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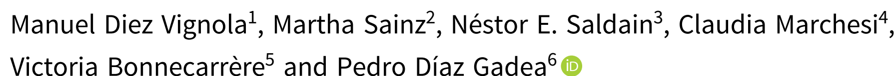
Limited induction of ethylene and cyanide synthesis are observed in quinclorac-resistant barnyardgrass (*Echinochloa crus-galli*) in Uruguay



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Abstract

Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv] is the foremost weed in rice (*Oryza sativa* L.) systems, and its control is crucial to successful rice production. Quinclorac, a synthetic auxin herbicide, has been used effectively to manage *E. crus-galli*. However, occurrences of quinclorac-resistant genotypes are frequently reported, and its resistance evolution has led to questions about the continued utility of quinclorac for grass control. Identification of the resistance mechanism(s) of resistant genotypes will facilitate development of integrated weed management strategies that sustain quinclorac use for management of *E. crus-galli*. We evaluated the responses to quinclorac of two contrasting genotypes: E7 (resistant, R) and LM04 (susceptible, S). Quinclorac induced ethylene and cyanide biosynthesis in the S-genotype. Both genotypes responded similarly to an increasing application of exogenous 1-carboxylic acid aminocyclopropane (ACC) and potassium cyanide, and their growth was inhibited at higher doses. The key mechanism for cyanide (HCN) detoxification in plants, β-cyanoalanine synthase (β-CAS) activity, was evaluated in both genotypes, and no significant difference was observed in the basal activity. However, quinclorac significantly induced β-CAS–like activity in the S-genotype, which is consistent with the increased synthesis of ethylene and cyanide. This work suggests that the resistance to quinclorac of the E7 R-genotype is likely related to an alteration in the auxin signal transduction pathway, causing a lower stimulation of ACC synthase and, therefore, limited synthesis of ethylene and HCN after quinclorac treatment.

Introduction

Rice (*Oryza sativa* L.) is one of the principal food crops worldwide. Weed interference with crop development is an important limiting factor in achieving high yields (Oerke 2006). Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv] is one of the most damaging weeds in rice (Leeson et al. 2005), and herbicides with different mechanisms of action have been used successfully to control it. However, several genotypes resistant to one of these herbicides, quinclorac, have been detected (Concenço et al. 2009; Heap 2019; López-Martínez et al. 1997). Quinclorac is a highly selective auxinic herbicide used to control weeds in rice crops (Grossmann and Kwiatkowski 2000). Although there is incomplete understanding of the mode and site of action of this herbicide, it is well known that it induces de novo synthesis of 1-carboxylic acid aminocyclopropane (ACC) from ACC synthase (EC 4.4.1.14) at the roots, resulting in increased ACC in susceptible plant species (Grossmann 1998). The excess ACC is translocated to the shoot tissue, where it is metabolized to ethylene and hydrogen cyanide by ACC oxidase (ACO; EC 1.14.17.4). This process is self-amplified, as ACC and cyanide (HCN) induce the activity of ACC synthase in the shoot tissue. Most cyanide produced in higher plants is detoxified primarily by the enzyme β-cyanoalanine synthase (β-CAS; EC 4.4.1.9). β-CAS, a mitochondrial enzyme, is dependent on pyridoxal phosphate and catalyzes the reaction of the formation of β-cyanoalanine and hydrogen sulfide from HCN and cysteine (Blumenthal et al. 1968). Another enzyme, cysteine synthase (CS; EC 4.2.99.8), is also capable of HCN detoxification in the cytoplasm (Liang and Li 2001; Machingura and Ebbs 2014; Maruyama et al. 1998, 2000), and when β-CAS activity is measured, the CS activity is also measured, and the term "β-CAS–like activity" is therefore used.

Due to the diversity and complexity of the responses to quinclorac, several possible mechanisms can explain resistance. These mechanisms range from differential absorption to translocation and metabolism to alteration in the route of transduction of auxinic signals and up to an increase in the activity of enzymes capable of eliminating excess HCN. Although differential absorption and translocation can cause resistance to herbicides in several species, no evidence has been found to indicate that these factors influence resistance to quinclorac (Grossmann and Kwiatkowski 2000; López-Martínez et al. 1997; Lovelace et al. 2007). Another factor that causes resistance to herbicides is differential metabolism. The metabolism of quinclorac in different monocot species is low and does not seem to influence the resistance, as quinclorac metabolism is similar between resistant and susceptible *E. crus-galli* genotypes (Grossmann and Kwiatkowski 2000; Lovelace et al. 2007). Some authors have suggested that resistance may occur through an alteration in the signal transduction of auxins, which must prevent, in the resistant genotypes, the de novo synthesis of the enzyme ACC synthase, and hence, the formation of ACC, ethylene, and cyanide (Abdallah et al. 2006; Grossmann 2000, 2010; Yasuor et al. 2011). Studies in *Arabidopsis thaliana* have linked mutations in the TIR and AFB auxin receptors with resistance to auxinic herbicides (Gleason et al. 2011; Walsh et al. 2006). Although the signal transduction caused by quinclorac has not been deeply studied yet, it has been shown that quinclorac can bind to the specific auxinic receptor AFB5 (Lee et al. 2014); therefore, a mutation in the gene that codes this receptor could cause resistance, as is the case with other auxinic herbicides (Gleason et al. 2011; Walsh et al. 2006). Moreover, an elevated basal activity of β-CAS has been proposed as a secondary resistance mechanism (Abdallah et al. 2006; Yasuor et al. 2011). β-CAS, which can eliminate excess cyanide, would have higher activity in resistant genotypes when compared with susceptible genotypes (Abdallah et al. 2006; Yasuor et al. 2011). Besides producing less cyanide, an augmented capacity to detoxify it would result in reduced activity of quinclorac in the resistant genotype.

