Advances in Weed Science
formerly Planta Daninha

RESEARCH ARTICLE

An Insight of Quinclorac Resistance Mechanism in Early Watergrass (*Echinochloa oryzoides*)

Muhammad Zia Ul Haq[a,b], Zheng Zhang[a], Sheng Qiang[a*], Ramala Masood Ahmad[c], Dina Abdulmajid[d] and Muhammad Fiaz[e]

[a] Weed Research Laboratory, College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China. Muhammad Zia Ul Haq (Ex-doctoral student). [b] Assistant Professor, Department of Agronomy, University of Agriculture, Faisalabad 38000, Pakistan. [c] Assistant Professor, Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad 38000, Pakistan. Ramala Masood Ahmad. [d] Assistant Researcher, Rice Research and Training Center, Field Crops Research Institute, Agricultural Research Centre, Kafr-El-Sheikh, 33717, Egypt. Dina Abdulmajid. [e] Ex-Doctoral student, Key Laboratory of Genetics and Fruit Development, College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China.

Abstract: Background: Quinclorac- main herbicide targeting to barnyard grass, has been used for decades in rice fields. *Echinochloa* species have been reported evolving into quinclorac resistance.

Objective: Quinclorac resistance and its mechanism remain undisclosed in *Echinochloa oryzoides* (Ard.) Fritsch that needs to be uncovered.

Methods: Dose-response assays were performed, followed by ethylene synthesis, and related enzyme activities along with gene transcription were studied. β-CAS activity and its molecular docking were investigated.

Results: *E. oryzoides* evolved into 21 times resistance to quinclorac from Jiangsu province of China. The increment in ethylene levels in this biotype was correlated negatively with the level of resistance and positively with quinclorac-induced growth inhibition. Ethylene response pathway determination showed that resistant biotype decreased 1-aminocyclopropane-1-carboxylic acid (ACC) contents, related enzyme activities, and transcription of *ACS* and *ACO* genes. These results indicated that ethylene biosynthesis inhibition and quinclorac resistance possessed a positive correlation. Resistant biotype exhibited ~ 2-fold more β-CAS activity than susceptible ones. Resistant *EcCAS* gene depicted nucleotide changes as compared to susceptible ones, which resulted in two amino acid substitutions (Met-287-Lys and Thr-352-Ala). Consequently, resistant β-CAS enzyme exhibited an increase in binding residue in active site (simulation modelling); that can be the probable reason for higher enzyme activity in the resistant biotype.

Conclusions: The study concludes that variation in response pathway of auxin and potentially improved cyanide degradation were plausible mechanisms endowing quinclorac resistance in *E. oryzoides*.

Keywords: *ACS* and *ACO* genes; auxin response pathway; β-cyanoalanine synthase; Ethylene biosynthesis.

Journal Information:
ISSN - 2675-9462
Website: http://awsjournal.org
Journal of the Brazilian Weed Science Society

How to cite: Haq MZU, Zhang Z, Qiang S, Ahmad RM, Abdulmajid D, Fiaz M. An Insight of Quinclorac Resistance Mechanism in Early Watergrass (Echinochloa oryzoides). Adv Weed Sci. 2022;40:e020220084. https://doi.org/10.51694/AdvWeedSci/2022;40:00009

Approved by:
Editor in Chief: Carlos Eduardo Schaedler
Associate Editor: Caio Brunharo

Conflict of Interest: The authors declare no conflict of interest regarding the publication of this manuscript.

Received: September 10, 2021
Approved: May 5, 2022

* Corresponding author:
<wrl@njau.edu.cn>

1. Introduction

The history of chemical weed control dates back to 1945 when 2,4-D {2,4-dichlorophenoxyacetic acid (synthetic auxin)} was first made available commercially. Multiple herbicide-resistant weed populations were selected owing to the long history of herbicidal weed control (Heap, 2021). Ultimately, the choices for alternative and effective herbicides are reduced. A 30 years long history of herbicidal control of *Echinochloa* weeds gave rise to herbicide resistance. *Echinochloa oryzoides* (Ard.) Fritsch is considered to be a noxious rice weed (Damalas et al., 2008). The herbicide resistance is swiftly evolving owing to the insistent herbicide usage. A study reported the first herbicide resistance case in *E. oryzoides* against lipid inhibitors in 2000 (Fischer et al., 2000). Furthermore, multiple herbicide resistance was reported in the same species against ALS (cyhalofobbutyl, bispyribac sodium) and ACCase (penoxsulam) inhibitors (Altop et al., 2014).

The widespread usage of quinclorac upsurged the herbicide resistance in *Echinochloa* species such as *E. colona*, *E. crus-galli* (Malik et al., 2010), *E. crus-galli* var. *zelayensis* and -var. *mitis* (Xu et al., 2013: Zia Ul Haq et al., 2020), *E. crus-pavonis* (Yang et al., 2021), *E. phyllopogon*, *E. oryzicola* and *E. hispidula* (Heap, 2021).

TIR1/AFB has been revealed as auxin co-receptors with Aux/IAA protein; hence the same are receptors for quinclorac (LeClere et al., 2018). It fits well in the auxin binding site of the TIR1 receptor, hence proving that target sites for quinclorac are auxin receptors. Quinclorac application contributes to the activation of auxin-responsive genes {1-aminocyclopropane-1-carboxylic acid (ACC) synthase}: hence, persuading the de novo synthesis of ACC synthase (ACS). In susceptible plants, ACS activity increases ACC contents; furthermore, ACC is oxidized by ACC oxidase (ACO) activity into ethylene and hydrogen cyanide (HCN). Higher HCN levels can impair several metabolic enzymes, eventually leading to plant death (Gao et al., 2017). HCN inhibits one of the essential respiratory chain enzymes- cytochrome c oxidase (Ikegaya et al., 2001). A multigene family encodes the ACS and ACO enzymes in

the ethylene biosynthesis pathway. Varied transcript levels of *ACS* and *ACO* genes were observed in quinclorac susceptible and resistant *E. crus-galli* var. *zalayensis* and -var. *mitis* (Gao et al., 2018; Zia Ul Haq et al., 2020).

β-cyanoalanine synthase (β-CAS) degrades the HCN produced during ethylene biosynthesis (Lai et al., 2009). Several studies have indicated that resistance to quinclorac could be instigated by high β-CAS activity (Gao et al., 2017). Defiantly, a study revealed that quinclorac resistance solely depended on ethylene biosynthesis inhibition instead of cyanide degradation in *E. phylopogan* (Chayapakdee et al., 2019). Though, mutations may increase the activity of the enzymes (Mdodana et al., 2019). Quinclorac resistant *E. crus-galli* var. *zelayensis* and -var. *mitis* were found to develop single and three nucleotides mutations in β-CAS, respectively (Gao et al., 2017; Zia Ul Haq et al., 2020).

A study recently reported differential herbicide translocation and metabolism in quinclorac resistant and susceptible *E. crus-pavonis* (Yang et al., 2021), contradictory to previous studies-those stating that in resistant and susceptible plants, the quinclorac absorption/uptake, transport, and metabolism were found indifferent. The resistant populations of *E. crus-galli* var. *zelayensis* and -var. *mitis* did not express a notable rise in ethylene level post quinclorac application. In resistant biotypes, the low level of ACS activity is caused by less susceptibility in the auxin receptor level or the modified auxin signal transduction pathway. As well as degradation of cyanide by β-CAS can offer an extra benefit to resistant biotypes in coping with quinclorac sensitivity (Xu et al., 2013; Zia Ul Haq et al., 2020)

Although *E. oryzoides*, as one of the main weeds in rice fields, has been subjected to decades of' quinclorac application, its quinclorac resistance has not been reported so far. One *E. oryzoides* biotype from Jiangsu province of China was found to develop quinclorac resistance. To elucidate the possible mechanism of quinclorac-resistance, ethylene production, ACC accumulation, related enzymes, and β-CAS activity and enzyme binding with cofactor- PLP (molecular simulation), along with gene transcript levels, were relatively assessed among one resistant and two susceptible biotypes of *E. oryzoides*.

2. Material and Methods

2.1 Collection of Plant Material

Seeds of *E. oryzoides* were collected from paddy fields of Jiangsu (Shuyang 33.97 N/118.89 E) in China during autumn 2016. This site from Jiangsu province was under quinclorac usage for over 15 years. At the same time, four more biotypes were collected from rice fields of Ningxia province {(Tongfu, 38.78 N/106.59 E; NX1-S), (Guanqu, 38.55 N/106.47 E; NX2-S), (Zhangzheng, 38.42 N/106.44 E; NX3-S), (Zhongning, 37.67 N/106.88 E; NX4-S)} and one biotype from Liaoning province (Dawa, 41.93 N/ 122.00 E; LN1-S). Two quinclorac susceptible biotypes (which were not exposed to quinclorac herbicide before), one from Heilongjiang province (Suihua, 46.73 N/126.98 E) and another from Zhejiang province (Jinhua, 29.17 N/119.88 E), were collected for comparative analysis. The collected seeds were screened against varying levels of quinclorac in Petri plates. These eight biotypes were sown in the field (30 and 60 plants for R and S biotypes, individually). At 3-4 leaf stage, the seedlings were sprayed with quinclorac (field rate: 300 g a.i. ha^{-1}); half of the S seedlings were left without spray (seeds from half un-treated plants were used in further studies). Cross-pollination was avoided by covering the inflorescence with a butter paper bag (35 days after emergence). Seeds of surviving biotype of quinclorac field dose were employed in further study.

