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CROSS RESISTANCE OF FENOXAPROP-P-ETHYL RESISTANT *PHALARIS MINOR* RETZ. TO CLODINAFOP-PROPARGYL: UNDERSTANDING PHYSIOLOGICAL AND MOLECULAR MECHANISM OF ACCase RESISTANCE

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Abstract

Spread of herbicide resistant *Phalaris minor* is a key issue for sustainable wheat production around the globe. The response of fenoxaprop-p-ethyl resistant *P. minor* biotypes to clodinafop-propargyl was evaluated using the classical bioassay. Results revealed fenoxaprop-p-ethyl resistant *P. minor* biotypes (PM FSD 1 and PM MBD 5) have developed cross resistance to clodinafop-propargyl. LD50 values of cross-resistant biotypes were 57.18 and 90.70 g a.i. ha^{-1} compared to the susceptible biotype (25.76 g a.i. ha^{-1}). The level of cross-resistance for PM FSD 1 and PM MBD 5 was 2.22 and 3.52. Physiological and molecular mechanisms of ACCase resistance in *P. minor* were studied by extracting ACCase enzyme followed by spectro-photometric assay and DNA extraction followed by SDS-PAGE and western blotting, respectively. The results revealed that more ACCase activity (2.49 nmol HCO^{-3} mg^{-1} protein min^{-1}) was recorded in PM MBD 5 followed by PM FSD 1 (resistant biotypes). In contrast, less ACCase activity (0.95 nmol HCO^{-3} mg^{-1} protein min^{-1}) was observed in susceptible biotypes. All the resistant biotypes had many folds more fenoxaprop-p-ethyl dose needed to inhibit 50% ACCase activity compared to susceptible ones. Among resistant biotypes, PM MBD 5 had the highest I50 value (9.35µM) and maximum protein concentration (0.69 mg ml^{-1}). Translation of the DNA sequence to the amino acid sequence showed that susceptible biotype had tryptophan (Trp, W) amino acid while resistant biotypes had cysteine (Cys, C) amino acid. Cysteine amino acid gave the resistance against ACCase-inhibiting herbicides. Confirmation of cross-resistance and novel insights into the physiological mechanisms of ACCase resistance will help to develop effective strategies for sustainable management of *P. minor*.

Key words: ACCase activity; ACCase-inhibiting herbicides; Classical assay; Cysteine amino acid; Resistance mechanism

Introduction

Phalaris minor Retz. is widely distributed in world's wheat grown areas and has become dominant mimicking weed after introduction of semi-dwarf wheat cultivars (Gherekhloo *et al*., 2021). It is very competitive and aggressive weed of wheat fields causing severe yield losses, even completely destroying wheat crop at high density (2000 plants m^{-1}) (Soni *et al*., 2023). Chemical control remains the most efficient and economical method to manage *P. minor*. However, in recent years, herbicide resistance has emerged as major issue for effective weed control in Pakistan and has gained considerable attention from weed scientists (Abbas *et al*., 2016; Raza *et al*., 2021; Abbas *et al*., 2024; Aslam *et al*., 2024). The rapid development and spread of herbicide resistance in *P. minor* and *Avena fatua* in Pakistan have made weed control in wheat more complicated (Abbas *et al*., 2016; Aslam *et al*., 2024). *P. minor* was efficiently managed with urea herbicide isoproturon till early 1990s, however lately unceasing and substantial spraying for longer periods, with low application rates, inefficient method and spray timing led to the development of isoproturon resistance in *P. minor* (Chhokar & Malik, 2002). Alternate herbicides including sulfosulfuron, clodinafop propargyl, fenoxaprop-P-ethyl and pinoxaden were utilized to control *P. minor* in wheat fields.

At present, *P. minor* has developed resistance against different herbicides around the globe, including multiple resistance against three modes of actions (Gherekhloo *et al*., 2021). Resistance to herbicides in weeds evolves through a complicated process influenced by various factors including the genetics and biology of the weeds, as well as herbicidal, operational, and other biological components, all of which shape its dynamics and impact (Vencill *et al*., 2012). Currently, *P. minor* has developed resistance against different herbicides in Australia, India, Iran, Israel, Mexico, Pakistan, South Africa and United States (Heap, 2025). Clodinafop propargyl got more popularity in growers due to its good efficiency to control *P. minor* and rare toxicity on wheat crop. The complete dependence on clodinafop propargyl resulted in development of resistance in *P. minor*. After first report of evolution of fenoxaprop-p-ethyl resistant *P. minor*

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from Pakistan in 2016 (Abbas *et al*., 2016a), clodinafop-propargyl was started as alternative herbicide to control *P. minor* in wheat fields.

