Research Article

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Quinclorac resistance: a concerted hormonal and enzymatic effort in *Echinochloa phyllopogon*

Hagai Yasuor,* Marco Milan, James W Eckert and Albert J Fischer

Abstract

BACKGROUND: Quinclorac (3,7-dichloro-quinoline-carboxylic acid) is a selective herbicide widely used to control annual grasses and certain broadleaf weeds. *Echinochloa phyllopogon* (Stapf) Koss. is the most noxious grass weed in California rice fields and has evolved resistance to multiple herbicides with different modes of action. A quinclorac-resistant (R) *E. phyllopogon* biotype found in a Sacramento Valley rice field where quinclorac has never been applied was investigated.

RESULTS: Resistant to susceptible (S) GR_{50} (herbicide rate for 50% growth reduction) ratios ranged from 6 to 17. The cytochrome P450 inhibitor malathion (200 mg L^{-1}) caused R plants to become as quinclorac susceptible as S plants. Quinclorac rapidly (6 HAT) stimulated ethylene formation in S plants, but only marginally in R plants. Malathion pretreatment did not reduce ethylene formation by quinclorac-treated S and R plants. Activity of β-cyanoalanine synthase (β-CAS) in tissue extracts was 2-3-fold greater in R than in S plants, and incubation of shoot extracts with 1 mM malathion reduced β-CAS activity by 40% in both biotypes.

CONCLUSION: Resistance to quinclorac in R *E. phyllopogon* involved at least two mechanisms: (a) insensitivity along the response pathway whereby quinclorac induces ethylene production; (b) enhanced β-CAS activity, which should enable greater HCN detoxification following quinclorac stimulation of ethylene biosynthesis. This unveils new resistance mechanisms for this multiple-resistant biotype widely spread throughout California rice fields.

Keywords: *Echinochloa phyllopogon*; herbicide resistance; malathion; β-cyanoalanine synthase; cyanide; ethylene

1 INTRODUCTION

Weed control in California rice paddies has become very problematic owing to the evolution of herbicide resistance in major weeds of this mostly non-rotated and continuously flooded crop. *Echinochloa phyllopogon* (Stapf) Koss. is an aquatic grass weed[1] that can cause severe yield losses if uncontrolled, and is thus the major weed problem of rice (*Oryza sativa* L.) in the Sacramento Valley of California.[2] The California rice culture system is heavily dependent on herbicides for weed control, and more than 40 years of thiocarbamate (molinate and thiobencarb) use for grass control has led to the evolution of herbicide resistance in *E. phyllopogon*. Populations of this species exhibit simultaneous resistance to herbicides from different chemical groups and with different modes of action: molinate and thiobencarb (thiocarbamates), fenoxaprop-ethyl and cyhalofop (aryloxyphenoxy propionate), bispyribac-sodium (pyrimidinyl benzoate), bensulfuron-methyl (sulfonylurea), penoxsulam (triazolopyrimidine sulfonamide) and clomazone (isoxazolidinone).[3–7] Thus, herbicide-resistant (R) *E. phyllopogon* is resistant to all herbicides available in California for grass weed control in rice except for propanil, although it is less susceptible to this herbicide than wild-type accessions (Fischer A, unpublished). Earlier studies using inhibitors of cytochrome P450 monooxygenases (P450s, EC 1.14.14.1) and P540 activity assays suggested that this multiple herbicide resistance was mostly endowed by enhanced P450-mediated herbicide degradation;[6,8,9] conjugation with glutathione and cysteine also contributed to resistance.[10] All R *E. phyllopogon* accessions collected throughout rice paddies in the Sacramento Valley exhibit the same herbicide cross-resistance patterns. In a previous study, involving responses to herbicides, morphological characterization and molecular markers, the present authors demonstrated that these R *E. phyllopogon* accessions descend from a single founder introduction that subsequently dispersed and was subjected to *in situ* selection by repeated thiocarbamate use.[11]

Quinclorac is a member of the quinolinecarboxylic acid family of auxinic herbicides that is highly effective in selectively controlling *Echinochloa* spp. and other annual grasses such as *Digitaria, Setaria* and *Brachiaria* spp., as well as certain dicot weeds including *Aeschynomene* spp., *Sesbania* spp. and *Monochoria* spp.[12,13] Quinclorac and other auxinic herbicides induce *de novo* synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (EC 4.4.1.14), resulting in increased levels of ACC in susceptible species (for a review, see Grossmann[13]). Recently, TIR1/AFB (transport

* *Correspondence to: Hagai Yasuor, Department of Vegetable Research, Gilat Research Center Agricultural Research Organization, Rural Delivery Negev, 85280 Israel. E-mail: hagai@volcani.agri.gov.il*