2.2 Quinclorac Resistance Confirmation (Screening in Petri Plates and Pot Assay)

Quinclorac was used in 5 varied concentrations (1200, 600, 300, 150, 75 mg L^{-1}), besides quinclorac free control was also maintained to screen seeds of R and S biotypes in Petri plate (9 cm diameter) bioassay using filter papers (Whatman no.1) and 15 seeds were incubated in each Petri plate. 5 mL herbicide solution or distilled water was applied to each Petri plate, and further rehydration was accomplished with the application of distilled water when required. The study was conducted in a growth chamber with 600 µmol photon m^{-2} s^{-1} PAR, 26/18 °C temperature, relative humidity at 60%, and a day/night period set at 14/10 h. Five random seedlings from each Petri plate were selected for shoot length measurement (9 days after incubation).

Five seedlings of individual biotypes were maintained in plastic pot having a diameter of 12 cm diameter for whole-plant bioassay. Pots were filled with organic soil and sand with 1:2 ratio. A laboratory sprayer (280 L ha^{-1} delivering capacity at 230 kPa) furnished with a flat fan nozzle (3WPSH-500D, Nanjing, China) was used to spray quinclorac at 3-4 leaf stage. For R biotype quinclorac was applied at 0, 75, 150, 300, 600, 1200 g a.i. ha^{-1} and for S biotypes the dose was 0, 37.5, 75, 150, 300 and 600 g a.i. ha^{-1}. The seedlings of S biotypes were treated only with the double field dose as it was understood that these seedlings would not survive on four-times field dose. Three weeks after quinclorac application, the above-ground plant parts were harvested for fresh weight measurement. Percent fresh weight reduction is the percent change in dry biomass at every dose with respect to un-treated control. The percent survival rate was calculated by subtracting the values of percent fresh weight reduction from 100.

2.3 Determination of Ethylene Production

Quinclorac was sprayed on all biotypes at 3-4 leaf stage with different concentrations (0, 150, 300, 600 g

a.i. ha^{-1}). At various time periods (0, 12, 24, 48, 96 h post spray), shoots from each treatment were harvested and incubated at 25 °C for five hours in 15 mL tared screw-neck vial. Abdallah's method was employed for the quantitative determination of ethylene using a GC system 7890A (Agilent Technologies). The injector, detector, and column were set at temperature of 70, 200, 250 °C, respectively. The flow rates of N_2, air, and H_2 were 30, 300, 30 mL min^{-1}, respectively. The ethylene contents were calculated in nmol g^{-1}fresh weight h^{-1} of incubation.

2.4 Endogenous ACC Levels Determination

Quinclorac-induced differential ACC levels in R and S biotypes were found in an experiment. Growth conditions, quinclorac application time and dose, as well as sampling times, were the same as ethylene determination. Shoot tissue (0.2 g) was powdered in liquid N2, and 5% sulfosalicylic acid (0.4 mL) was used for extraction. Chemical conversion of ACC into ethylene in samples was carried out by $HgCl_2$ (Bulens et al., 2011). The reaction was carried out in 5 mL tared screw neck vials. The escaped ethylene gas from the reaction was quantitatively determined by using a GC system 7890A (Agilent Technologies). ACC contents were calculated from ethylene by using the formula of Bulens et al. (2011).

2.5 ACS and ACO and β-CAS activities determination

Varying quinclorac doses (0, 150, 300, 600 g a.i. ha^{-1}) were applied to plants at 3-4 leaf stage. Root tissues were collected 24 h after quinclorac application for ACS activity (pmol g^{-1} root fresh weight h^{-1} of incubation) determination. ACC levels in extracted samples were monitored by their conversion into ethylene to find out ACS activity. Shoot samples from the same experiment were collected 24 h after quinclorac application for ACO activity (pmol g^{-1} root fresh weight h^{-1} of incubation) determination. Crushed frozen sample (0.5 g) was extracted by 1 mL MOPS extraction buffer {add MOPS (8.37 g) + 10 mL glycerol + ascorbic acid sodium salt (0.594 g) and make final volume to 100 mL by adding distilled water}. The samples were vortexed and incubated at 4 °C for 10 min at thermo mixer. After incubation, the samples were centrifuged for 30 min at 22000 × g and 4 °C. The supernatants (400 µl) were permitted to react with 3.6 mL of MOPS reaction buffer {add MOPS (1.046 g) + 10 mL glycerol + ascorbic acid sodium salt (0.099 g) + sodium bicarbonate (0.168 g) + iron sulphate (0.3 mg) + ACC (0.01 g) + DDT (0.015 g) and make final volume to 100 mL by adding distilled water}. After incubation for 1 h, the headspace gas was withdrawn to measure ethylene levels by GC system (Bulens et al., 2011).

Potential cyanide degradation by β-CAS activity in R and S biotypes was determined. Kasugai nutrient solution was prepared by adding $(NH_4)_2$ So_4 (188.7 mg), Na_2HPO_4 (40 mg), $CaCl_2$ (7.9 mg), Fe-EDTA (27.4 mg), $MnCl_2$ (0.75 mg), KCl (47.5), SiO_2 (137.3 mg), N (40 mg), P_2O_5 (20 mg), CaO (4 mg), MgO (6 mg), K_2O (30 mg) in 1 L of distilled water. Seedlings were grown till 3-4 leaf stage in Kasugai nutrient solution. Plant sample collection time was 1, 12, and 24 h after addition of quinclorac {50 µ mol L^{-1} (µM)} in growth medium. Enzyme extract was incubated for 30 min at 35 °C after extracting with 0.1 M Tris-HCl buffer. Absorbance was recorded at 650 nm using Na_2S as standard (Chon et al., 2008).

2.6 *EcCAS* Gene Sequence

Primers sequence (F-CCGTCCTTCAGCGTCAAA, R-ATGCCAACCAAAAGACCCT, TM = 55°C) was employed to amplify partial sequence of *EcCAS* in *E. oryzoides*. PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Otsu, Japan) was used to synthesize cDNA from RNA of samples that was extracted by using Plant Easy Spin RNA Miniprep Kit (Biomiga, San Diego, USA). TaKaRa PCR Thermal Cycler (TaKaRa, Otsu, Japan) was used for amplification in 50 µl total volume that consisted of TaKaRa ExTaqTM 0.4 µl, 10X Taq Buffer 5 µl, 4 µl of MgCl2 and dNTP each, forward and reverse primers 2 µl (10 µM) each, ddH2O 30.6 µl, and cDNA (2.5 ng). Gel/PCR Extraction Kit (Biomiga, San Diego, USA) was used to gel-purify PCR product. pMDTM 19-T Vector Cloning Kit (Clontech, USA) was employed to clone purified cDNA into E. coli DH5α Competent Cells (Clontech, USA), and then further sequencing was carried out by Sangon Biotech (Shanghai, China).

Rapid Amplification of cDNA Ends (RACE) was accomplished by SMARTer® RACE 5'/'3' Kit (Clontech, USA) with the use of primers (5'-RACE-CCCTCCTCTCAAGACTGGTGTATGACGGCA, 3´-RACE-CTGAAGCAAATGTTCTGAATGGTGGAAAGCC, TM= <70°C). After gel purification, PCR product was sequenced from the company. BioEdit Sequence Alignment Editor was used to align the obtained sequences. NCBI website's ORF Finder Function (https://www.ncbi.nlm.nih.gov/orffinder/) was employed to find *EcCAS* gene open reading frame (ORF). The ORF amplification was done by the primers (F-ATGGAGAGGATGCTGGCG, R- TTAGTCTACTGGCACTGGT

TCC, TM = 57°C) and sent to Sangon Biotech, Shanghai, China, for sequencing (4 individual plants and 6 clones from each replicate).

2.7 Computational Simulation of β-CAS Binding with its cofactor- PLP

Quinclorac resistant and susceptible β-CAS structures underwent homology modelling. 3D structure of β-CAS was constructed by Protein Homology/analogY Recognition Engine (Phyre2) (Kelley et al., 2015). Energy minimization of the structure was carried out using YASARA, and further structure validation was carried out by ProSA. The structure of PLP was obtained from PubChem (CID: 1051). Both

structures were prepared for docking by AutoDock Tools v. 1.5.6. and further AutoDock Vina v. 1.1.2. was used for docking of β-CAS and PLP. Docking grid box size (Å) was X:40, Y:60, and Z:44 and X:43, Y:51, and Z:44 for quinclorac susceptible and resistant β-CAS enzymes, respectively. Results obtained from docking were visualized and mined by PyMOL v. 2.3.3.