The resistance might be due increased ACCase enzyme expression as a target site mechanism in weed biotype. This physiological process confers resistance in weeds to number of herbicides (Bo *et al*., 2017). An increased activity of ACCase has been recognized as the resistance mechanism in different weed species (Kaundun, 2014; Takano *et al*., 2020). This study was performed to check the cross-resistance status of fenoxaprop-p-ethyl resistance in *P. minor* to clodinafop-propargyl after confirmation of fenoxaprop-p-ethyl resistance. Further, novel insights into the physiological mechanisms of ACCase resistance in *P. minor*, previously unexamined, have been clarified.

Material and Methods

Bioassay for confirmation of cross resistance in *P. minor* biotypes: Pot experiment was performed twice at wire house, College of Agriculture, University of Sargodha, Sargodha, Punjab, Pakistan. The seeds of fenoxaprop-p-ethyl resistant *P. minor* biotypes were used. The susceptible *P. minor* seeds were collected from non-cultivated area having no history of application of clodinafop-propargyl. Experiment comprised of two factors one was *P. minor* biotypes (resistant biotypes and susceptible standard) other was different doses of clodinafop-propargyl that were [27.5 g a.i. ha^{-1} (0.5X), 55 g a.i. ha^{-1}(1X) and 110 g a.i. ha^{-1}(2X)].

The wire house experiment was designed in completely randomization (CRD) with 4 replications. Twenty-one days after emergence (at the 3–4 leaf stage), *Phalaris minor* plants (5 plants per pot) were treated with commercial formulation of clodinafop-propargyl (Topik 240EC) as per treatment. Three weeks after spraying, mortality percentage was recorded using 0-100% rating scale where 0 indicates no effect of treatment and 100 complete control. On the basis of mortality percentage, LD_{50} and resistance level in the form of resistant index were determined (Abbas *et al*., 2016a). The alive plants were uprooted and oven-dried at 70°C till the constant weight was achieved. The data regarding dry biomass has been expressed in percentage of the control (Gherekhloo *et al*., 2012).

Mortality % data were subjected to probit analysis by using inverse prediction of logistic 3P in JMP 13 to calculate the herbicide rate necessary to kill 50% of (LD50) of each biotype.

$$LD50 = c/(1+exp\{-a(dose-b)\})$$

where a is growth rate, b is an inflection point, c is an asymptote.

Resistance level of various resistant *P. minor* biotypes is presented as resistance index (RI), it was determined as ratio of LD_{50} of resistant biotype by the LD_{50} of the susceptible biotype (Abbas *et al*., 2016a).

Studying the physiological mechanism of ACCase resistance in *P. minor*

Experimental detail: This experiment was executed twice under completely randomized design in plastic pots (13×10×6 cm) under wire house condition. Nine *P. minor* biotypes (PM MBD 1, PM MBD 2, PM MBD 4, PM MBD 5, PM FSD 2, PM SGD 1, PM FSD 1, PM NS, PM SH) resistant to fenoxaprop-p-ethyl along with susceptible were used in this study. Soil taken from field was air-dried and farmyard manure was also mixed in 2:1 w/w. Seeds of each biotype were sown uniformly in pots and covered with light layer of soil (1 cm depth). The photoperiod of wire house was ten hours. Fenoxaprop-p-ethyl (Puma super 750 EW) at 67.5 g a.i. ha^{-1} was sprayed after 3 weeks of *P. minor* sowing at 3-4 leave stage. The leave samples of biotypes were harvested at three weeks after herbicide application and were used for extraction of enzyme and ACCase activity. Observations including ACCase activity (nmol HCO^{-3} mg^{-1} protein min^{-1}), fenoxaprop-p-ethyl dose needed to inhibit 50% ACCAse activity (I_{50} µM) and protein concentration of *P. minor* biotypes were recorded.