Weed Science Program, Department of Plant Sciences, University of California, Davis, CA, USA

inhibitor response 1 and auxin-binding F-box) have been identified as receptors for indole-3-acetic acid (IAA), 2,4-D, 1-naphthalene acetic acid (1-NAA) and picloram. In addition, computer modeling with TIR1 crystal structure showed that the quinoline carboxylic acids quinclorac and quinmerac also fit well into the auxin-binding cavity of the receptor, strongly suggesting that the auxin receptor(s) are the target sites of quinclorac action, leading to gene expression of ACC synthase and 9-*cis*-epoxycarotenoid dioxygenase (NCED) in the abscisic acid (ABA) biosynthesis pathway.[13] ACC is subsequently oxidized to ethylene, CO_2 and HCN by ACC oxidase (EC 1.14.17.4). Thus, following treatment with quinclorac, ethylene levels increase and HCN accumulates to toxic levels in susceptible grasses. In addition to the auxinic-induced stimulation of ethylene production, response to quinclorac in susceptible dicots also involves increased ABA biosynthesis, which is linked to overproduction of reactive oxygen species (ROS), cell damage and foliar senescence.[13,14] Resistance to quinclorac has been reported for *D. ischaemum* [(Schreb. ex Schweigg) Schreb. ex Muhl],[15] *E. crus galli* (L.) Beauv.,[16–21] *E. crus-pavonis*,[22,23] *E. colona*,[18,24] *E. oryzoides* (Ard.) Fritsch, *E. oryzicola* (Vasing.) Vasing. (syn. *E. phyllopogon*), *E. hispidula* (Retz.) Nees[25] and *Gallium spurium* L.[14] In the cases where the mechanism of resistance was studied, resistance could not be related to differences in quinclorac uptake, translocation or metabolism between R and susceptible (S) plants.[14,16,26,27] Unlike susceptible plants, tolerant rice and resistant grass species or biotypes do not respond to quinclorac treatment with enhanced ethylene and HCN production.[15,28] In the dicot *G. spurium*, S plants produced threefold more ethylene and 14-fold more ABA than R plants after quinclorac treatment.[14] Also, greater activity of the HCN-detoxifying enzyme β-cyanoalanine synthase (β-CAS, EC 4.4.1.9) has been found in tolerant rice and quinclorac-resistant grasses compared with S plants.[15,28] These results led to the conclusion (a) that selectivity and resistance to the auxinic herbicide quinclorac involve a target-site-related mechanism whereby differential sensitivity at the level of auxin receptors or in the auxinic signal transduction pathway results in lower ACC synthase activity in resistant versus sensitive plants following quinclorac treatment, and (b) that R plants can also detoxify quinclorac-induced HCN better than S plants.[13–15]

Although *Echinochloa* spp. have already evolved resistance to quinclorac elsewhere, quinclorac was considered a potential tool for controlling multiple-herbicide-resistant *E. phyllopogon* because quinclorac has never been available for use in California rice. Thus, direct selection for resistance by repeated use of this herbicide has not occurred. Target-site resistance to quinclorac in *E. phyllopogon* populations is highly unlikely to have been selected for by the usual herbicide use practices,[29] because the chemical structure and mode of action of quinclorac are different to those of the other herbicides against which *E. phyllopogon* has already evolved resistance, and other auxin-type herbicides have not been used for grass control in rice. Herbicide metabolism is the prevailing herbicide resistance mechanism in R *E. phyllopogon*, but, as stated earlier, quinclorac resistance or selectivity has not been attributed to enhanced detoxification.

In order to evaluate the potential usefulness of quinclorac to control R *E. phyllopogon*, a study was made of quantified responses by R and S plants to various quinclorac rates. On the basis of the observed responses, ethylene production, β-CAS activity and interactions between quinclorac and the P450 inhibitor malathion in R and S plants were further compared. Thus, mechanisms of quinclorac resistance that were new to multiple-resistant *E. phyllopogon* from California and had presumably evolved in the absence of direct selection by quinclorac use were elucidated.

2 MATERIALS AND METHODS

2.1 Plant materials and common procedures

An R *E. phyllopogon* accession was collected in 1997 in the Sacramento Valley of California from rice fields (39° 40′ N/122° 09′ W latitude/longitude) where this weed had evolved resistance to multiple herbicides mainly through malathion-sensitive enhanced P450 detoxification.[3,6,8,30] Quinclorac had never been used in these fields, and is not yet available for use in California. A control (S) accession known to be susceptible to the herbicides currently used for grass control in rice also originated from a Sacramento Valley rice field (39° 08′ N/122° 69′ W latitude/longitude). Mass-collected seeds (spikelets) of the R and S accessions were used in dose–response studies, and 3× selfed inbred strains derived from single seeds of these R and S accessions were used in the assay of mechanisms of resistance to ensure genetic uniformity. Relative responses to molinate, thiobencarb, fenoxaprop-ethyl and bispyribac-sodium, plant morphology, AFLP fingerprinting and P450 activity of these strains have been described in detail elsewhere.[8,11]

For all experiments, seeds were germinated on wet Whatman No. 1 paper in a growth chamber set at 26/10 °C day/night temperature and a 16 h photoperiod under 500 µmol m^{-2} s^{-1} photsynthetic photon flux density (PPFD) delivered by a mixture of incandescent and fluorescent lights. For whole-plant dose–response experiments, uniform R and S seedlings with approximately 1 mm long radicle and coleoptile were transplanted into plastic pots (0.5 L) filled with Yolo clay loam (fine-silty, mixed, non-acid, thermic Typic Xerorthents, 1.02% organic matter) and placed in a greenhouse where natural light was supplemented by 900 µmol m^{-2} s^{-1} PPFD; average temperatures ranged from 22 to 31 °C, and day length was 16 h. Emerged seedlings were thinned to four uniform and equidistantly spaced plants per pot. Pots were immersed in water and fertilized after establishment with 12.6 kg ha^{-1} nitrogen (ammonium sulfate). For all other experiments, plants of R and S inbred strains were grown hydroponically in a growth chamber after placing one germinated seedling of each accession in a 6 mL glass tube containing 2 mL of half-strength Hoagland's solution. Growing conditions were as described earlier.