In Uruguay, most of the 35 resistant *E. crus-galli* genotypes evaluated originated in different locations with different field histories. One of them, E7, was particularly interesting, because it expressed a high level of resistance to quinclorac, as indicated by a GR_{50} (dose of herbicide at which a 50% reduction in the response occurred) higher than the quinclorac label rate (375 g ha^{-1}) and higher than the maximum dose evaluated (3,000 g ha^{-1}). The E7 genotype also showed intermediate levels of resistance to propanil (Marchesi and Saldain 2019). Despite the high level of quinclorac resistance observed, studies were carried out to confirm or refute a mechanism of metabolic resistance. Dose–response assays with quinclorac, using malathion, an inhibitor of the cytochrome oxidase P450, were performed. No reduction of the high level of quinclorac resistance was observed when the herbicide was applied after malathion spraying (Saldain and Sosa 2016). In this context, the present work aimed to contribute to the knowledge of the mechanisms of resistance to quinclorac of *E. crus-galli* through the analysis of ethylene and cyanide synthesis induction, and β-CAS–like activity, in the resistant genotype E7 and the susceptible genotype LM04.

Materials and Methods

Plant Material

Echinochloa crus-galli seeds were supplied by the Instituto Nacional de Investigación Agropecuaria (INIA), Estación Experimental del Este, Treinta y Tres, Uruguay. Three genotypes sensitive to quinclorac (LM04, CL44, and CB01), and three genotypes with suspected resistance to quinclorac (ZA01, E7, and RB282) were subjected to dose–response experiments. The resistant genotypes were multiplied twice by selfing isolated from one another. Based on these results, the LM04 (S) and E7 (R) genotypes were selected to proceed with the physiological and biochemical studies.

For plant growth in pots for cyanide and β-CAS–like activity (including β-CAS and CS enzyme) determination in quinclorac-treated plants, 5-mm-long seedlings were placed in 300-ml volume pots (5 seedlings per pot) in a sand–vermiculite (1:1) mix in a growth chamber with a 16/8-h (light/dark) photoperiod at 30/25 C day/night temperatures and watered with nutrient solution (Hoagland and Arnon 1950). At the 2- to 3-leaf stage, the treated plants were watered with 300 ml of a 10 μM quinclorac solution. At 1, 3, 5, and 7 d post–quinclorac application, the roots and shoots of 3 pots per genotype (S and R), and treatment (quinclorac treated and nontreated controls) were harvested separately. The material was immediately snap frozen in liquid nitrogen and stored at −80 C until use.

For all experiments, seeds were treated with H_2SO_4 18 M for 8 min with gentle shaking and rinsed several times with sterile water. Treated seeds were placed in 10 g L^{-1} agar-water petri dishes and kept in the previously described growth chamber for 2 to 3 d until 5-mm shoots developed.

Herbicide

Plants were treated with a 95% quinclorac (3,7-dichloro quinoline-8-carboxylic acid) analytical standard provided by Cibeles S.A. (12 de Diciembre 767, Montevideo, Uruguay). A 50 mM stock solution was prepared by diluting the herbicide in a dimethylsulfoxide:methanol (1:10) mixture.

Dose–Response Experiments in Agar Medium

Seedlings (5-mm long) were further grown in square petri dishes (5 seedlings per dish) containing 100 ml of nutrient solution (Hoagland and Arnon 1950) with 10 g L^{-1} agar and various concentrations of quinclorac (0, 0.6, 1.2, 2.4, 4.8, 10, 20, 40, 80 μM), ACC (0, 0.01, 0.1, 1 mM) and potassium cyanide (KCN) (0, 0.2, 0.4, 0.8, 1.6, 3.2 mM). The effects of ACC and KCN exogenous application in the growth of shoot and root were evaluated to investigate the relationship between ethylene and cyanide production and cytotoxicity in the S- and R-genotypes. Each experimental treatment had two replicates, and the experiment was conducted three times. The petri dishes were placed in the previously described growth chamber, and a photographic register using a Nikon D40 camera was performed on day 7. The photographs were analyzed digitally. Quantitative data on root and shoot length were recorded using the software ImageJ (National Institute of Mental Health, Bethesda, MD, USA). The level of resistance was determined using the GR_{50} (herbicide rate required for 50% root growth reduction), which was estimated using nonlinear regression analysis.

Dose–Response Experiments in Pots

Seedlings (5-mm long) were transplanted into 300-cm^3 pots (5 seedlings per pot) filled with a sand–vermiculite (1:1) mix in the growth chamber and watered with nutrient solution as previously described. The experiment was conducted twice using a

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completely randomized design with four replicates per quinclorac dose (0, 80, 160, 320, 640, 1,280 and 2,560 g ha^{-1}). The quinclorac dilutions were applied at the 2- to 3-leaf stage, and 3 wk later the shoot tissue was cut at ground level and used for cyanide and chlorophyll determination, antioxidant enzyme activity analysis, and β-CAS–like activity determination. The details for these procedures are provided in the following sections. Aboveground fresh biomass weight (FW) was determined. The effect of each herbicide treatment was calculated as the percentage of the FW obtained with respect to the FW of the nontreated control samples. The level of resistance was determined using the GR_{50} (herbicide rate required for 50% aboveground fresh biomass weight reduction), which was estimated using nonlinear regression analysis.

Ethylene, Cyanide, Protein, Chlorophyll, and Antioxidant Enzyme Activity Determination

Hydroponic devices were used for ethylene determination. Seedlings (5-mm long) were placed in 1.05-L sterile flasks containing 450 ml of nutrient solution (Hoagland and Arnon 1950). The flasks were placed in a growth chamber under the previously described conditions until the 2- to 3-leaf stage.

Ethylene production was determined using a Shimadzu GC-2010 Plus (Shimadzu Corporation, Kyoto, Japan) gas chromatograph equipped with a flame ionization detector. Following the quinclorac application (10 μM), the flasks were sealed with rubber septa and incubated for 5 h at 25 C. After incubation, ethylene was measured by withdrawing a 0.5-ml gas sample with a Hamilton Gastight syringe and injecting it into the gas chromatograph. This procedure was performed daily up to 4 d after application. The ethylene concentration was expressed as the nanomoles produced per milligram of FW.

Cyanide extraction was performed following the procedure by Abdallah et al. (2006), and quantified by the Lambert method (Lambert et al. 1975), with modifications (Yip and Yang 1988). The cyanide concentration was expressed as nanomoles produced per gram of FW. Total protein was determined following the procedures by Bradford (1976), using bovine serum albumin as the standard. Chlorophyll was quantified according to Wellburn (1994), and chlorophyll concentration was expressed as milligrams of chlorophyll a and b per milligram of FW. The activity of the APX, CAT, and SOD enzymes was determined according to Chen and Asada (1989), Beers and Sizer (1952), and Dhindsa and Matowe (1981), respectively.