Enzyme extraction: The ACCase enzyme extraction was done by using the procedure of De Prado *et al*., (2005). Three weeks after herbicide application, fresh leaves weighing 6 g were harvested from each biotype. The leaves were ground using liquid N in a mortar and then 24 mL buffer (pH 7.5) was added. The suspension was mixed and kept for 10 min on magnetic stirrer. After 10 min it was filtered sequentially using 4 layers of cheese cloth. Then extract was centrifuged at 24000 *g* for 20 min. The resulted material was fractionated using ammonium sulphate. The fraction of protein containing ACCase was undergone for precipitation. The resulted supernatant was discarded, and pellet was again suspended in buffer (1 mL). PD-10 column was used for re-suspension. The resultant protein extract was instantly taken to chamber to measure activity of ACCase enzyme (Gherekhloo *et al*., 2012).

Spectro-photometric assay: ACCase activity was determined by calculating the ADP production for NADH oxidation by PK and LDH (Rendina *et al*., 1988). In a spectrophotometer, the changes in A340 for 5 min. at 34°C were recorded. The mixture of standard reaction comprised of 0.5 mM acetyl-COA, 0.4 M glycerol, 0.1 M Tricine-KOH, 5 mM $MgSO_4$, 0.5 mM DTT, 2.5 m~ ATP, 0.5 m~ PEP, 15 mM $NaHCO_3$, 1.5 units of LDH, 1.25 units of PK, 0.32 mM NADH 50 mM KCI, pH 8.3 and Mono Q-purified ACCase at 0.025 mL. LDH and PK were used and background activity was recorded during first 5 min. To start the reaction, Acetyl-COA was poured and kept for 5 min. For each assay, background activity <5% was deducted from rate of reaction. All assays remained linear with enzyme concentration and time. The enzyme quantity which catalyzes carboxylation of 1 p mol acetyl COA is called enzyme activity (Gherekhloo *et al*., 2012).

Studying molecular mechanism of ACCase resistance in *P. minor*

Experimental detail: All the resistant biotypes (PM SGD 1, PM FSD 1, PM FSD 2, PM MBD 1, PM MBD 2, PM MBD 4, PM MBD 5, PM NS and PM SH) and susceptible

were grown in wire house using plastic pots (13×10×6 cm). Soil used in experiment was air-dried, mixed with farmyard manure (2:1 w/w) pots were filled. Seeds were sown at 1 cm depth near to surface of pots. Approximate photoperiod at experimental site was 10 h. After 21 days of sowing, at 3-4 leave stage, plants were sprayed with fenoxaprop-p-ethyl (Puma super 750 EW) at 67.5 g a.i. ha^{-1} (field dosage). After three weeks of herbicide application, the leaf samples were collected for ACCase resistance. DNA was extracted from the plant samples and PCR was run to study the genetic variability.

DNA extraction: Fresh leaves of *P. minor* were used for extraction of genomic DNA. The DNA was quantified immediately by using PCR during spring season. To intensify region in CT domain, two primers were used to confer ACCase sensitivity against herbicides. PCR was initiated in a 25-µL, which comprised of genomic DNA (300 ng), 0.5 l M primer and 12.5 µL of 2×GoTaq Green Master mix. The PCR was initiated for 4 min at 94°C in a thermo cycler and then at 94°C for 30 seconds (35 cycles). Further it was run at 48°C and 72°C for 30 seconds. From agarose gel, PCR products were purified and used (Gherekhloo *et al*., 2012).