2.2 Chemicals

Plants were treated with commercial quinclorac (75% dry flowable, Facet® 75) provided by BASF Corporation (Research Triangle Park, NC). A commercial formulation (500 g L^{-1}, EC) of the P450 inhibitor malathion was purchased from Ace Hardware Corporation (Oak Brook, IL). Technical malathion was purchased from ChemService Inc. (West Chester, PA).

2.3 Whole-plant dose–response experiments

The extent of quinclorac resistance was quantified by comparing responses of the R and S accessions to various quinclorac rates. Seedlings were treated at the three-leaf stage of growth with a commercial formulation of quinclorac; rates were 0X, X/128, X/64, X/32, X/16, X/8, X/4, X/2 and X (X = 560 g AI ha^{-1}) for S seedlings and 0, X/32, X/16, X/8, X/4, X/2, X, 2X and 4X for R seedlings. All treatments were applied using a cabinet sprayer equipped with an 8001E-VS (Spraying Systems, Wheaton, IL) flat-fan even-spray nozzle to deliver 380 L ha^{-1} at 275 kPa

pressure. Pots were immersed in separate containers, and, 1 day after spraying quinclorac, the immersion water in the greenhouse was raised to 3 cm above the pots' soil surface, as is the normal practice in California rice fields. Above-ground fresh weight per pot was measured 21 days after spraying. Experimental treatments (factorial combinations of quinclorac rates, and *E. phyllopogon* accessions) were replicated 4 times, and each experiment was conducted twice.

2.4 Quinclorac and malathion interaction

The effects of interactions between quinclorac and the P450 inhibitor malathion on plant growth were evaluated to detect the possible involvement of malathion-sensitive P450 metabolism as a mechanism of quinclorac resistance, because this is the prevailing mechanism of resistance to other herbicides in this R *E. phyllopogon* accession.[5,6,8] Seeds from both R and S inbred strains were germinated and grown hydroponically as described earlier. When plants reached the three-leaf stage of growth, quinclorac was applied to the hydroponic solution to achieve 0, 1, 10 and 100 μM concentrations, and plants were either immersed directly into these solutions or were first treated with a commercial formulation of malathion (200 mg L^{-1} in half-strength Hoagland's solution). In this case, roots were first submerged in 3 mL of the malathion solution for 2 h, then gently washed with deionized water and finally placed in the corresponding quinclorac solution. Seven days after immersion in quinclorac, chlorophyll fluorescence (maximum quantum yield of PSII, F_v/F_m) was measured on the second fully developed leaf of dark-adapted plants using a multimode chlorophyll fluorometer (Opti Sciences, Inc., Hudson, NH); plant fresh weight was also measured. Experimental treatments (combinations of quinclorac rates, P450 inhibitor and *E. phyllopogon* accessions) were replicated 4 times, and each experiment was conducted twice.

2.5 Ethylene production in response to quinclorac and malathion

This experiment was conducted to assess whether *E. phyllopogon* resistance to quinclorac results from differential sensitivity along the response pathway whereby quinclorac induces ethylene production, and further to assay for the possible involvement of differential quinclorac detoxification via malathion-sensitive P450 metabolism as a mechanism of resistance. Plants of R and S *E. phyllopogon* inbred strains were hydroponically treated at the three-leaf stage of growth with 0, 1, 10 and 100 μM of quinclorac, either directly or following a 2 h malathion (200 mg L^{-1}) pretreatment as described earlier. Two sets of plants were assayed. Shoots from one set were excised 6 h after herbicide application and placed in tared glass syringes with the plunger set to 20 mL. The syringe was reweighed to ascertain the tissue's fresh weight, capped with a rubber septum stopper and incubated in the dark for 5 h at 25 °C. After incubation, ethylene was measured by withdrawing a 1 mL gas sample with a syringe and injecting it into a Shimadzu GC 8A gas chromatograph (Kyoto, Japan) equipped with a 1.2 m stainless steel column packed with Porapak Q 50/80 mesh (Alltech, Unterhaching, Germany) and a flame ionization detector for the quantitative determination of ethylene. The column temperature was 80 °C, and the injector and detector oven temperature was 150 °C. Gas flow rates were 90 mL min^{-1} for N_2 and 75 mL min^{-1} for air and H_2. Another set of plants was used to measure fresh weight 7 days after exposure to quinclorac alone, as described earlier. Experimental treatments were combinations of quinclorac rates, P450 inhibitor and *E. phyllopogon* accessions and were replicated 4 times; the experiment was conducted twice.

2.6 Long-term ethylene production

Hydroponically grown plants of R and S *E. phyllopogon* inbred strains were treated at the three-leaf stage of growth with either 0 or 10 μM of quinclorac added to the growth solution. Ethylene was measured as described in the preceding section at 2, 4, 6, 8, 24, 30 and 48 h after plant exposure to quinclorac in the growth medium, to detect differences between R and S plants in basal ethylene production in the absence of quinclorac and in long-term ethylene production following quinclorac treatment. Growing conditions and ethylene measurements were as described in the preceding section. Experimental treatments (*E. phyllopogon* accessions, quinclorac rates and measuring intervals) were replicated 5 times, and the experiment was conducted twice.