β-CAS–like Activity Determination and Visualization in Native Gel

Preparation of Crude Extracts

The crude extracts were prepared according to the procedures of Liang and Li (2001). The frozen leaves or roots were homogenized using TEC buffer (0.1 M Tris–HCl, 2 mM EDTA-Na_2, 10 mM Cys, pH 9.5) (10 ml g^{-1} FW for leaves, 5 ml g^{-1} FW for roots) in a prechilled mortar and pestle at 4 C. The homogenate was filtered through four layers of gauze. The filtrate was centrifuged at 25,000 × *g* for 10 min at 4 C, and the supernatant was used as crude extract.

Subcellular Fractionation

Three subcellular compartments—chloroplast, mitochondria, and cytosol—were further analyzed to determine the presence of β-CAS isozymes (or CS enzyme) in the leaves of the E7-R genotype. These three compartments were isolated according to Liang and Li (2001). Leaves were homogenized using STEC buffer (0.33 M sucrose, 0.1 M Tris–HCl, 2 mM EDTA-Na_2, 10 mM Cys, pH 9.5) (10 ml g^{-1} FW) in a prechilled mortar and pestle at 4 C. The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at 200 × *g* for 1 min at 4 C. The resulting supernatant was centrifuged at 1,300 × *g* for 3 min at 4 C. The pellet was collected to obtain chloroplast using additional procedures (1) and the supernatant was centrifuged at 6,000 × *g* for 2 min. The resulting supernatant was centrifuged at 23,000 × *g* for 10 min. The pellet was used to obtain mitochondria through additional procedures (2) and the supernatant was used to prepare cytosol by following additional procedures (3). Additional procedures (1): the pellet was washed two times with 10 ml STEC buffer and was centrifuged at 1,300 × *g* for 3 min at 4 C. The pellet was used as chloroplast. Additional procedures (2): the pellet was washed two times with 10 ml STEC buffer and was centrifuged at 23,000 × *g* for 10 min at 4 C. The pellet was used as mitochondria. Additional procedures (3): the supernatant was centrifuged at 25,000 × *g* for 30 min, and the supernatant was used as cytosol preparation. Before being loaded to non-denaturing polyacrylamide gel electrophoresis (PAGE) for β-CAS–like gel activity determination or to SDS-PAGE for western blot analysis, the isolated mitochondria and chloroplast were lysed by adding 400 μl of TEC buffer. The protein concentration of each fraction was determined using the Bradford protein assay (Sigma-Aldrich, St. Louis, MO, USA).

Fractionation Validation Using Western Blotting

Validation of the purity of the subcellular fractions was determined by SDS-PAGE analysis of the three compartmental protein markers. Protein samples (20 μg per fraction) were separated using a 12% SDS-PAGE for 90 min at 100 V, transferred onto polyvinylidene fluoride membranes (Sigma-Aldrich, St. Louis, MO, USA) for 90 min at 80 mA, and then probed using polyclonal antibodies for PsbA/D1 protein of photosystem II (Agrisera, Vännäs, Sweden; 1:10,000), alternative oxidase 1/2 (Agrisera, 1:1,000), and actin (Agrisera, 1:3,000) for the chloroplast, mitochondrial, and cytosolic fractions respectively. Proteins were visualized after application of specific secondary horseradish peroxidase–conjugated antibodies and exposure to ECL substrate (Clarity™ Western ECL Substrate, Bio-Rad, Hercules, CA, USA) by use of a C-DiGit blot scanner (Li-Cor, Lincoln, NE, USA). All antibodies were incubated for 1 h at room temperature with mild agitation, and the membranes were washed with Tris-buffered saline-Tween-20 three times for 10 min each time following each antibody incubation.

β-CAS–like Activity

β-CAS–like activity was determined using the methylene blue method according to Yasuor et al. (2011). Na_2S was used as the standard. Enzymatic activity was expressed as nanomoles of H_2S per mg of protein per minute. For the visualization of β-CAS–like activity, non-denaturing PAGE was performed on 12% separating and 5% stacking gel according to Sambrook et al. (1989). The electrophoresis was run at 4 C, 20 mA, 90 min.

For leaf crude extracts 50 μg of total protein was used, and for root crude extracts 30 μg of total protein was used. The protein concentration of each sample was determined using the Bradford protein assay (Sigma). The β-CAS–like gel activity was measured according to Maruyama et al. (1998). The substrates, KCN (5 mM), and Cys (10 mM) were dissolved in TE buffer (0.1 M Tris-HCl, 2 mM EDTA-Na_2), which was preincubated at 30 C in a plastic tray with a lid. After electrophoresis, the gel was immersed in the buffer solution and incubated for 15 min. The reaction was

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stopped by adding 5 mM lead acetate into the incubation buffer. β-CAS isozymes were visualized by the appearance of brown bands (PbS) on the gel after about 30 min.

Statistical Analysis

All experiments were conducted as completely randomized designs. Data from repeated experiments were pooled for analysis. For the whole-plant dose–response experiments, the variable analyzed was FW, while for the seedling dose–response experiments, the lengths of shoot and roots were analyzed. A nonlinear regression model with four parameters was fit to the data using the statistical program R (Knezevic et al. 2007; R Development Core Team 2010; Ritz 2010; Ritz et al. 2015):

$$f(x,(b,c,d,e)) = c + \frac{d-c}{(1+\exp(b(\log(x)-\log(e))))} \quad [1]$$

where d represents the maximum limit, c represents the minimum limit, b represents the slope around the inflexion point, and e is the dose of herbicide at which a 50% reduction in the response occurred (GR_{50}). A goodness-of-fit analysis was performed using the *modelFit* function of the DRC package to verify that the adjusted nonlinear method gives a satisfactory view of the data variability. An analysis of the homogeneity of variance and normality of error distribution was performed using residuals and Q-Q plots.

The data for ethylene production, cyanide accumulation, and the β-CAS–like activity assay were subjected to a factorial analysis for the determination of the significance of the source of variation (genotype and days after quinclorac treatment [DAT]). All effects were considered as fixed. Significant interaction was observed between genotype and DAT. After ANOVA, Duncan's multiple range test was performed to identify significant differences between treatment means. ANOVA was conducted using InfoStat software (Di Rienzo et al. 2011).