2.8 Transcriptional studies of *ACS*, *ACO*, and *CAS* Genes by Real-time PCR

Seedlings of R and S biotypes were grown, and samples were collected following the same procedure for β-CAS activity determination. Primers for *EcACS7*, *EcACS-like*, *EcACO-like*, *EcACO homolog 4*, *EcACO1*, and *EcACO5-like* (Table 1) were designed by using Primer Premier 5. *EcActin* {HQ395760; Table 1} was used as reference gene (Li et al., 2013). cDNA was synthesized as described earlier. Gene expression analysis of three biotypes of *E. oryzoides* was carried out on real-time PCR (Eppendorf Metercycler® Realplex2) with SYBR® Premix Ex TaqTM (Tli RNAaseH Plus; TaKaRa, Otsu, Japan). The volume of the reaction mixture was 10 µl, containing 5 µl SYBR® Premix Ex TaqTM, 0.4 µl primers (10 µM), 0.8 µl cDNA (2.5 ng), and 3.8 µl ddH_2O. PCR profile was as follows; 95°C for 30 s, 40 cycles at 95°C for 5 s, and 60°C for 30 s. A standard curve (Ct value-based) against cDNA template serial dilution was constructed to evaluate each primer set's PCR amplification efficiency. Target and internal control were confirmed for approximately equal amplification efficiencies. ΔΔCt method was employed to calculate relative mRNA levels (3 biological and 4 technical replicates). Up- or down-regulation of gene expressions was carried out by the t-test ($P < 0.05$) and fold change.

2.9 Statistical Analysis

Dose-response bioassays were conducted in triplicate. The concentration of ethylene, ACC contents, ACS, ACO, and β-CAS activity-related experiments were carried out in quadruplicate. All the experiments were repeated twice by using a completely randomized design. Percent reduction of fresh weight/shoot length related to untreated control was calculated. Dose-response function (growth/sigmoidal) of Origin Pro 8 SR0 {v 8.0724 (B724), Origin Labs Corporations. MA 01060. USA.} was used to calculate EC50 and GR50 (effective concentration to inhibit growth by 50%). Primer Premier 5 (PREMIER Biosoft International, Canada) was used to design primers. Graphs were drawn by Microsoft Excel 2019 and Sigma Plot. Correlation analysis and ANOVA construction were accomplished by SPSS version 24 (SPSS, Chicago, IL, USA).

3. Results and Discussion

3.1 Dose-response Analysis Confirmed Quinclorac Resistance in *Echinochloa oryzoides*

Biotypes from Ningxia and Liaoning viz; NX1-S, NX2-S, NX3-S, NX4-S, and LN1-S, respectively, exhibited quinclorac sensitivity after Petri plate bioassay (Table 2). Hence, these five biotypes were not used in further study. HLJ-S and ZJ-S depicted lower values of EC50, GR50, and RI, as on increased quinclorac rates, both biotypes were not able to survive (Figure 1; Table 3). However, JS-R biotype was able to survive at higher dose of quinclorac field rates, and it showed 21-32-fold increase in RI compared to HLJ-S- proving this biotype was resistant to quinclorac. The quinclorac resistance outbreak in *Echinochloa* species in Chinese rice fields mainly occurred by an exaggerated use of this herbicide as *E. oryzoides* biotype exhibited quinclorac resistance from Jiangsu province of China. Resistance evolution in this biotype was caused by the continuous use of quinclorac. As per earlier projections, those stated that continuous use of herbicide with the same mode of action could lead to resistant weed species (Malik et al., 2010).

3.2 Effect of Quinclorac Application on Ethylene Biosynthesis

In susceptible plants, ethylene biosynthesis is induced by the application of quinclorac (Zia Ul Haq et al., 2020). Therefore, to determine the relationship between quinclorac

Table 1 - Primers set for *ACS*, *ACO* and *CAS* genes amplification in *Echinochloa oryzoides*.

Gene	Forward primer	Reserve Primer	Annealing Temperature (°C)	Length of Product
EcActin	5´-TACTCCTTCACCACAACCGC-3´	5´-TGATGACCTGTCCATCAGGC-3´	60	154
EcACS7	5´-TCGCGAGGATGAGCAGATTC-3´	5´-ACCCAAGGTAGTATTATTTACCCTC-3´	60	124
EcACS-like	5´-GATGCTGTCGGACCACGAG-3'	5´-GTCCATCCACGAGAAGAGCC-3	60	148
EcACO homolog 4	5´-CATCTTCCCGCACACGGAC-3´	5´-GATGGAAACCCTTGGCTCGT-3´	60	250
EcACO1	5´-AGTCCCAGGTTTGGAGTTTCTG-3´	5´-ATTATGGCGTCAGCACCAGG-3´	60	207
EcACO-like	5´-CCGGAGTTCAAGGAGACCAT-3´	5´-TGACCTTGGTGCCGAAGAAG-3´	60	163
EcACO5-like	5´-GCACATGGCGGTGAACTACT-3´	5´-CACGCTCTTGTAGCGGTCAT-3´	60	229
EcCAS	5´- ATGCCGTCATACACCAGTCTT-3´	5´- CAGCAGGTTCAACTCCATAGAT-3´	57	253

resistance and ethylene biosynthesis, three biotypes were subjected to the measurement of the ethylene levels. Ethylene levels increased more rapidly and exhibited higher values in susceptible biotypes. However, the resistant biotype showed a little increase in ethylene levels even at 2 times of quinclorac field dose (Figure 2a, b). This biotype showed 4 times higher ethylene levels than the untreated control at a quinclorac dose of 600 g a.i. ha^{-1}, verifying that ethylene production correlated negatively with resistance against quinclorac (Figure 3a). 24 h after quinclorac application, a peak in ethylene production was observed; afterward, a gradual decrease was evident in three biotypes at the quinclorac rate of 300 g a.i. ha^{-1} (Figure 2a, b).

Nevertheless, HLJ-S and ZJ-S biotypes showed nearly 2 times increment in ethylene levels measured at 96 h after quinclorac application than their respective controls. Additionally, the quinclorac-induced ethylene level increment triggered toxicity in plants and reduced fresh weight in both susceptible biotypes. Stimulation of ethylene biosynthesis and quinclorac-induced growth inhibition were found to possess a positive correlation. JS-R biotype exhibited a relatively slight increase (0.5 times compared to untreated control) after quinclorac spray, hereafter verifying that quinclorac resistance levels and ethylene biosynthesis correlated negatively. In susceptible plants, an increase in ethylene and cyanide contents due to de novo synthesis of ACS are evident from the application of quinclorac- as it is an auxin herbicide (Abdallah et al., 2006; Xu et al., 2013). Senescence and plant death are governed by a major plant hormone- ethylene. Several studies reported that ethylene biosynthesis inhibition in barnyard grass could be a possible mechanism endowing quinclorac resistance (Abdallah et al., 2006; Xu et al., 2013; Gao et al., 2018).

This study showed distinct levels of ethylene in resistant and susceptible biotypes post quinclorac

Table 2 - EC_{50}, and RI of quinclorac sensitive biotypes of *Echinochloa oryzoides* from Ningxia and Liaoning Provinces.

Populations	Dose-Response Analysis		
	EC_{50} (mg L^{-1})	r^2	RI (EC_{50} Ratio)
NX1-S	401.18 (3.32)	0.97	3.57
NX2-S	282.93 (7.33)	0.99	2.52
NX3-S	197.71 (1.63)	0.99	1.76
NX4-S	196.62 (0.46)	0.98	1.75
LN1-S	196.71 (0.55)	0.99	1.75

EC_{50} = effective dose causing 50 % inhibition, RI (Resistance Index) = ratio of EC_{50} R over EC_{50} S, r^2 = Coefficient of determination, $P < 0.05$, Standard errors are in parenthesis. Biotypes showing RI less than 10 are considered as herbicide sensitive.

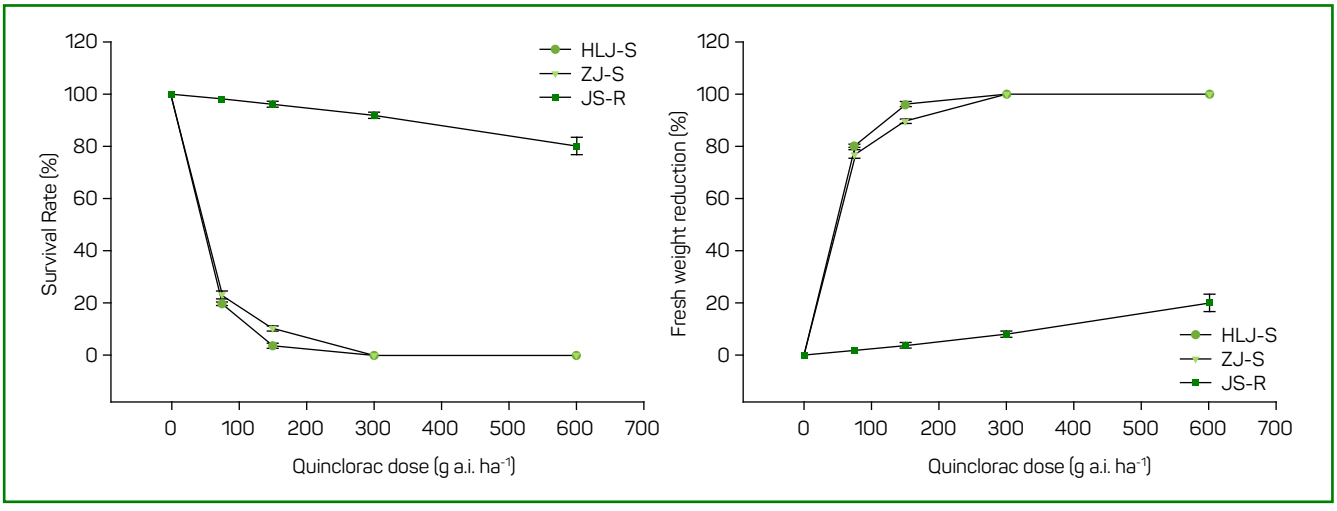

Figure 1 - Dose-response curve of *Echinochloa oryzoides* possessing varied levels of quinclorac sensitivity.