2.7 Differential cyanide toxicity and detoxification in R and S plants

An investigation was conducted to establish whether the lower quinclorac sensitivity of R plants could be related to an enhanced ability to avoid quinclorac-induced cyanide accumulation in plant tissues compared with S plants. An assay for β-cyanoalanine synthase (β-CAS) activity was conducted on the basis of the reduction of methylene blue by hydrogen sulfide,[31] following methodologies by Goudey *et al.*[32] and Abdallah *et al.*[15] Plants of R and S *E. phyllopogon* inbred lines were grown hydroponically as described up to the 2–3-leaf stage, and the assay was conducted separately for shoots and roots. Shoots and roots were homogenized with 2.5 mL of 100 mM Tris buffer (pH 8.5) per gram of tissue. After centrifugation at 10 000 × *g* for 10 min at 4 °C, the supernatant was employed for enzyme assay. The enzyme extract and the freshly prepared substrate solution [NaCN and L-cysteine dissolved in 50 mM Tris-HCl (pH 8.5) to a final concentration of 25 and 5 mM respectively] were equilibrated separately at 30 °C for 10 min. The assay was started in a sealed test tube by adding 0.5 mL of extract to the tube containing 4 mL of substrate solution. In addition, an extract duplicate was incubated in substrate solution containing technical malathion (1 mM, dissolved in 100% ethanol). After incubation at 30 °C for 60 min, the reaction was stopped and color was developed by adding 1.0 mL aliquots of the reaction mix (enzyme extract + substrate solution) to 1.5 mL microfuge tubes containing 0.1 mL of 0.03 M $FeCl_3$ in 1.2N HCl, followed by the addition of 0.1 mL of 0.02 M *N,N*-dimethyl-*p*-phenylenediamine sulfate in 7.2N HCl. Samples were agitated in a vortex and left in the dark at room temperature for color development. After 20 min, the samples were centrifuged at 1020 × *g* for 2 min to remove precipitated protein. The enzyme activity was determined colorimetrically after conversion of the released H_2S (from cysteine) to methylene blue, reading absorbance at 650 nm with a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA), and using Na_2S as the standard reference. Enzyme activity was expressed in nmol H_2S g^{-1} fresh weight min^{-1}. Experimental treatments (factorial combination of *E. phyllopogon* accessions and malathion exposure) were replicated 7 times, and the experiment was conducted twice.

2.8 Statistical analysis

Treatments were arranged in a completely randomized design, and data from repeated experiments were pooled for analysis.

Responses to continuous quantitative variables were subjected to regression and Pearson's correlation analysis. Four- and three-parameter log-logistic regressions and exponential models were used to describe dose–response data and the effects of ethylene on growth.[33,34] The regression models chosen in each case were those with the lowest mean square errors and the highest significance in their coefficients. Herbicide rates to inhibit plant growth by 50% with respect to the untreated control (GR_{50}) were calculated from the regressions, and a resistance index (R/S ratio) was computed as $GR_{50}(R)/GR_{50}(S)$. Values of GR_{50} were considered to be statistically different when their respective 95% confidence intervals did not overlap. Regression analysis was conducted using SigmaPlot (v.11.0, 2008) statistical software (Systat Software, Inc., San Jose, CA). Data of ethylene production by untreated plants and from the β-CAS activity assay were subjected to ANOVA; a Box–Cox transformation was conducted on the β-CAS activity data to stabilize variances and comply with normality prior to analysis. Means from significant effects were compared using orthogonal contrasts. ANOVA was conducted using JMP software (v.8.0, 2008 Academic; SAS Institute Inc., Cary, NC).

3 RESULTS AND DISCUSSION

To elucidate whether the evolution of resistance to multiple herbicides in R *E. phyllopogon* from Sacramento Valley rice fields has also endowed mechanisms for resistance to quinclorac, the authors compared growth, chlorophyll fluorescence, ethylene production, cyanide detoxification and responses to the P450 inhibitor malathion in quinclorac-treated R and S plants.

3.1 Resistance characterization

Growth responses by whole plants sprayed with incremental quinclorac rates, or treated hydroponically with quinclorac, demonstrated that the R *E. phyllopogon* accession was resistant to quinclorac (Fig. 1). Ratios (R/S) of the GR_{50} values ranged from approximately 6 to 17 (Table 1). Previous studies had reported that resistance to other herbicides in the same R and S *E. phyllopogon* accessions from California[3,5–7] was mostly mediated by enhanced P450 metabolism.[6,8,9] This metabolic basis of resistance had been readily detected when application of the P450 inhibitor malathion drastically reduced resistance levels.[5,6,30]