The data for chlorophyll a and b concentration determination were subjected to a factorial analysis for the determination of the significance of the source of variation (genotype, herbicide, and DAT). No significant interaction was observed among genotype, herbicide, and DAT (Supplementary Table S1). After ANOVA, the Duncan's multiple range test was performed to identify significant differences between treatment means. ANOVA was conducted using InfoStat software (Di Rienzo et al. 2011).

Results and Discussion

The high frequency with which *E. crus-galli* genotypes with quinclorac resistance are currently found in Uruguayan rice fields raises questions about the continued utility of quinclorac for grass control. Because quinclorac is one of the main herbicides for integrated control of weeds associated with rice production, not using this herbicide would mean the loss of an important tool for controlling *E. crus-galli*. Given this situation, studies were initiated to characterize one genotype with suspected resistance in order to provide information to identify the resistance mechanism(s). Understanding of the mechanisms underlying this genotype's resistance will allow development of a better approach to delay resistance appearance, increase economic benefits, and reduce environmental impact.

Table 1. Quinclorac rates required for 50% reduction (GR_{50}) of root growth or aboveground fresh biomass weight for experiments in agar medium or pots, respectively.

		Dose–response experiment in agar medium[a]		Dose–response experiment in pots[b]	
	Genotype	GR_{50} (μM)[c]	RF[d]	GR_{50} (g ha^{-1})[c]	RF[d]
S	LM04	1.07 ± 0.18	—	94.8 ± 5.44	—
	CL44	1.79 ± 0.25	1.7	ND	ND
	CB01	1.90 ± 0.24	1.8	ND	ND
R	E7	56.5 ± 12.2	52.8	>2560	>27
	ZA01	40.8 ± 13.7	38.1	>2560	>27
	RB282	38.7 ± 10.2	36.1	>2560	>27

[a]In the initial dose–response experiments in agar medium, three S-genotypes (LM04, CL44, CB01) and three genotypes with suspected resistance to quinclorac (E7, ZA01, RB282) were assayed.
[b]In the dose–response experiment in pots, only the LM04 genotype was used as the susceptible control.
[c]GR_{50} values were calculated from the regression curves presented in Figure 1 for LM04 and E7 genotype and in Supplementary Figure S1 for all assayed genotypes. ND: not determined.
[d]Resistance factor (RF) corresponds to the ratio between GR_{50} values of resistant (R)-genotype plants to GR_{50} values of the susceptible (S)-genotype plants.

Resistance Characterization

Dose–response assays were performed to determine the degree of resistance of the genotypes. Initially, dose–response assays in agar medium were performed for three susceptible genotypes (LM04, CL44, and CB01) and three genotypes with suspected resistance (E7, RB282, and ZA01) to quinclorac (Supplementary Figure S1A). Both shoot and root length were recorded, and root growth was determined to be the adequate parameter to obtain a resistance relationship between resistant and susceptible genotypes. The data for shoot growth were not satisfactory to make the nonlinear adjustment. These assays showed that the three genotypes with suspected resistance to quinclorac were indeed resistant, with resistance factor ($GR_{50}R/GR_{50}S$) values ranging from approximately 36 to 52 (Table 1). Dose–response assays in pots were also performed, but used only the LM04 genotype as the susceptible control (Supplementary Figure S1B). The GR_{50} value of the S-biotype was 94.8 g ha^{-1}, but could not be calculated for the R-genotypes (due to the insignificant reduction in the shoot growth that the herbicide caused) and was estimated to be greater than 2,560 g ha^{-1} (Table 1). From the six genotypes assayed, the most sensitive, LM04 (S), and the most resistant, E7 (R) (Figure 1), were selected to proceed with the physiological and biochemical studies.

Ethylene and Cyanide Production of E7-R Genotype

The main action of quinclorac in sensitive species involves the induction of ACC synthase, the stimulation of ethylene biosynthesis, and concomitantly, cyanide accumulation (Grossmann 2010). The ranges of ethylene production in nontreated plants of the S- and R-genotypes were 0.03 to 0.07 nmol g^{-1} FW and 0.05 to 0.08 nmol g^{-1} FW, respectively. There was no difference in ethylene production between nontreated S- and R-genotype plants. In the treated plants, the range of ethylene production was 0.11 to 0.26 nmol g^{-1} FW for the S-genotype and 0.07 to 0.11 nmol g^{-1} FW for the R-genotype. At 2 d after quinclorac application (10 μM), a peak of ethylene production of 500% relative to nontreated plants was observed for plants of the S-genotype (Figure 2). At 3 and 4 d after quinclorac treatment, ethylene

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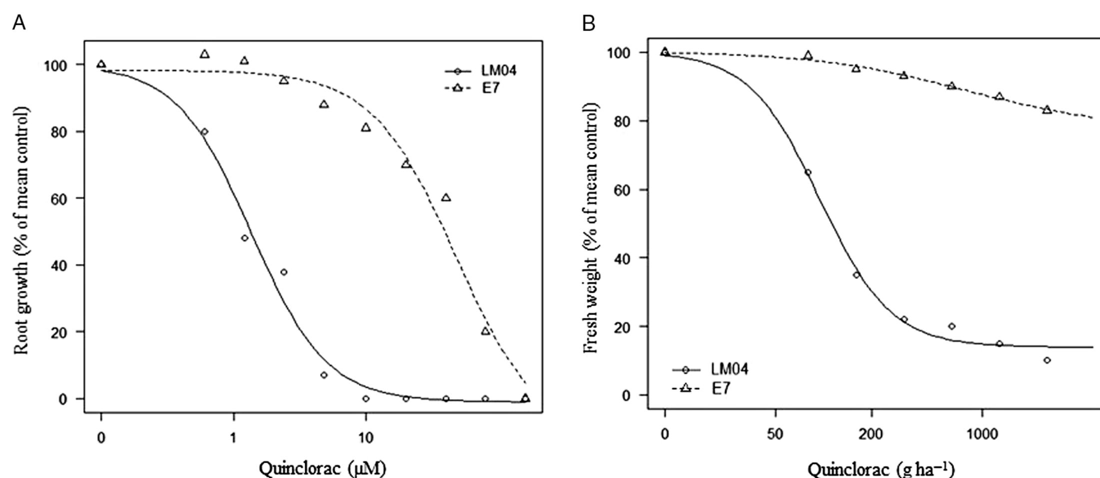