Table 3 - EC_{50}, GR_{50}, and RI of *Echinochloa oryzoides* possessing varied levels of quinclorac sensitivity.

Populations	Dose-Response Assay					
	EC_{50} (mg L^{-1})	r^2	RI (EC_{50} Ratio)	GR_{50} (g a.i. ha^{-1})	r^2	RI (GR_{50} Ratio)
HLJ-S	112.46(16.24)	0.99	1	29.54(4.60)	0.99	1
ZJ-S	177.95(18.56)	0.99	1.58	47.61(5.28)	0.99	1.61
JS-R	3570.21(290.84)	0.97	32	625.86(70.04)	0.99	21

EC_{50} = effective dose causing 50 % inhibition, GR_{50} = effective dose causing 50% growth inhibition, RI (Resistance Index) = ratio between R and S (EC_{50} or GR_{50}), r^2 = Coefficient of determination, $P < 0.05$, Values in parenthesis are standard errors.

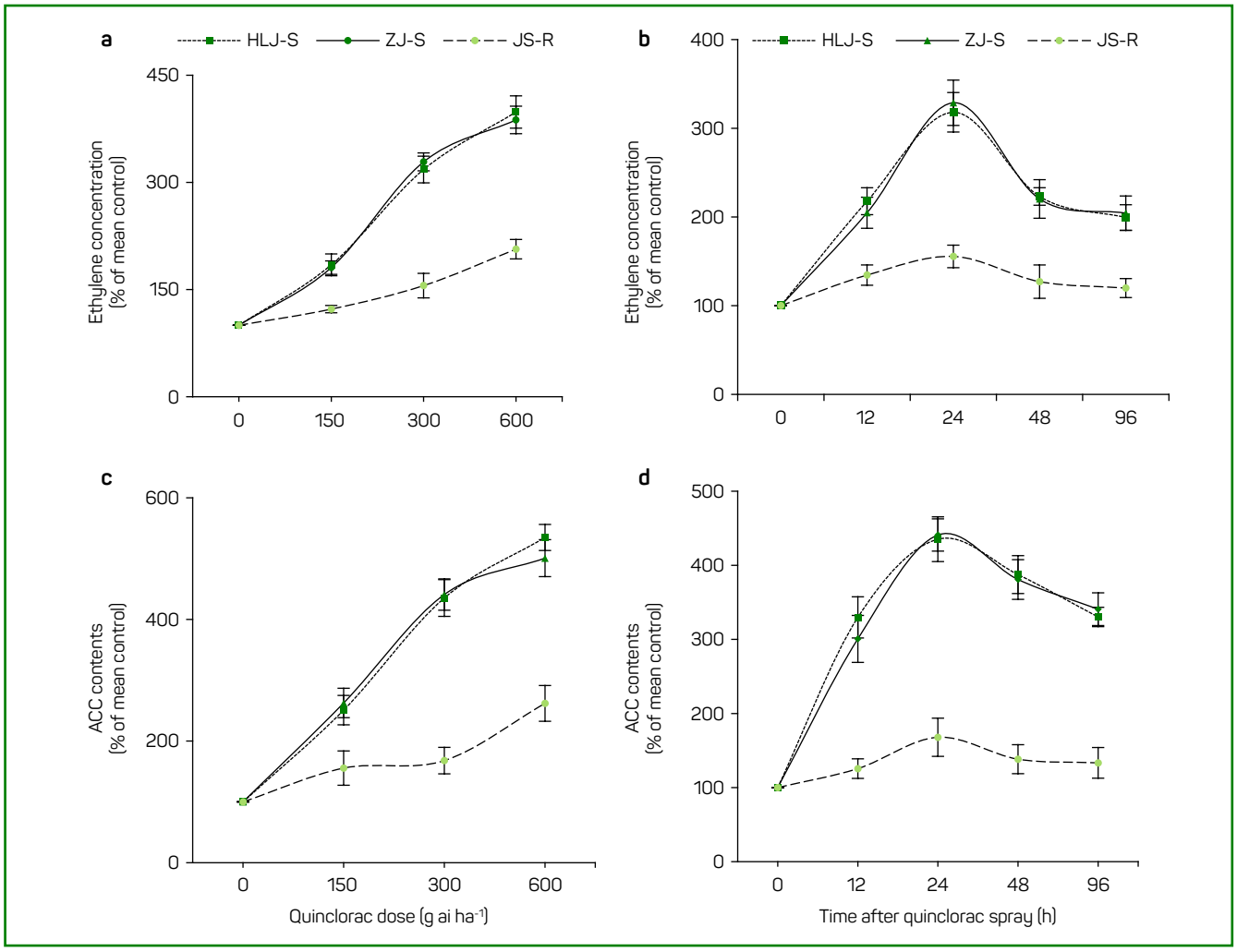

Figure 2 - Ethylene concentration (a, b; % of mean untreated plant in same biotype) and ACC contents (c, d; % of mean control) depicted by *Echinochloa oryzoides* biotypes (quinclorac resistant and susceptible) effected by various quinclorac doses (0, 150, 300, 600 g a.i. ha^{-1}) at 24 HAT and sampling times (0, 12, 24, 48, 96 HAT) at quinclorac dose of 300 g a.i. ha^{-1}.

application, which validates the link between ethylene biosynthesis inhibition and quinclorac resistance (Xu et al., 2013; Gao et al., 2018). In this study, quinclorac-induced hormone homeostasis imbalance resulted in phytotoxic growth inhibition confirming the positive relationship among ethylene biosynthesis and growth inhibition. Hereby, this validates the protagonist of ethylene in quinclorac phytotoxicity (Abdallah et al., 2006; Xu et al., 2013; Zia Ul Haq et al., 2020).

3.3 Differential Effect of Quinclorac Application on ACC, ACS, and ACO enzymes activities

ACC accumulation was studied in all three biotypes of *E. oryzoides* at 24 h after treatment with different quinclorac rates (0, 150, 300, 600 g a.i. ha^{-1}; Figure 2c, d). A slight ACC contents increase (1.5-1.9 fold) was observed in JS-R biotype at 150 and 300 g a.i. ha^{-1}, while at 600 g a.i ha^{-1}, this biotype exhibited 2.8-fold increase in ACC contents. HLJ-S and ZJ-S being quinclorac susceptible biotypes, recorded the highest ACC contents (5 times more as compared to untreated control and 2 times more than resistant biotype) at quinclorac dose of 600 g a.i. ha^{-1} (Figure 2c, d). ACC contents spiked in all biotypes till 24 h after quinclorac application (300 g a.i. ha^{-1}), while a downward trend in ACC contents was observed beyond 24 h (Figure 4). The application of 300 g a.i. ha^{-1} quinclorac caused a slight increment of ACC contents in resistant biotype irrespective of sampling times. While ACC contents in susceptible biotypes, the ACC contents were around 4 times higher than control (0 h after spray) at 24 h after quinclorac application. A negative correlation was found between ACC contents and resistance levels of all biotypes, the same as found in ethylene levels.