3.2 Role of ethylene

The primary target of quinclorac toxicity involves induction of ACC synthase, stimulated ethylene biosynthesis and concomitant accumulation of toxic HCN in sensitive grasses.[13] The present study found more ethylene formation in S than in R plants 6 h after quinclorac had been applied hydroponically to three-leaf plants (Fig. 2A). R plants exhibited little stimulation of ethylene production compared with S plants; by 48 h after treatment with 10 µM of quinclorac, ethylene levels were close to control values in R plants, while in S plants ethylene production was fivefold that of untreated plants (F-ratio for orthogonal contrast = 7.17, $P < 0.01$, df = 1, 251) (Fig. 2B). This agrees with other studies where quinclorac-resistant species and biotypes do not exhibit major changes in ACC synthase activity and ethylene levels after quinclorac treatment.[15,17,27] Enhanced ethylene production in response to quinclorac was clearly associated with *E. phyllopogon* growth reduction (Fig. 3). Similar correlations between ethylene production and quinclorac toxicity have been reported for sensitive *Echinochloa* spp. and grass weeds.[12,15,35]

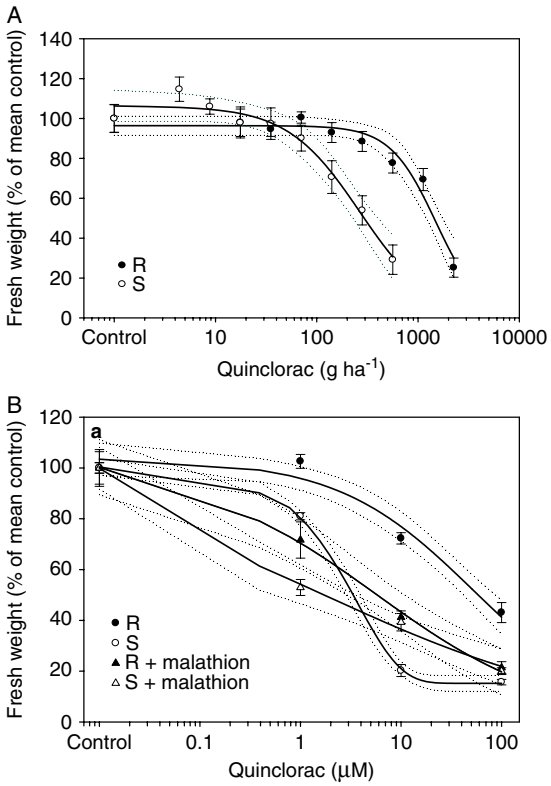

Figure 1. Above-ground fresh weights of resistant (R, ●) and susceptible (S, ○) *Echinochloa phyllopogon*. (A) 3 weeks after foliar treatment with 0, 4.375, 8.75, 17.5, 35.0, 70.0, 140.0, 280.0, 560, 1120 and 2240 g ha^{-1} of quinclorac at the three-leaf stage of growth. Fitted equations are: $Y(\bullet) = 96.3/(1 + \exp\{4.4 \times [\log(X) - \log(1496.6)]\})$; $Y(\circ) = 106.3/(1 + \exp(2.7 \times [\log(X) - \log(266.2)]\})$; $P < 0.0001$. (B) 1 week after hydroponic treatment with quinclorac (0, 1, 10 and 100 µM) alone (●, ○ for R and S plants respectively) or following a malathion (200 mg L^{-1}) pretreatment (▲, △ for R and S plants respectively) at the three-leaf stage of growth. Fitted equations are: $Y(\bullet) = 103.5/(1 + \exp(1.5 \times [\log(X) - \log(52.7)]\})$; $Y(\circ) = 101.1/(1 + \exp(2.4 \times [\log(X) - \log(3.4)]\})$; $Y(\blacktriangle) = 100.3/(1 + \exp(1.1 \times [\log(X) - \log(5.7)]\})$; $Y(\triangle) = 100/(1 + \exp(0.7 \times [\log(X) - \log(1.8)]\})$; $P < 0.0001$. Data were expressed as percentage of the mean fresh weight of untreated control plants; absolute fresh weights ± standard error (g $plant^{-1}$) for the controls are: (A) S = 6.7 ± 0.3, R = 6.3 ± 0.4; (B) S = 0.24±0.01, S+M = 0.15±0.01, R = 0.22±0.01, R+M = 0.16±0.01). Vertical bars represent the standard error of the mean ($n = 8$); dotted lines represent 95% CI; calculated GR_{50} values and R/S ratios are presented in Table 1.

3.3 β-CAS activity differs in R and S plants

In addition to the differences in ethylene production by quinclorac-treated S and R *E. phyllopogon* plants (Figs 2A and 2B), ethylene exerted greater growth inhibition in S than in R plants (Table 2). Thus, a given amount of ethylene released following quinclorac treatment was associated with more growth reduction in S than in R plants (Fig. 3A), suggesting that R plants were better able to cope with the extra HCN released during the quinclorac-stimulated ethylene biosynthesis. Cyanide can be produced from various precursors,[36] but most plants derive their endogenous cyanide mainly from ethylene biosynthesis,[13] and toxicity results from cyanide inhibition of enzymes involved in key metabolic processes.[37–41] A key enzyme for detoxifying endogenous cyanide is the mitochondrial β-CAS, which catalyzes the conjugation of HCN with cysteine to form hydrogen sulfide and β-cyanoalanine, which is further metabolized to asparagine.[32,38,39] More β-CAS activity was found in R than in S *E. phyllopogon* plant

Table 1. Quinclorac rates required for 50% reduction (GR_{50}) of above-ground fresh biomass and ratios (R/S) of the GR_{50} values of resistant (R) to susceptible (S) *Echinochloa phyllopogon* plants; quinclorac was applied at various rates, either as a foliar spray or hydroponically either alone or in combination with 200 mg L^{-1} of malathion (M)

	Foliar spray		Hydroponic	
Accession	GR_{50} (g ha^{-1}) (±95% CI)[a]	R/S	GR_{50} (μM) (±95% CI)[a]	R/S
S	266 ± 79	–	3.5 ± 1.0	–
R	1497 ± 279	5.6	58.5 ± 21.1	16.9
S + M			1.7 ± 1.9	–
R + M			5.8 ± 3.9	3.4

[a] GR_{50} values were calculated from the regression curves presented in Fig. 1; CI values are the 95% confidence interval ($n = 8$).