Figure 1. Dose–response experiments of susceptible (LM04) and resistant (E7) *Echinochloa crus-galli* genotypes. (A) Root growth at 7 d after herbicide treatment with 0, 0.6, 1.2, 2.4, 4.8, 10, 20, 40, and 80 µM of quinclorac. Results from three separate experiments were combined, and data were expressed as percent of the mean root growth of nontreated control seedlings. (B) Aboveground fresh weight at 3 wk after herbicide treatment with 0, 80, 160, 320, 640, 1,280, and 2,560 g ha^{-1} of quinclorac at the 3-leaf stage of growth. Results from two separate experiments were combined, and data were expressed as percent of the mean fresh weight of nontreated control plants. Calculated GR_{50} (dose of herbicide at which a 50% reduction in the response occurred) and resistance factor values are presented in Table 1.

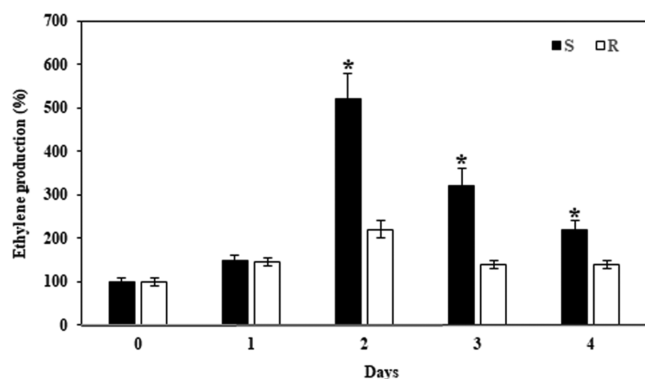

Figure 2. Ethylene production in LM04 (S) and E7 (R) genotypes after quinclorac application (10 µM). The data are expressed as a percentage with respect to the average of the nontreated control samples. The vertical lines represent the SEs of the average of the measurements. Asterisks (*) indicate significant differences at the 0.01 level (Duncan's test) between genotypes at each time point. 100% = 0.05 nmol g^{-1} FW. FW, aboveground fresh biomass weight.

production values declined to 300% and 200% relative to the nontreated S-genotype plants, respectively (Figure 2). R-genotype plants exhibited little accumulation of ethylene production (0.07 to 0.11 nmol g^{-1} FW) compared with S-genotype plants (0.11 to 0.26 nmol g^{-1} FW); in the course of 4 d of observation following treatment with quinclorac, ethylene levels (0.07 to 0.11 nmol g^{-1} FW) were close to those of nontreated plants (0.05 to 0.08 nmol g^{-1} FW) (Figure 2).

HCN is ubiquitous and can be formed from a variety of precursors (Miller and Conn 1980). Most plant species are cyanogenic. In addition to the HCN produced from the degradation of cyanogenic glycosides, the oxidation of ACC, catalyzed by ACO, leads to ethylene and HCN formation (Machingura et al. 2013). This process is the main source of HCN in several plant tissues (Goudey et al. 1989). In the S-genotype, the HCN concentration in the shoots increased significantly at 5 d post–quinclorac application and starting at 3 d in the roots (Figure 3). However, in the R-genotype, HCN concentration in the shoot or root did not change with time after quinclorac treatment (Figure 3). These results corroborate

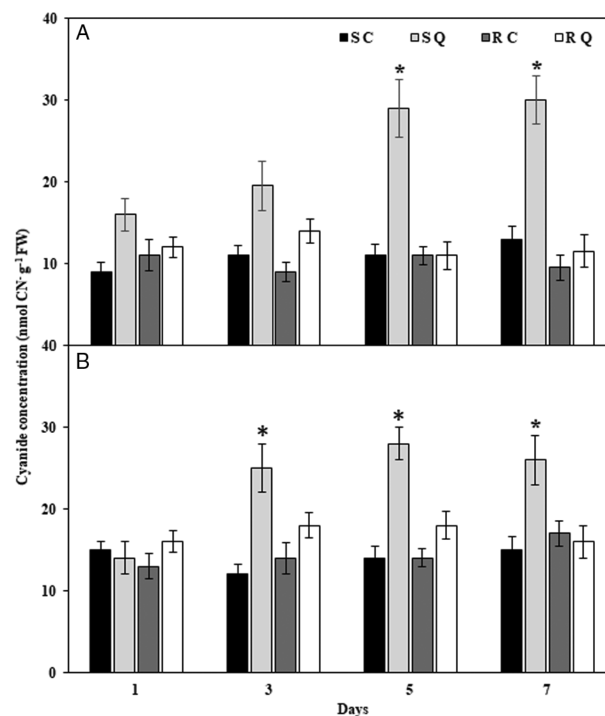

Figure 3. Cyanide accumulation in LM04 (S) and E7 (R) after quinclorac application (10 µM) in (A) shoots and (B) roots. SC, S-genotype in control (nontreated) conditions; SQ, S-genotype + quinclorac; RC, R-genotype in control (nontreated) conditions; RQ, R-genotype + quinclorac; FW, aboveground fresh biomass weight. The vertical lines represent the SEs of the average of the measurements. Asterisks (*) indicate significant differences at the 0.01 level (Duncan's test) between each treated sample and its nontreated control sample at each time point.

those of other studies on *Echinochloa*, *Digitaria*, *Brachiaria*, and *Setaria* quinclorac-resistant species in which the activity of ACC synthase, the production of ethylene, and the accumulation of HCN did not change after herbicide application (Abdallah et al. 2006; Grossmann 2000; López-Martínez et al. 1997; Yasuor et al. 2011). Quinclorac induces the activity of ACC synthase in the roots of sensitive species such as *E. crus-galli* (Grossmann and

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Table 2. Chlorophyll a and b concentration in shoots of LM04 (S) and E7 (R) genotypes after quinclorac application (10 µM) expressed in milligrams of chlorophyll per gram of aboveground fresh biomass weight (FW).