Ethylene production rate is controlled by two important enzymes (ACS and ACO) in its biosynthesis pathway. Differential ACS and ACO enzymes activities induced by quinclorac application in resistant and susceptible biotypes of *E. oryzoides* were investigated under varying quinclorac doses. Results revealed that the ACS and ACO activities were

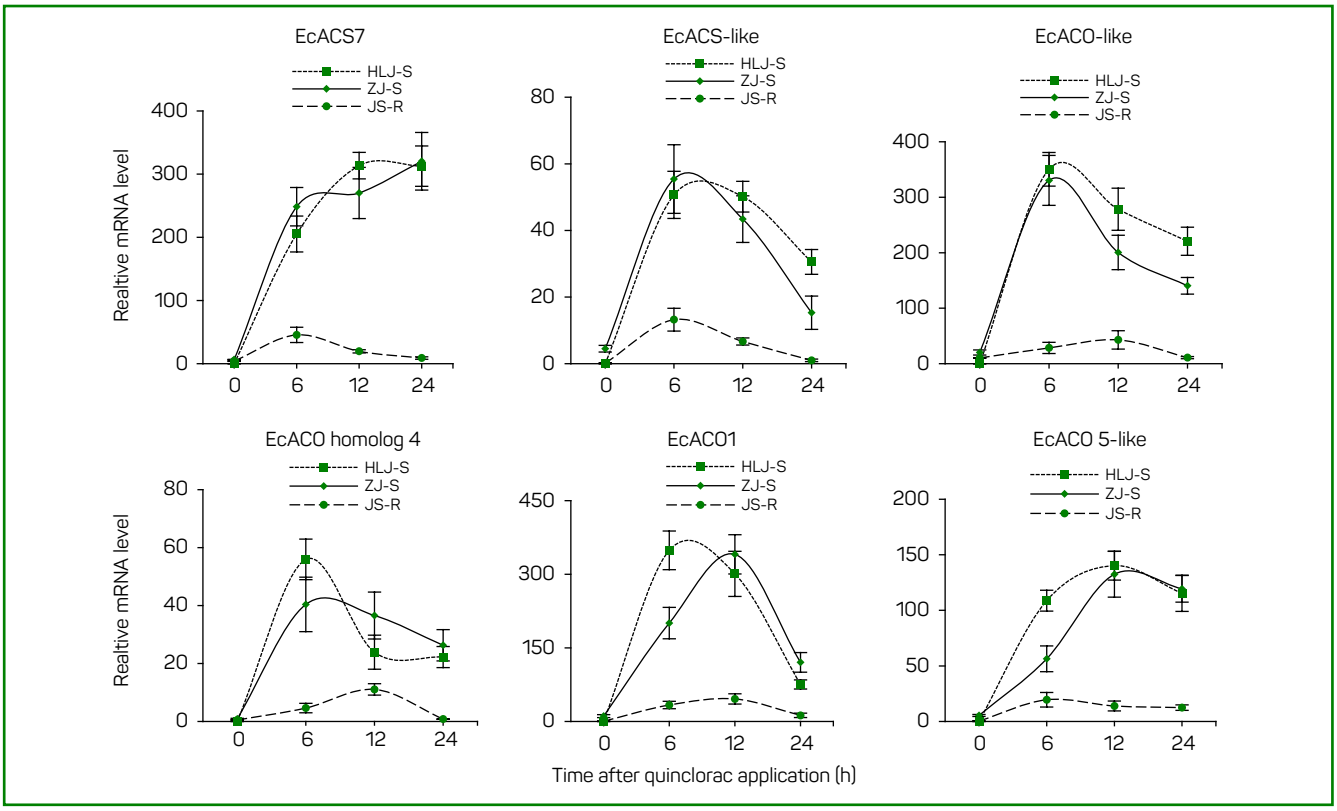

Figure 3 - The *ACS* (*EcACS7*, *EcACS-like*) and *ACO* (*EcACO-like*, *EcACO homolog 4*, *EcACO*, *EcACO5-like*) genes relative transcription in *Echinochloa oryzoides* biotypes calculated at 0-, 6-, 12-, and 24-hours post quinclorac treatment.

```
EOCAS(Q.Sensitive)      MERMLARLMRRRSSLLHQGAAPPIPLGA--SSSLFSTTQQ-----QQADPGVTPGLKIRD    53
EOCAS(Q.Resistant)      MERMLARLMRRRSSLLHQGAAPPIPLGA--SSSLFSTTQQ-----QQADPGVTPGLKIRD    53
OSCAS                   MFRALMSLMRRRSLLQSGGRAPPAMAAAAGGSPFFSTLQQAAAADPVQSPGILPGLKIRD    60

EOCAS(Q.Sensitive)      SASQLIGRTPMVYLNKVTEGCGARIAAKLEFLQPSFSVKDRPAISMLEDAENKGLITPGK    113
EOCAS(Q.Resistant)      SASQLIGRTPMVYLNKVTEGCGARIAAKLEFLQPSFSVKDRPAISMLEDAENKGLITPGK    113
OSCAS                   SASQLIGRTPMVYLNKVTEGCGARIAAKLEFLQPSFSVKDRPAISMLEDAEKKGLITPGK    120

EOCAS(Q.Sensitive)      TTLIEPTSGNMGIGLAFMAALKGYELILTMPSYTSLERRVTMRAFGANLVLTDPTKGMGG    173
EOCAS(Q.Resistant)      TTLIEPTSGNMGIGLAFMAALKGYELILTMPSYTSLERRVTMRAFGANLVLTDPTKGMGG    173
OSCAS                   TTLIEPTSGNMGIGLAFMAALKGYELILTMPSYTSLERRVTMRAFGAKLVLTDPTKGMGG    180

EOCAS(Q.Sensitive)      TVRKAAELYEKHPSAYMLQQFQNPANVKIHYETTGPEIWEDTLGQVDIFVMGIGSGGTVT    233
EOCAS(Q.Resistant)      TVRKAAELYEKHPSAYMLQQFQNPANVKIHYETTGPEIWEDTLGQVDIFVMGIGSGGTVT    233
OSCAS                   TVRKAAELYENHPSAFMLQQFENPANVKVHYETTGPEIWEDTLGQVDIFVMGIGSGGTVT    240

EOCAS(Q.Sensitive)      GVGKYLKEKNPNTKIYGVEPAEANVLNGGKPGPHLITGNGVGFKPDILDMDVMEMVLEVK    293
EOCAS(Q.Resistant)      GVGKYLKEKNPNTKIYGVEPAEANVLNGGKPGPHLITGNGVGFKPDILDMDVMEKVLEVK    293
OSCAS                   GVGKYLKEKNPNAKIYGVEPAEANVLNGGKPGPHLITGNGVGFKPEILNMDIMEKVLEVK    300

EOCAS(Q.Sensitive)      SEDAVKMARELAVKEGLLVGISSGANTVAALELAKKPENKGKLIVTVLPSLGERYLSSTL    353
EOCAS(Q.Resistant)      SEDAVKMARELAVKEGLLVGISSGANTVAALELAKKPENKGKLIVTVLPSLGERYLSSAL    353
OSCAS                   GEDAVKMARELALKEGLLVGISSGANTVAALELAKKPENKGKLIVTVLPSLGERYLSSAL    360

EOCAS(Q.Sensitive)      FEELRKEAEAMEPVPVD       370
EOCAS(Q.Resistant)      FEELRKEAEAMEPVPVD       370
OSCAS                   FEELRAEAEAMQPVPVD       377
```

OSCAS: *Oryza sativa* CAS

Figure 4 - Amino acid sequence (ORF generated) of *EcCAS* gene from quinclorac resistant {EOCAS (Q. Resistant)} and susceptible {EOCAS (Q. Sensitive)} *Echinochloa oryzoides*. Two nucleotide mutations (Met-287-Lys and Thr-352-Ala) between both biotypes are specified by boxed codon.

negatively correlated with quinclorac resistance. ACS activity was the same in three biotypes understudy at 0 and 150 g a.i. ha^{-1} herbicide dose (Table 4). However, the difference between ACS activity extended between quinclorac resistant and susceptible biotypes with increased quinclorac dose. Nearly 2 times higher ACS activity was observed in HLJ-S and ZJ-S biotypes as compared to JS-R at 600 g a.i. ha^{-1} quinclorac dose. ACO activity was higher in HLJ-S and ZJ-S biotypes as compared to JS-R before treatment with quinclorac. JS-R biotype depicted the slightest increase (77% higher than control) in ACO activity at quinclorac rate of 600 g a.i. ha^{-1}. Whereas HLJ-S and ZJ-S being quinclorac susceptible biotypes, exhibited much higher ACO activity, as ~60, 120, and 230% increment in ACO activity was found at quinclorac doses of 150, 300, and 600 g a.i. ha^{-1} (Table 4). A reasonable upsurge in ACC contents, ACS, and ACO activities were recorded in a susceptible biotype of *E. oryzoides* after quinclorac application. While increment of these parameters was relatively low in the resistant biotype. Subsequently, resistant biotype produced a lower level of ethylene as compared to susceptible ones. These results follow previous studies where susceptible biotypes showed a high increase in ACC, ACS, and ACO activities than resistant biotypes (Xu et al., 2013; Zia Ul Haq et al., 2020).

3.4 *ACS* and *ACO* Genes Transcription Varied among Resistant and Susceptible Biotypes

The relative transcript levels of *ACS* and *ACO* genes were calculated at 0, 6, 12, 24 HAT in all biotypes under study. The data exhibited that the susceptible biotypes expressed a significant upsurge in relative transcript levels of *ACS* and *ACO* genes post quinclorac application (Figure 3). The relative transcript levels of six genes (*EcACS7*, *EcACS-like*, *EcACO-like*, *EcACO homolog4*, *EcACO1*, and *EcACO5-like*) under study were upregulated by 39, 12, 18, 8, 67, and 24 folds, respectively at 6 HAT in HLJ-S. Expression levels of *EcACS7* and *EcACO5-like* increased at 12 HAT in HLJ-S, with a decreasing trend till 24 HAT. *EcACS-like* maintained its expression levels at 12 HAT in the same biotype. The remaining genes under study depicted a decreasing trend afterward in HLJ-S (Figure 3). Relative transcription of *EcACS7*, *EcACS-like*, *EcACO-like*, *EcACO homolog4*, *EcACO1*, and *EcACO5-like* were amplified to some extent in JS-R; nevertheless, it was significantly lower than the increment in HLJ-S. Except for *EcACO1*, all other genes exhibited 10-folds upregulation in gene transcription level at either calculation time. Less inducible expressions for *ACS* and *ACO* genes triggered a diminution in ACS and ACO enzyme activities in quinclorac-resistant *E. oryzoides*. These expression levels correlated negatively with quinclorac resistance.