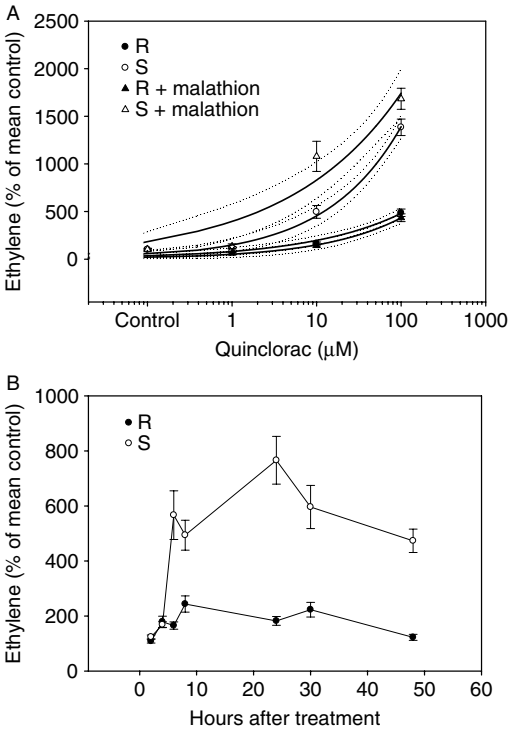

Figure 2. Quinclorac stimulation of ethylene production in resistant (R) and susceptible (S) *Echinochloa phyllopogon* plants. (A) 6 h after hydroponic treatment with quinclorac (0, 1, 10 and 100 μM) alone (●, ○ for R and S plants respectively) or following a pretreatment with 200 mg L^{-1} of malathion at the three-leaf stage of growth (▲, △ for R and S plants respectively). Fitted equations are as follows: $Y(●) = 80.6X^{0.39}$; $Y(○) = 148.2X^{0.49}$; $Y(▲) = 51.6X^{0.46}$; $Y(△) = 395X^{0.32}$; $P < 0.0001$; dotted lines represent 95% CI. (B) At different hours after R (●) and S (○) plants were hydroponically treated with 10 μM of quinclorac. Data were expressed as percentage of the mean ethylene content of untreated control plants; absolute ethylene concentrations ± standard error (μL L^{-1} g^{-1}) are: (A) S = 0.20 ± 0.03, S + M = 0.15 ± 0.01, R = 0.34 ± 0.02, R + M = 0.48 ± 0.06; (B) R = 0.54 ± 0.28, S = 0.28 ± 0.01 (48 h average). Vertical bars represent the standard error of the mean ($n = 8$ and 10 in A and B respectively).