Treatment	Days after quinclorac application[a]			
	1	3	5	7
		Chlorophyll a mg g^{-1} FW		
S	50 a	45 a	44 a	40 a
S+quinclorac	45 a	30 b	22 b	11 b
R	42 a	40 a	44 a	38 a
R+quinclorac	41 a	35 a	35 a	34 a
		Chlorophyll b mg g^{-1} FW		
S	22 a	20 a	18 a	19 a
S+quinclorac	19 a	16 a	13 b	6 b
R	18 a	17 a	18 a	16 a
R+quinclorac	17 a	18 a	16 a	14 a

[a]Values within each type of chlorophyll and within each day after quinclorac application labeled with the same letter are not significantly different at the 0.01 level (Duncan's test).

Kwiatkowski 1995). Possibly, the accumulated ACC from the increased activity of ACC synthase in the roots is transported acropetally to the shoot, where, together with the cyanide produced by oxidation, it acts as a signal, stimulating ACC synthase activity and finally triggering the release of ethylene and HCN (Grossmann and Scheltrup 1997).

Because ethylene is a gaseous molecule, it can easily diffuse between cells, causing local responses. Also, the presence of aerenchyma facilitates rapid long-distance transport of ethylene in plant organs (Van de Poel and Van Der Staeten 2014). However, local and long-distance ethylene responses can also be achieved by transport of ACC, its precursor. It has been shown under different stress conditions that ACC is transported from the roots to the shoots (McManus 2012). In tomato (*Solanum lycopersicum* L.) roots exposed to waterlogging, only a fraction of the ACC can be converted into ethylene due to the lack of oxygen, which is one of ACO's substrates (Bradford and Yang 1980a). The remaining ACC is transported via xylem to the shoot tissue (Bradford and Yang 1980a). Once there, the ACC is converted into ethylene and HCN by the ACO present in the shoots (English et al. 1995). These authors also showed an induction of ethylene biosynthesis via increased ACC synthase activity in tomato plant roots that were mechanically wounded (Bradford and Yang 1980b). A similar situation could occur in *E. crus-galli*, where although the main action of quinclorac is at the root level, its effects are observed in the shoots with increased levels of ACC and HCN (Grossmann and Scheltrup 1997). That is, the shoot tissue of *E. crus-galli* plants treated with quinclorac is more susceptible to damage than the roots (Grossmann and Kwiatkowski 1993). Our data, which agree with those obtained by Grossmann and Kwiatkowski (1993) and Grossmann and Scheltrup (1997), showed that damage and tissue decay were most evident in the shoots of treated S-genotype plants. In these plants, chlorotic regions with chlorophyll loss (Table 2) and growth reduction (data not shown) were observed. However, in roots of treated S-genotype plants, the main effect of quinclorac was growth arrest without obvious visual damage. R-genotype plants did not present any damage or tissue decay in the shoots; in the roots, growth arrest was only evidenced at high doses of quinclorac. The low ethylene and cyanide biosynthesis in plants of R-genotype after quinclorac treatment suggest that the resistance observed could be associated with a low activation of the auxin response pathway. This may occur due to mutations in specific auxin receptors such as AFB5, which has been shown to interact with quinclorac (Lee et al. 2014). Other authors have demonstrated in *Arabidopsis* that different auxinic herbicides such as dicamba and picloram mediate their response by interacting with several specific receptors and that mutations in some of these receptors generated resistance (Gleason et al. 2011; Walsh et al. 2006).

ACC and KCN Effect on the Growth of Root and Shoot Tissues

In petri dish assays, the addition of ACC (Figure 4A and B) and KCN (Figure 4C and D) to the growth medium significantly reduced shoot and root growth of both genotypes. The growth reduction was of similar magnitude in both genotypes (Figure 4). As in the quinclorac dose–response assays (Figure 1A), the exogenous application of ACC and KCN caused a greater reduction in root growth, but without causing visible tissue damage. In the shoots, areas with chlorophyll loss were observed, and at the higher concentrations, even necrotic areas were evident (data not shown). In *Digitaria* spp. and *Echinochloa* spp., quinclorac phytotoxicity has been attributed to HCN accumulation, dependent on ACC accumulation (Abdallah et al. 2006; Grossmann and Kwiatkowski 1995).

An increase in ACC concentration in the growth medium would be comparable to the initial stages of the response to quinclorac in susceptible species. The concentration of ACC in the roots would increase, then excess ACC would be transported to the shoot tissue, where it would be converted to ethylene and HCN by ACO. The addition of KCN in the growth medium would be comparable to late stages of the response to quinclorac in susceptible species. The growth of S- and R-genotypes did not differ even at high KCN concentrations (Figure 4C and D), which indicates that the resistance of the R-genotype was not due to a differential detoxification mechanism. Instead, it could be due to a lack of activation of auxin perception/transduction signaling pathways resulting in a low stimulation of ACC synthase and, consequently, reduced ethylene synthesis (Abdallah et al. 2006; Yasuor et al. 2011).

Another common effect of auxinic herbicides, in addition to ethylene-induced abscisic acid (ABA) synthesis (Grossmann et al. 2001), is the overproduction of reactive oxygen species (ROS) that causes oxidative stress and tissue damage (Grossmann 1996; Grossmann et al. 2001; Romero-Puertas et al. 2004). Following an increase in ABA levels, ABA is distributed throughout the plant, causing growth inhibition, stomatal closure, reduction in carbon assimilation, and ROS production (Grossmann 1996, 2000; Hansen and Grossmann 2000; Scheltrup and Grossmann 1995). Thus, a differential antioxidant capacity could be a determining factor in quinclorac-resistant genotypes (Sunohara et al. 2011). We evaluated the antioxidant capacity of the S- and R-genotypes through the activity of some antioxidant enzymes (ascorbate peroxidase, catalase, and superoxide dismutase) without finding significant differences in their basal (nontreated) activities (Supplementary Figure S2). In the S-genotype, the activity of the three enzymes increased at 5 d post–quinclorac application (10 µM) and remained high until the end of the experiment. These data suggest that the S-genotype increased its antioxidant enzyme activity in response to a high concentration of ROS. By comparison, no significant differences were found for the R-genotype after quinclorac treatment (Supplementary Figure S2). We consider that the oxidative stress caused by quinclorac would be a late response of the herbicide, thus ruling out that an augmented capacity of ROS detoxification