Quinclorac susceptible *E. crus-galli* var. *mitis* and -var. *zelayensis* exhibited the increment in ethylene production due to *ACS* and *ACO* genes upregulation (Gao et al., 2018; Zia Ul Haq et al., 2020). In this study, quinclorac susceptible and resistant *E. oryzoides* showed the differential relative transcript levels of *EcACO5-like*, *EcACO1*, *EcACO homolog4*, *EcACO-like*, *EcACS-like*, and *EcACS7* genes. Quinclorac susceptible biotypes of *E. oryzoides* in the present study exhibited > 10-fold increase in transcript levels of *EcACO5-like*, *EcACO homolog4*, *EcACO-like*, *EcACS-like* and *EcACS7*. Contrarily, the resistant biotype did not show any remarkable increment in transcript levels of the above-mentioned genes. Alteration in ACS and ACO activity induction can be correlated to quinclorac resistance in the present study. As it is clear from *ACS* and *ACO* genes transcription data, increased ACS and ACO (~2-3 times, respectively) activity levels in susceptible than resistant biotype of *E. oryzoides* can reinforce the protagonism of ethylene biosynthesis inhibition causing quinclorac resistance in the resistant biotype.

3.5 Sequence Description, Evaluation, and Mutation Verification of *EcCAS* gene

EcCAS gene ORFs in all biotypes were comprised of 1113 bp, encoding 370 amino acids polypeptide. The sequences were submitted to NCBI website (MT007245, MT007246). *Echinochloa oryzoides EcCAS* gene was found to be highly similar (>90%) with *EcCAS* gene of *E. crus-galli* var. *mitis* (MN815009-10) and -var. *zelayensis* (KY922855.1). Four copies of *EcCAS* gene were found from all biotypes of *E. oryzoides* (data not presented). Nucleotide sequences depicted two-point mutations found in all copies of resistant biotype as compared to susceptible ones. The

Table 4 - ACS and ACO activities by *Echinochloa oryzoides* biotypes possessing varied quinclorac sensitivity after 24 hours under various quinclorac doses.

Dose (g a.i. ha^{-1})	ACS activity (pmol. g^{-1} fresh weight h^{-1})			ACO activity (pmol. g^{-1} fresh weight h^{-1})		
	HLJ-S	ZJ-S	JS-R	HLJ-S	ZJ-S	JS-R
0	50.58±0.65$^{d\ NS}$	48.08±2.99$^{c\ NS}$	44.03±1.44$^{c\ NS}$	290.66±6.27$^{d\ A}$	289.16±10.92$^{d\ A}$	207.52±15.44$^{c\ B}$
150	64.78±2.71$^{c\ NS}$	60.28±6.87$^{c\ NS}$	50.72±1.87$^{bc\ NS}$	490.54±21.65$^{c\ A}$	500.54±31.22$^{c\ A}$	263.08±8.09$^{bc\ B}$
300	81.92±1.35$^{b\ A}$	84.67±3.77$^{b\ A}$	55.59±1.75$^{ab\ B}$	652.05±18.78$^{b\ A}$	667.05±30.97$^{b\ A}$	303.74±21.71$^{b\ B}$
600	113.55±2.65$^{a\ A}$	117.80±6.77$^{a\ A}$	61.34±2.15$^{a\ B}$	981.19±20.54$^{a\ A}$	1006.19±36.95$^{a\ A}$	367.59±11.35$^{a\ B}$

Data are four replicate's means ± standard error. Significant difference among various doses in one biotype, and among various biotypes under the same dose are represented by $^{a\text{-}d}$ and $^{A\text{-}B}$, respectively, ^{NS} = non-significant under the same quinclorac dose. ACS = 1-Aminocyclopropane-1-carboxylic acid synthase, ACO = 1-Aminocyclopropane-1-carboxylic acid oxidase

mutations of ATG to AAG and TAC to TGC caused amino acid substitutions of Met-287-Lys and Thr-352-Ala, respectively (Figure 4). Non-target enzyme activity can be instigated by gene mutations and alteration in their expression levels (Iwakami et al., 2014).

3.6 Enhanced β-CAS Activity, Expression Pattern of *EcCAS* Gene, Effect of Mutations on β-CAS Docking with Cofactor- PLP

The β-CAS activity in susceptible biotypes was significantly lower than the resistant one prior to quinclorac treatment (Figure 5a). A decreasing trend in β-CAS was observed in susceptible biotypes post quinclorac treatment, as 24 hours after treatment (HAT), around 25% decline was recorded in β-CAS activity. The resistant biotype exhibited significantly ($P < 0.05$) higher enzyme activity than susceptible biotypes regardless of the sampling time under study (Figure 5a). Untreated resistant biotype of *E. oryzoides* depicted higher ($P < 0.05$) relative transcript levels of *EcCAS* gene than susceptible ones, as it was 1.8-fold higher than susceptible ones (Figure 5b). *EcCAS* transcript levels were declined post quinclorac application in susceptible biotypes, as at 24 HAT, it decreased 0.70-fold in the untreated plants. Resistant biotype was succeeded to maintain *EcCAS* transcript levels; nevertheless, no significant increase was observed in its transcript levels than untreated plants. An increment of 2.6-fold was observed in *EcCAS* transcript levels of resistant biotype than susceptible ones at 24 HAT (Figure 5b). Resistant and susceptible biotypes varied regarding β-CAS activity in this study. The higher β-CAS activity was observed in quinclorac-resistant *E. oryzoides* than in susceptible ones, so this biotype was potentially capable of detoxifying produced cyanides due to the quinclorac application. The increased β-CAS activity in quinclorac-resistant *E. crus-galli* var. *zelayensis* and -var. *mitis* has been documented already (Gao et al., 2017; Zia Ul Haq et al., 2020). Increased cyanides accumulated in susceptible biotypes led to cyanide poisoning. Elevated cyanides in plants disrupted cytochrome c oxidase (Ikegaya et al., 2001). On the other hand, ACS activity can be triggered by cyanides produced, ultimately intensifying the ethylene and cyanide levels in plants (Grossmann and Scheltrup, 1997). Therefore, resistant biotype was escaped from more ethylene production because cyanides were potentially degraded by β-CAS activity. Maintenance of *EcCAS* relative expression levels in quinclorac resistant *E. oryzoides* and downregulation in susceptible ones post quinclorac application proved that β-CAS activity and quinclorac resistance were correlated positively. It can be inferred that quinclorac resistance in *E. oryzoides* was instigated by variation in ethylene response pathway and potential cyanide degradation by β-CAS activity, as found earlier in *E. crus-galli* var. *zelayensis* and -var. *mitis* (Xu et al., 2013; Gao et al., 2018; Zia Ul Haq et al., 2020).

Figure 6a, b shows the 3D structures of β-CAS, and active sites in the structure are shown at the point of binding of PLP (Figure 6c, d). The PLP binding with β-CAS was affected due to point mutations as in susceptible β-CAS, Ser-121, Gln-193, Gly-271, and Asn-272 amino acids bounded the PLP, though it was bound to Ser-121, Gln-193, Ile-269, Gly-271, and Asn-272 in resistant one. Molecular docking was carried out around nine modes (Table 5) in both resistant and susceptible β-CAS 3D enzyme structures. Results depicted a slight decrease in binding free energy (-0.61 to -0.62 kcal mol^{-1}; Table 5), making resistant β-CAS to bind with PLP further conveniently. β-CAS binds with cysteine with the help of a cofactor. CN- attachment with cysteine