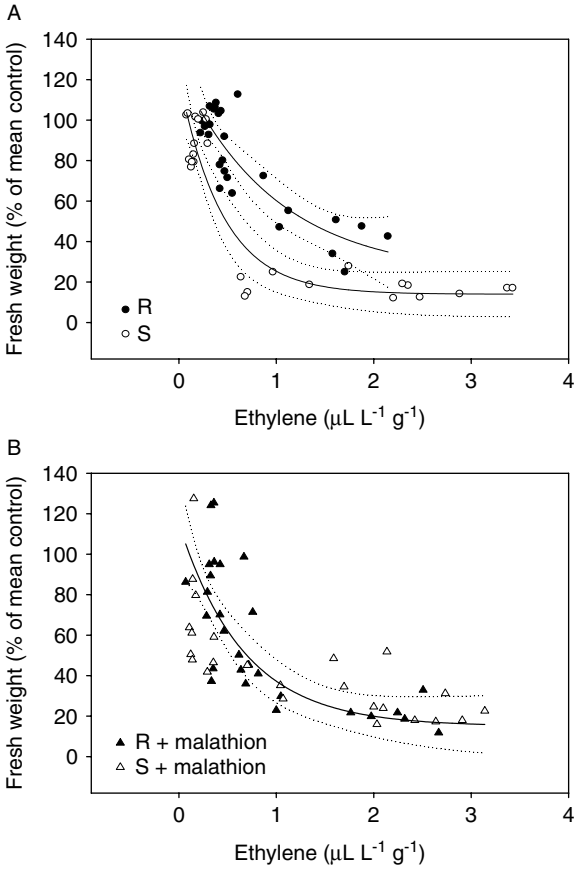

Figure 3. Relationship between ethylene produced in shoots and shoot fresh weight of resistant (R) and susceptible (S) *Echinochloa phyllopogon* plants after hydroponic treatment at the three-leaf stage of growth with (A) 0, 1, 10 and 100 μM of quinclorac (●, ○ for R and S plants respectively) or (B) the same quinclorac rates applied after pretreatment with 200 mg L^{-1} of malathion (▲, △ for R and S plants respectively). Ethylene production (μL L^{-1} per g of plant fresh weight and 5 h incubation) was determined 6 h after quinclorac application, and fresh weights were measured 1 week after herbicide treatment. Fresh weight data were expressed as percentage of the untreated control plants. A lack-of-fit test at $P = 0.05$ suggested that data in (A) should be described by two different regression lines [$Y(●) = 23.4 + 101.9 \times \exp(-1.01X)$; $Y(○) = 13.9 + 107.2 \times \exp(-2.25X)$; $P < 0.0001$] and in (B) by a single model [$Y(▲, △) = 15.0 + 100.0 \times \exp(-1.5X)$; $P < 0.0001$].[71] Dotted lines represent 95% CI; calculated GR_{50} values and R/S ratios are presented in Table 2.

tissues (Table 3), suggesting that R plants cope better than S plants with the toxic effects associated with quinclorac-stimulated ethylene production (Figs 2 and 3A) owing to a greater ability to detoxify the concomitantly released HCN. Similarly, in an earlier study it was demonstrated that quinclorac toxicity to smooth crabgrass, *D. ischaemum* (Schreb.) Muhl., was caused by an accumulation of cyanide derived from quinclorac-stimulated ethylene biosynthesis,[15] and that quinclorac-resistant *D. ischaemum* plants had significantly greater levels of β-CAS activity than S plants, as was also found by Grossmann and Kwiatkowski[28] working with rice and other grass weeds.

3.4 Resistance involves a malathion-sensitive HCN detoxification mechanism

Malathion and other P450 inhibitors have been used in various studies as tools for detecting the involvement of enhanced P450-mediated herbicide degradation as a mechanism conferring herbicide resistance in plants.[42–46] In the present study, malathion (200 mg L^{-1}) applied alone to R and S *E. phyllopogon* did not affect plant growth. However, when malathion was used in combination with quinclorac, R plants became almost as

Table 2. Quinclorac-induced endogenous ethylene concentrations required for 50% reduction (GR_{50}) of above-ground fresh biomass and ratios (R/S) of the GR_{50} values of resistant (R) to susceptible (S) *Echinochloa phyllopogon* plants treated hydroponically with various rates of quinclorac alone or after pretreatment with 200 mg L^{-1} of malathion

Quinclorac treatment	Accession	GR_{50} (μL L^{-1} g^{-1}) (±95% CI)[a]	R/S
Alone	S	0.5 ± 0.2	–
	R	1.3 ± 0.4	2.7
After malathion	Joint R and S model[b]	0.7 ± 0.2	–

[a] GR_{50} values were calculated from regression curves presented in Figs 3A and 3B; CI values are the 95% confidence interval ($n = 28$).
[b] Data for the treatment with quinclorac followed by malathion pretreatment were described by a single model ($n = 56$).

quinclorac sensitive as S plants (Fig. 1B), causing GR_{50} values for the R accession to drop by a factor of 10 and the R/S ratio to shift from approximately 17 to 3 compared with the treatment with quinclorac alone (Table 1). According to earlier studies involving other herbicides and these same *E. phyllopogon* accessions, such responses would suggest that resistance is due to enhanced P450-mediated herbicide detoxification, because R plants become sensitive to the herbicide once this detoxification ability is blocked by malathion.[3,5,6] However, quinclorac is reportedly quite resilient to plant metabolism, and resistance or selectivity has not been related to differential quinclorac detoxification.[28,47–49] Moreover, malathion did not cause an increase in ethylene formation by quinclorac-treated R *E. phyllopogon* plants (Fig. 2A), as would be expected if P450 metabolism had impaired quinclorac activity in those R plants. Alternatively, it was found that malathion strongly inhibited the enhanced β-CAS activity of R plants (Table 3), which helps explain why, after pretreatment with malathion, R plants were no longer more tolerant than S plants to the toxicity associated with quinclorac stimulation of ethylene production (Fig. 3B and Table 2). These results suggest that malathion synergizes quinclorac toxicity to R *E. phyllopogon* (Fig. 1B) by inhibition of a resistance mechanism involving HCN detoxification by enhanced β-CAS activity.

Cyanide blockage of electron flow in chloroplasts and mitochondria can lead to the formation of reactive oxygen species (ROS) and cell damage.[50–52] Damage by ROS in plants can be detected by a reduction in chlorophyll fluorescence ($F_v/F_m < 0.8$) emission, and damage to fresh weight depicted in Fig. 1B was strongly correlated ($r = 0.933$, $n = 55$) with F_v/F_m reduction (not shown).[53,54] Plants have enzymatic antioxidative protection mechanisms to prevent photobleaching by ROS under normal daylight conditions.[55] Inhibition by malathion of similar antioxidant defense enzymes can reportedly enhance peroxidative ROS damage to animal cells;[56,57] Therefore, a malathion enhancement of quinclorac toxicity could have also occurred in R plants (Fig. 1B) if resistance had been due to an upregulated antioxidant protection system sensitive to malathion in those plants.[58] However, basal chlorophyll fluorescence was similar in R and S plants and was not affected by malathion treatment ($P > 0.115$; mean $F_v/F_m = 0.729 \pm 0.006$). This suggests that an enhanced ROS protection mechanism sensitive to inhibition by malathion, such that it would explain the responses in Fig. 1B, does not seem to operate in R *E. phyllopogon* plants.