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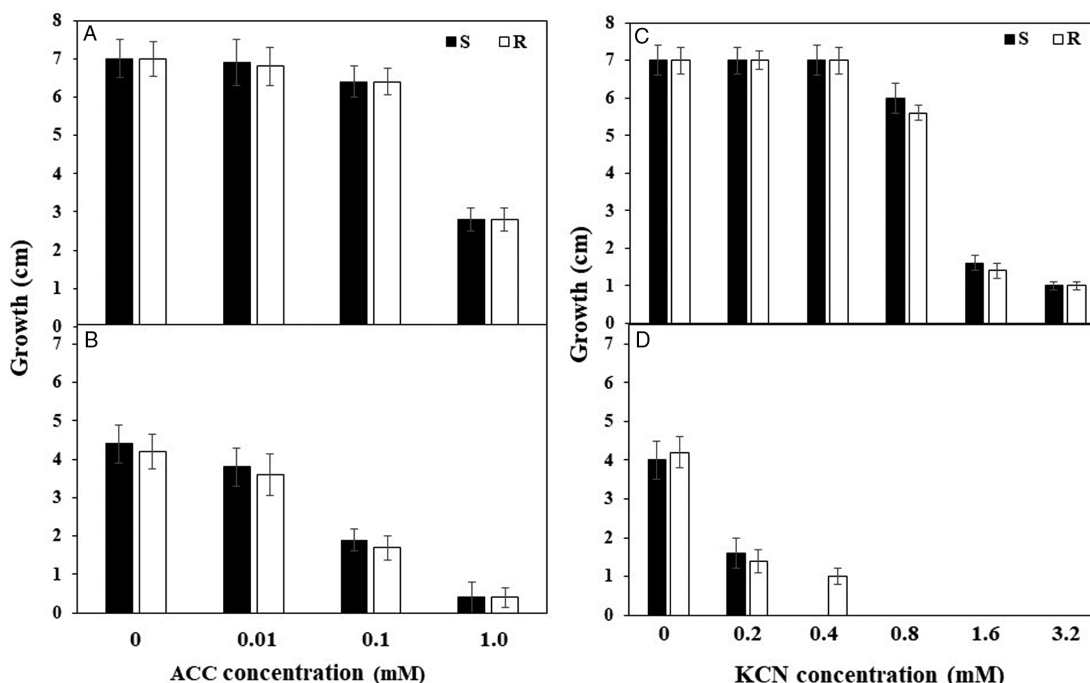

Figure 4. Effect of the addition of 1-carboxylic acid aminocyclopropane (ACC) (A and B) or potassium cyanide (KCN) (C and D) to seedling growth medium on the shoot (A and C) and root (B and D) growth of LM04 (S) and E7 (R) genotypes. The vertical lines represent the SEs of the average of the measurements. No significant differences between genotypes at any ACC or KCN dose were found.

via increased antioxidant enzyme activity was related to the resistance of the E7 genotype.

HCN Accumulation and Detoxification

HCN is a cytotoxic agent that inhibits various enzymes, particularly metalloenzymes, involved in major plant metabolic processes such as respiration, carbon and nitrogen assimilation, and carbohydrate metabolism, among others (Miller and Conn 1980; Solomanson 1981). Plants have developed different mechanisms to metabolize HCN and prevent its accumulation to toxic levels (Grossmann 1996). The key mechanism to detoxify endogenous HCN is via the mitochondrial enzyme β-CAS, which catalyzes the reaction of HCN and cysteine to form hydrogen sulfide and β-cyanoalanine (Gupta et al. 2010). The latter is subsequently metabolized to asparagine in a reaction catalyzed by β-cyanoalanine hydrolase (Goudey et al. 1989). The CS enzyme is also capable of catalyzing HCN detoxification. This enzyme has high amino acid sequence homology with β-CAS and catalyzes the formation of cysteine from *O*-acetyl-L-serine and hydrogen sulfide (Maruyama et al. 1998). Our data show that the β-CAS–like activity (which includes the activity of β-CAS and CS enzymes) in the shoots of the S-genotype increased on day 5 post–quinclorac application (10 μM) and remained high until the experiment was terminated (Figure 5A), and from day 3 until day 5 in the roots (Figure 5B). In contrast, in the R-genotype, quinclorac did not stimulate β-CAS–like activity (Figure 5). Unlike the results of previous studies (Abdallah et al. 2006; Yasuor et al. 2011), the basal (nontreated) β-CAS–like activity of S- and R-genotypes was similar (Figure 5). This result was consistent with that of the addition of exogenous KCN (Figure 4C and D). The same basal level of β-CAS–like activity could result in a similar ability to detoxify KCN, and this could

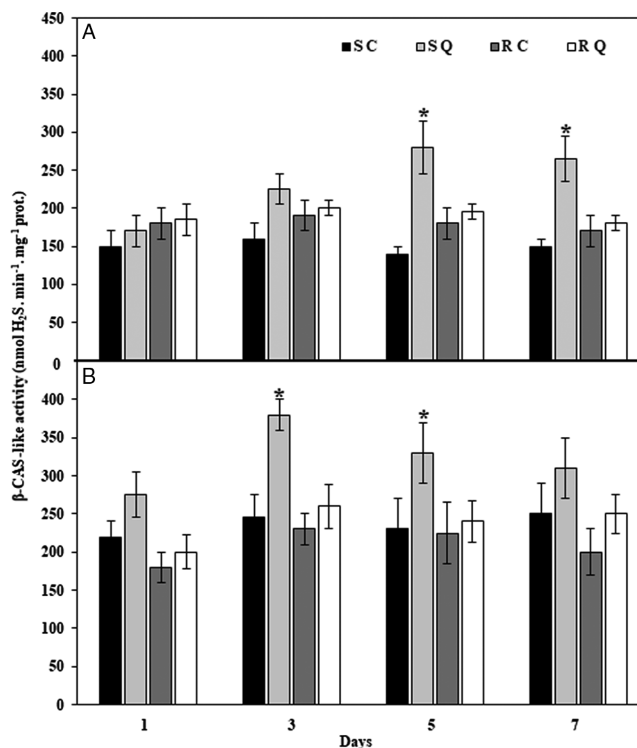

Figure 5. β-cyanoalanine synthase (β-CAS)-like activity in LM04 (S) and E7 (R) genotypes after quinclorac application (10 μM) in (A) shoots and (B) roots. SC, S-genotype in control (nontreated) conditions; SQ, S-genotype + quinclorac; RC, R-genotype in control (nontreated) conditions; RQ, R-genotype + quinclorac. The vertical lines represent the SEs of the average of the measurements. Asterisks (*) indicate significant differences at the 0.01 level (Duncan's test) between each treated sample and its nontreated control sample at each time point.