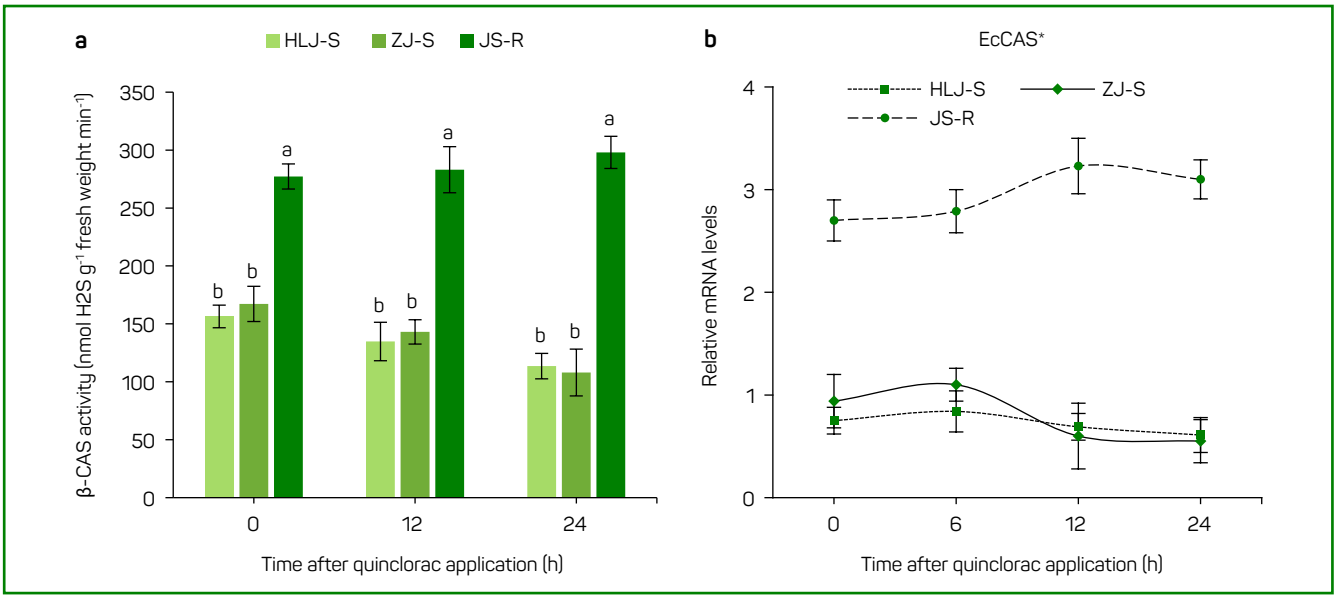

Figure 5 - β-CAS activity (a), and *EcCAS* gene relative transcription (b) depicted by *Echinochloa oryzoides* biotypes (quinclorac resistant and susceptible) at various time intervals post quinclorac treatment.

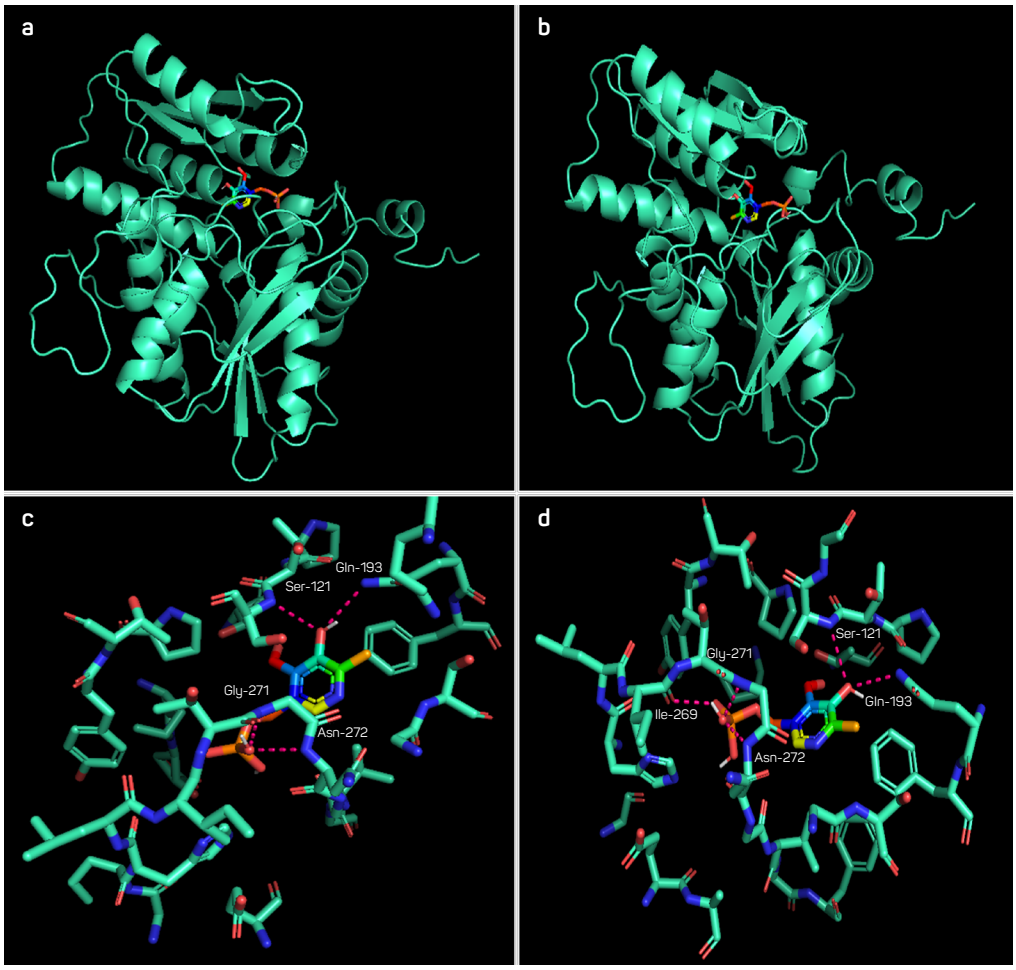

Figure 6 - Ribbon diagram demonstrating the β-CAS structure of *Echinochloa oryzoides* (a) quinclorac susceptible, and (b) resistant. Molecular simulation of active sites of (c) quinclorac susceptible, and (d) resistant β-CAS docked with PLP.

Table 5 - Molecular docking carried out around different modes in quinclorac-resistant and susceptible β-CAS enzyme 3D structure.*

Modes	Quinclorac-susceptible β-CAS enzyme			Quinclorac-resistant β-CAS enzyme		
	Affinity (kcal mol^{-1})	Distance from best mode (Å)		Affinity (kcal mol^{-1})	Distance from best mode (Å)	
		RMSD lb	RMSD ub		RMSD lb	RMSD ub
1	-6.1	0.000	0.000	-6.2	0.000	0.000
2	-6.0	4.126	6.617	-5.8	2.077	2.903
3	-5.9	2.898	5.553	-5.8	5.317	7.857
4	-5.7	1.984	2.840	-5.7	4.231	6.560
5	-5.6	5.659	7.559	-5.5	2.650	3.958
6	-5.5	5.842	7.115	-5.5	3.480	5.697
7	-5.5	5.886	6.498	-5.4	3.530	5.418
8	-5.4	6.444	7.669	-5.2	5.615	7.375
9	-5.3	4.383	6.317	-5.1	5.037	6.399

RMSD = root mean square deviation, lb = lower bound, ub = upper bound, Å = Angstrom

*Auto-generated table from AutoDock Vina v. 1.1.2 during molecular docking.

is completed due to the repositioning of amino acid; this binding initiates the β cyanoalanine production (Yi et al., 2012). The understanding of binding variation between quinclorac susceptible and resistant β-CAS enzymes was accomplished by molecular docking studies of PLP and β-CAS. In the active site, five amino acid subunits made polar bonds with resistant β-CAS; this led to a slight decline in binding free energy. This provided a base for easier and firm binding of β-CAS with PLP. Resultingly efficient β-CAS-PLP binding ultimately led to the increased overall efficiency of β-CAS to degrade cyanides produced in plants during the ethylene biosynthesis pathway in response to quinclorac application (Zia Ul Haq et al., 2020). Protein-protein interactions are responsible for governing cell biological processes.

β-*CAS* gene in quinclorac-resistant *E. phyllopogon* showed single nucleotide polymorphism (SNP) in the intron region of the gene, and there was no link between quinclorac resistance and β-CAS mutation (Chayapakdee et al., 2019). Consequently, the translated amino acid sequence did not depict any change due to SNP. Recently, a study on *E. crus-pavonis* excluded the role of β-CAS in endowing quinclorac resistance (Yang et al., 2021).

Though, in the present study, *EcCAS* gene of *E. oryzoides* depicted two-point mutation that led to amino acid substitutions. Recently three point-mutations in *EcCAS* gene have already been reported in *E. crus-galli* var. *mitis* (Zia Ul Haq et al., 2020). It is a well-known fact that β-CAS enzyme is responsible for HCN degradation that is produced during ethylene synthesis (Lai et al., 2009). Subsequently, the enzymatic activity was possibly maintained due to mutation in β-CAS gene post quinclorac application. Hence, maintenance of β-CAS activity levels still played a role in endowing resistance against quinclorac. Increased enzymatic activity due to mutation in gene is evident in various studies (Mdodana et al., 2019; Zia Ul Haq et al., 2020). Molecular simulation study revealed the positive role of these mutations in enzyme and cofactor binding by various means such as binding site residues increment, the decline in binding free energy, and structural stability. The affinity of an enzyme and substrate proved to be enhanced by mutations (Wu et al., 2017). Hence, it can be concluded that maintenance in β-CAS enzyme activity post quinclorac application was endowed by said mutations.

Therefore, in light of the above-mentioned results, a decline in ACO and ACS activities was instigated by the decrease in transcript levels of *EcACO1*, *EcACO homolog4*, *EcACO-like*, *EcACO5-like*, *EcACS-like*, and *EcACS7* genes. Besides, *EcCAS* gene mutations endowed varied transcript levels between resistant and susceptible *E. oryzoides*, which led to the maintenance of β-CAS activity. Resultingly, a reduction in ethylene biosynthesis combined with potentially enhanced cyanide degradation caused quinclorac resistance.