3.5 Conceptualizing the evolution of resistance

Herbicide resistance evolves in response to the selection exerted by repeated use of herbicides that share a common mode of action or mechanism of inactivation by plants.[29,59] The factors leading to the evolution of quinclorac resistance in R *E. phyllopogon* are unclear. It seems unlikely that repeated herbicide use could have selected for quinclorac resistance in R *E. phyllopogon*, because quinclorac has never been used in California rice fields and the malathion-sensitive enhanced P450 metabolism endowing R *E. phyllopogon* with resistance to other herbicides could not be detected. Because R *E. phyllopogon* originates from a single founder introduction from Asia,[11] repeated exposure to quinclorac could have occurred in Asian rice fields prior to introduction to California. This also seems unlikely, because quinclorac began to be marketed in Asia by 1989,[60] yet, by the early 1990s, R *E. phyllopogon* had already caused concerns in California.[61] Alternatively, quinclorac resistance may reflect a different kind of adaptation, perhaps one that favored survival of plants with enhanced basal ethylene production. During a 48 h time course, and in the absence of quinclorac, R plants produced an average of 0.54 ± 0.28 μL L^{-1} g^{-1} ethylene versus

Table 3. Activity of β-cyanoalanine synthase in shoot and root tissue of 2–3-leaf-stage resistant (R) and susceptible (S) *Echinochloa phyllopogon* plants. Enzyme activity assays involved incubation with and without 1 mM malathion. Data are presented as means ± SE of 14 observations from two separate experiments

Tissue	Effect[a]	nmol H_2S g^{-1} fresh weight min^{-1}	*F*-ratio (df = 1, 52)	*F*-ratio (df = 1, 110)	$P > F$
Shoot	R	321.6 ± 22.0			
	S	156.5 ± 15.9	100.63		<0.0001
	No malathion	316.9 ± 20.7			
	Malathion	161.2 ± 18.9	89.69		<0.0001
	Mean	239.0 ± 17.4			
Root	R	760.1 ± 45.0			
	S	255.7 ± 16.4	293.15		<0.0001
	No malathion	614.4 ± 65.3			
	Malathion	401.4 ± 43.7	47.9		<0.0001
	Mean	507.5 ± 41.5		1356.2	<0.0001

[a] Data were subject to Box–Cox transformation for ANOVA; no interactions were significant, untransformed means ± SE of main effects are presented.

0.28 ± 0.01 μL L^{-1} g^{-1} for S plants (F-ratio = 159.7, $P < 0.0001$, df = 1, 125; data not shown). Greater accumulation of ethylene in tissue would require the concomitant evolution of enhanced β-CAS activity to detoxify the additional stoichiometric build-up of HCN (Table 3).[62] Would the greater ethylene production of R plants reveal an adaptive advantage under the continuously flooded conditions of rice culture? Ethylene biosynthesis can be stimulated by flooding, resulting in plant elongation and the formation of aerenchyma cells in roots under hypoxia,[13,63,64] which is key for aquatic plant survival in flooded environments.[65–68] Would such plants need less additional ethylene when exposed to a flooding event compared with plants with constitutively lower ethylene levels, and would this result in reduced 'target site' sensitivity to quinclorac, because both flooding and quinclorac stimulate ACC synthase?[13,65] These kinds of questions remain to be answered. However, it is clear that understanding the forces leading to the evolution of this adaptation could provide the basis for environmental manipulations to delay the evolution of quinclorac resistance. This knowledge could even lead to a broader understanding of the evolution of multiple herbicide resistance in R *E. phyllopogon*, because it is also known that exposure of deepwater rice to ethylene can upregulate genes coding for P450 monooxidases,[69] which belong to the same enzymatic system conferring resistance to several herbicides in this same R *E. phyllopogon* accession.

4 CONCLUSIONS

The study shows that at least two relevant mechanisms contribute towards *E. phyllopogon* resistance to the auxinic herbicide quinclorac. The first involves an alteration along the normal auxin reception-signal transduction pathway, causing lower stimulation of ACC synthase activity in R than in S plants after quinclorac treatment. The second mechanism involves a greater constitutive β-CAS activity in R than in S plants. Greater ability to detoxify HCN via β-CAS would explain why quinclorac-stimulated ethylene release was associated with less growth inhibition in R than in S plants. Malathion inhibition of β-CAS activity enhanced quinclorac toxicity and rendered R *E. phyllopogon* susceptible to quinclorac. Because malathion did not reduce the stimulation of ethylene production in quinclorac-treated R and S *E. phyllopogon*, it is concluded that the enhanced malathion-sensitive P450 herbicide metabolism that confers resistance to other herbicides in this R *E. phyllopogon* biotype is not a relevant mechanism of quinclorac resistance. Thus, new mechanisms of herbicide resistance have been uncovered in a biotype already known to be endowed with metabolism-mediated resistance to multiple herbicides. It is not clear how resistance to quinclorac has evolved in *E. phyllopogon*. Further research is clearly needed on R *E. phyllopogon* to elucidate the extent to which multiple resistance involving quinclorac and other herbicides represents a concerted upregulation of a network of hormonal and enzymatic systems evolved to counteract certain environmental stresses,[70] or whether it is just the result of selection by repeated herbicide use.

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