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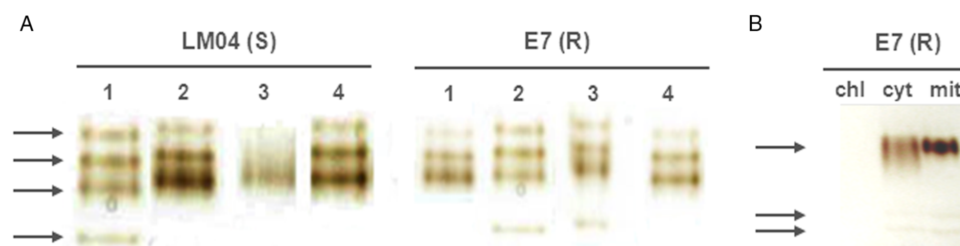

Figure 6. β-cyanoalanine synthase (β-CAS)-like gel activity in (A) roots and shoots of LM04 (S) and E7 (R) genotypes at 5 d post–quinclorac application (10 μM) and (B) enriched subcellular fractions from E7 (R) shoot tissue. 1, nontreated control roots; 2, roots + quinclorac; 3, nontreated control shoots; 4, shoots + quinclorac. Arrows indicate different isoforms with β-CAS–like activity. chl, chloroplastic; cyt, cytosolic; mit, mitochondrial.

explain why both genotypes had similar growth rates. Several bands with β-CAS or β-CAS–like activity were detected in the S- and R-genotypes (Figure 6A). These bands could correspond to different isoforms of β-CAS or CS enzyme. A marked increase in the intensity of the bands in quinclorac-treated roots, and in the intensity and number of bands in quinclorac-treated shoots was observed for the S-genotype (Figure 6A), proving that quinclorac stimulated β-CAS–like activity in this genotype. Conversely, in the R-genotype, there were only minimal changes in the intensity of bands after treatment with the herbicide (10 μM) in both roots and shoots (Figure 6A).

We also performed a subcellular fractionation to obtain enriched chloroplast, mitochondrial, and cytosolic fractions from the shoot tissue of the R-genotype to assay β-CAS–like gel activity in these fractions (Figure 6B). The validation of the purity of the subcellular fractions is presented in Supplementary Figure S3. The western blot analysis revealed that the chloroplast fraction was the purest, as little mitochondrial contamination was observed (weak band at approximately 34 kDa corresponding to AOX1/2). The cytoplasmic fraction had organellar contamination, as weak bands at approximately 38 kDa (PsbA/D1) and 34 kDa (AOX1/2) were observed. The mitochondrial fraction also demonstrated contamination with the other two compartments, but mainly from chloroplasts. A dense band for PsbA/D1 protein and a weak band for ACT (45 kDa) were observed. This fraction was the least pure. As expected, the crude extract showed reactivity against all tested antibodies. In Figure 6B, we observed β-CAS–like activity mainly in the mitochondrial fraction, which is consistent with the β-CAS enzyme location, but also in the cytosolic fraction, which could be due to the CS enzyme (Liang and Li 2001). No β-CAS–like activity was found in the chloroplast fraction (Figure 6B). Although the mitochondrial fraction was highly contaminated with chloroplasts (Supplementary Figure S3A), these organelles do not have either β-CAS or CS enzymes, so they could not be responsible for the observed β-CAS–like activity in the mitochondrial fraction (Figure 6B). In addition, and as mentioned before, no β-CAS–like activity was observed in the highly purified chloroplast fraction (Figure 6B).

These results support those obtained previously on ethylene production and cyanide accumulation (Figures 2 and 3). The low production of ethylene and cyanide accumulation by the R-genotype after the application of quinclorac coincides with the unaffected β-CAS–like activity in the treated samples. As mentioned earlier, in contrast to the results obtained by others (Abdallah et al. 2006; Yasuor et al. 2011), our data show that the basal β-CAS–like activity was similar in both genotypes. Previous studies suggested that a higher basal β-CAS–like activity in a resistant genotype could endow greater tolerance to quinclorac than a sensitive genotype with lower basal β-CAS–like activity. Our data, which agree with those obtained by Peng et al. (2019) and Chayapakdee et al. (2019), indicate that the resistance mechanism of the E7 genotype to quinclorac is not due to increased detoxification capacity of HCN.

Several weed populations around the world have evolved resistance to herbicides by metabolizing herbicide active ingredients to nonphytotoxic metabolites (Nandula et al. 2019). This metabolic capacity to detoxify herbicides (metabolic resistance) is mediated by enzyme systems such as cytochrome P450 monooxygenases, glutathione *S*-transferases, and glucosyl transferases (Yu and Powles 2014). As mentioned in the "Introduction," previous studies carried out by our group with the E7 R-genotype to assess whether the metabolic resistance was involved in resistance indicated that this mechanism cannot explain resistance in the R-genotype (Saldain and Sosa 2016). Other authors (Yasuor et al. 2011) working with rice barnyardgrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.] plants also concluded that the resistance to quinclorac is not caused by enhanced metabolism.

In the present study, we compared the responses to quinclorac of two contrasting *E. crus-galli* genotypes. Our results suggest that the resistance of the R-genotype is likely related to an alteration in the auxin signal transduction pathway, provoking a lower stimulation of the enzyme ACC synthase and, therefore, reduced ethylene production. Alternative resistance mechanisms such as an increased HCN detoxification capacity by the β-CAS enzyme or an enhanced capacity for ROS detoxification would not be involved in this resistance case. Consequently, we deduced that the difference in the response to quinclorac of the two genotypes occurs before ethylene synthesis. A better understanding of the early response to quinclorac, which includes a possible mutation in auxinic receptor(s) or a differential expression of auxin response genes, is essential to fully understand the resistance mechanisms. In addition, although the activity of β-CAS does not seem to be involved in the resistance of the R-genotype, it would be interesting to investigate its physiological role in HCN detoxification and its possible relationship with the CS enzyme.

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