poa annua* L.) (GenBank accession number KJ754027.1), foxtail millet [*Setaria italica* (L.) P. Beauvois] (GenBank accession number AY219175.1), American sloughgrass [*Beckmannia syzigachne* (Steud.) Fernald] (GenBank accession number KJ580619.2), johnsongrass [*Sorghum halepense* (L.) Pers.] (GenBank accession number KF885933.1), Japanese foxtail (*Alopecurus japonicus* L.) (GenBank accession number KF683622.1), and red sprangletop [*Leptochloa chinesis* (L.) Nees] (GenBank accession number AY803783.1). ACCase gene amplification for both resistant and susceptible biotypes using DNA and cDNA templates was not successful. Therefore, to verify the coding regions of the ACCase gene of the Amazon sprangletop biotypes, RNA sequencing was conducted. Transcriptional studies for the detection of amino acid substitutions endowing herbicide target-site based resistance (TSR), e.g. acetolactate synthase (ALS), have been conducted in several non-model organisms