4. Conclusions

ACS and ACO activities were enhanced post herbicide treatment in quinclorac susceptible *E. oryzoides* due to the transcription induction of respective genes. Consequently, ethylene biosynthesis was increased in these plants. Contrarily, quinclorac treatment did not lead to a rise in relative transcript levels of *ACS* and *ACO* genes in quinclorac resistant *E. oryzoides*; hence, ACS and ACO activities kept at low levels that commanded less ethylene production. The auxin-induced genes need further study to find the underlying mechanism; those studies might include gene-specific response and Aux/IAA protein sequencing. Quinclorac susceptible *E. oryzoides* showed a decline of *EcCAS* gene expression and ultimately lower β-CAS activity after herbicide treatment. Whereas quinclorac-resistant *E. oryzoides* exhibited higher β-CAS activity, facilitated HCN degradation. In addition, molecular simulations revealed that two-point mutations in *EcCAS* gene altered the enzyme structure that helped to improve its binding ability with HCN substrate, ultimately resulting in efficient cyanide degradation. Nevertheless, further study is needed to explore whether quinclorac acts as a signalling compound to induce the transcription of *ACS* and *ACO* genes or it induces a minute ethylene production.

Author's contributions

Conceptualization of the manuscript and development of the methodology: M.Z.U.H., S.Q.; data collection and curation: M.Z.U.H., Z.Z., R.M.A., D.A., and M.F.; data analysis: M.Z.U.H, Z.Z., R.M.A., D.A., and M.F.; data interpretation: M.Z.U.H., Z.Z., and R.M.A.; funding acquisition and resources: S.Q.; project administration: S.Q., and M.Z.U.H; supervision: S.Q.; writing the original draft of the manuscript: M.Z.U.H., D.A., and M.F.; writing, review, and editing: S.Q. All authors read and agreed to the published version of the manuscript.

Acknowledgements

The authors are thankful to Wei Jiajia (Ph.D. student, Weed Research Laboratory, College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China) for helping in the identification of species.

Funding

National Key Research and Development Program of China (2016YFD0200805).

Conflict of Interest

The authors declare no conflict of interest.

References

Abdallah I, Fischer AJ, Elmore CL, Saltveit ME, Zaki M. Mechanism of resistance to quinclorac in smooth crabgrass (*Digitaria ischaemum*). Pestic Biochem Phys. 2006;84(1):38–48. https://doi.org/10.1016/j.pestbp.2005.05.003

Altop EK, Mennan H, Streibig JC, Budak U, Ritz C. Detecting ALS and ACCase herbicide tolerant accession of *Echinochloa oryzoides* (Ard.) Fritsch. in rice (*Oryza sativa* L.) fields. Crop Protect. 2014: 65: 202–206. https://doi.org/10.1016/j.cropro.2014.07.011

Bulens I, de Poel B, Hertog ML, De Proft MP, Geeraerd AH, Nicolai BM. Protocol: an updated integrated methodology for analysis of metabolites and enzyme activities of ethylene biosynthesis. Plant Methods. 2011; 7:17. https://doi.org/10.1186/1746-4811-7-17

Chayapakdee P, Sunohara Y, Endo M, Yamaguchi T, Fan L, Uchino A, et al. Quinclorac resistance in *Echinochloa phyllopogon* is associated with reduced ethylene synthesis rather than enhanced cyanide detoxification by β-cyanoalanine synthase. Pest Manag Sci. 2019;76(4):1195–1204. https://doi.org/10.1002/ps.5660

Chon NM, Nishikawa-Koseki N, Takeuchi Y, Abe H. Role of ethylene in abnormal shoot growth induced by high concentration of brassinolide in rice seedlings. Pestic Sci. 2008;712250003. https://doi.org/10.1584/jpestics.G07-20

Damalas CA, Dhima KV, Eleftherohorinos IG. Bispyribac-sodium efficacy on early watergrass (*Echinochloa oryzoides*) and late watergrass (*Echinochloa Phyllopogon*) as affected by coapplication of selected rice herbicides and insecticides. Weed Technol. 2008;22(4):622–627. https://doi.org/10.1614/WT-08-033.1

Fischer AJ, Ateh CM, Bayer DE, Hill JE. Herbicide-resistant *Echinochloa oryzoides* and *E. phyllopogon* in California *Oryza sativa* fields. Weed Sci. 2000;48(2):225–230. https://doi.org/10.1614/0043-1745(2000)048[0225:HREOAE]2.0.CO;2

Gao Y, Li J, Pan X, Liu D, Napier R, Dong L. Quinclorac resistance induced by the suppression of the expression of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase genes in *Echinochloa crus-galli* var. *zelayensis*. Pestic Biochem Phys. 2018;146:25–32. https://doi.org/10.1016/j.pestbp.2018.02.005

Gao Y, Pan L, Sun Y, Zhang T, Dong L, Li J. Resistance to quinclorac caused by the enhanced ability to detoxify cyanide and its molecular mechanism in *Echinochloa crus-galli* var. *zelayensis*. Pestic Biochem Phys. 2017;143:231–238. https://doi.org/10.1016/j.pestbp.2017.08.009

Grossmann K, Scheltrup F. Selective induction of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity is involved in the selectivity of the auxin herbicide quinclorac between barnyard grass and rice. Pestic Biochem Phys. 1997;58(2):145–153. https://doi.org/10.1006/pest.1997.2290

Heap I. International survey of herbicide resistant weeds. 2021. Available at: http//www.weedscience.org (accessed 05-05-2021).

Ikegaya H, Iwase H, Hatanaka K, Sakurada K, Yoshida K, Takatori T. Diagnosis of cyanide intoxication by measurement of cytochrome c oxidase activity. Toxicol Lett. 2001;119:117–123. https://doi.org/10.1016/S0378-4274(00)00297-6

Iwakami S, Endo M, Saika H, Okuno J, Nakamura N, Yokoyama M. et al. Cytochrome P450 CYP81A12 and CYP81A21 are associated with resistance to two acetolactate synthase inhibitors in *Echinochloa phyllopogon*. Plant Phys. 2014;165(2):618–629. https://doi.org/10.1104/pp.113.232843

Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc. 2015;10:845–858. https://doi.org/10.1038/nprot.2015.053

Lai KW, Yau CP, Tse YC, Jiang L, Yip WK. Heterologous expression analyses of rice *OsCAS* in *Arabidopsis* and in yeast provide evidence for its roles in cyanide detoxification rather than in cysteine synthesis in vivo. J Exp Bot. 2009;60(3):993–1008. https://doi.org/10.1093/jxb/ern343

LeClere S, Wu C, Westra P, Sammons RD. Cross-resistance to dicamba, 2, 4-D, and fluroxypyr in *Kochia scoparia* is endowed by a mutation in an AUX/IAA gene. PNAS. 2018;115(13): E2911–E2920. https://doi.org/10.1073/pnas.1712372115

Li G, Wu S, Cai L, Wang Q, Zhao X, Wu C. Identification and mRNA expression profile of glutamate receptor-like gene in quinclorac-resistant and susceptible *Echinochloa crus-galli*. Gene. 2013;531(2):489–495. https://doi.org/10.1016/j.gene.2013.09.013

Malik MS, Burgos NR, Talbert RE. Confirmation and control of propanil-resistant and quinclorac-resistant barnyardgrass (*Echinochloa crus-galli*) in rice. Weed Technol. 2010;24(3):226–233. doi:10.1614/WT-09-053.1

Mdodana NT, Jewell JF, Phiri EE, Smith ML, Oberlander K, Mahmoodi S, et al. Mutations in glucan, water dikinase affect starch degradation and gametophore development in the moss *Physcomitrella patens*. Sci Rep. 2019;9:15114. https://doi.org/10.1038/s41598-019-51632-9

Wu J, De Paz A, Zamft BM, Marblestone AH, Boyden ES, Kording KP, et al. DNA binding strength increases the processivity and activity of a Y-Family DNA polymerase. Sci Rep. 2017;7:4756. https://doi.org/10.1038/s41598-017-02578-3

Xu J, Lv B, Wang Q, Li J, Dong L. A resistance mechanism dependent upon the inhibition of ethylene biosynthesis. Pest Manag Sci. 2013;69(12):1407–1414. https://doi.org/10.1002/ps.3524

Yang X, Han H, Cao J, Li Y, Yu Q, Powles SB. Exploring quinclorac resistance mechanisms in *Echinochloa crus-pavonis* from China. Pest Manag Sci. 2021;77(1):194–201. https://doi.org/10.1002/ps.6007

Yi H, Juergens M, Jez JM. Structure of soybean β-cyanoalanine synthase and the molecular basis for cyanide detoxification in plants. Plant Cell. 2012;24(6): 2696–2706. https://doi.org/10.1105/tpc.112.098954

Zia Ul Haq M, Zhang Z, Wei J, Qiang S. Ethylene biosynthesis inhibition combined with cyanide degradation confer resistance to quinclorac in *Echinochloa crus-galli* var. *Mitis*. Int J Mol Sci. 2020;21(5):1573. https://doi.org/10.3390/ijms21051573