SDS-PAGE and western blotting: PhastGels 4 to 15% (w/v) was used to separate purified and crude fractions of ACCase. Before electrophoresis, sample buffer was used to dilute (2-fold) the samples and then kept for boiling for 2 mins. On SDS-PAGE, the distance covered by susceptible and resistant ACCases was calculated by regression analysis with respect to distance covered by biotinylated protein standard (high molecular weight) and ferritin of horse spleen. Proteins, which were present on PhastGels, moved to Immobilon P through capillary movement (Braun & Abraham, 1989). Buffer comprised of NaCl (50 m) and 3 (cyclohexylamino)-l- propanesulfonic acid with pH of 11. Polysorbate 20 and tris-buffered saline were used for blots to block for one night at normal temperature. Avidin dilution (1:4000) was mixed to solution and blots were further agitated for next two hours at 25°C. The blots were rinsed and washed for 10 mins in tris-buffered saline and Tween 20. Nitroblue tetrazolium and chromogenic substrate were used to detect AP activity (Blake *et al*., 1984). The blots were air dried after washing with water (Gherekhloo *et al*., 2012). SDS-PAGE is an electrophoresis method which separate protein contents based on their mass. The medium is a polyacrylamide-based discontinuous gel. In addition, SDS (sodium dodecyl sulfate) is used. About 1.4 grams of SDS bind to a gram of protein, corresponding to one SDS molecule per two amino acids. SDS-PAGE was done to confirm the required protein (ACCase) on molecular weight basis.

Results

Bioassay for confirmation of cross resistance in *P. minor* biotypes

Mortality (%), dry biomass reduction, LD50 and RI of *P. minor* biotypes: Fenoxaprop-p-ethyl resistant biotypes (PM FSD 1 and PM MBD 5) showed difference in percent mortality. Maximum mortality (100%) for both biotypes was noted at 2X dose of clodinafop while lowest mortality was recorded in PM FSD 1 (22%) and PM MBD 5 (14%) at 0.5X. While susceptible biotype showed 79% mortality at 0.X and 100% at 1X (Fig. 1).

Cross resistant biotypes showed less reduction in dry biomass (34 to 100%) of *P. minor*, while more dry biomass reduction in susceptible biotype was observed (91 to 100%). LD_{50} values of cross-resistant biotypes PM FSD 1 and PM MBD 5 were calculated 57.18 and 90.70 g a.i. ha^{-1} (Table 1). While the value of LD_{50} for susceptible biotype was 25.76 g a.i. ha^{-1}. Resistant index of PM FSD 1 and PM MBD 5 were recorded 2.22 and 3.52.

Studying the physiological mechanism of ACCase resistance in *P. minor*

ACCase activity (nmol HCO^{-3} mg^{-1} protein min^{-1}): Data regarding ACCase activity of different biotypes of *P. minor* revealed the higher ACCase activity in resistant biotypes compared to susceptible biotype (Fig. 2). Among resistant biotypes, maximum ACCase activity (2.49 nmol HCO^{-3} mg^{-1} protein min^{-1}) was recorded in PM MBD 5 followed by PM FSD 1. While minimum ACCase activity (0.95 nmol HCO^{-3} mg^{-1} protein min^{-1}) was recorded in susceptible biotype. Among resistant biotypes, minimum ACCase activity was recorded in PM FSD 2.

Fenoxaprop-p-ethyl dose needed to inhibit 50% ACCAse activity (I_{50} µM): Data regarding the fenoxaprop-p-ethyl dose needed to inhibit 50% ACCase activity are given (Fig. 3). The data showed that all the resistant biotypes had many folds more fenoxaprop-p-ethyl dose needed to inhibit 50% ACCase activity compared to susceptible. Among resistance biotypes, PM MBD 5 had highest I_{50} value (9.35µM) followed by PM FSD 1 while minimum I_{50} (0.75 µM) was recorded in susceptible biotype. However, among resistant biotypes, minimum I_{50} was documented in PM FSD 2 and PM SGD 1 (7.21 and 7.23 µM, respectively).

Table 1. Percent dry biomass reduction, LD50 and resistance level of different *Echinochloa colona* populations against bispyribac-sodium in dose-response bioassay.

P. minor biotypes	Clodinafop-propargyl doses (g a.i. ha^{-1})				LD50[a]	Resistance level ([b]RL)
	0	0.5X (27.5)	1X (55)	2X (110)		
PM FSD 1	0	40	80	100	57.1856	2.22
PM MBD 5	0	34	58	100	90.7042	3.52
Susceptible	0	91	100	100	25.7645	0

[a]LD_{50} was determined with the Quest Graph™ LD50 Calculator. [b]RL was calculated by dividing the LD50 dose (g a.i. ha^{-1}) of resistant biotype by the LD50 dose of susceptible biotype

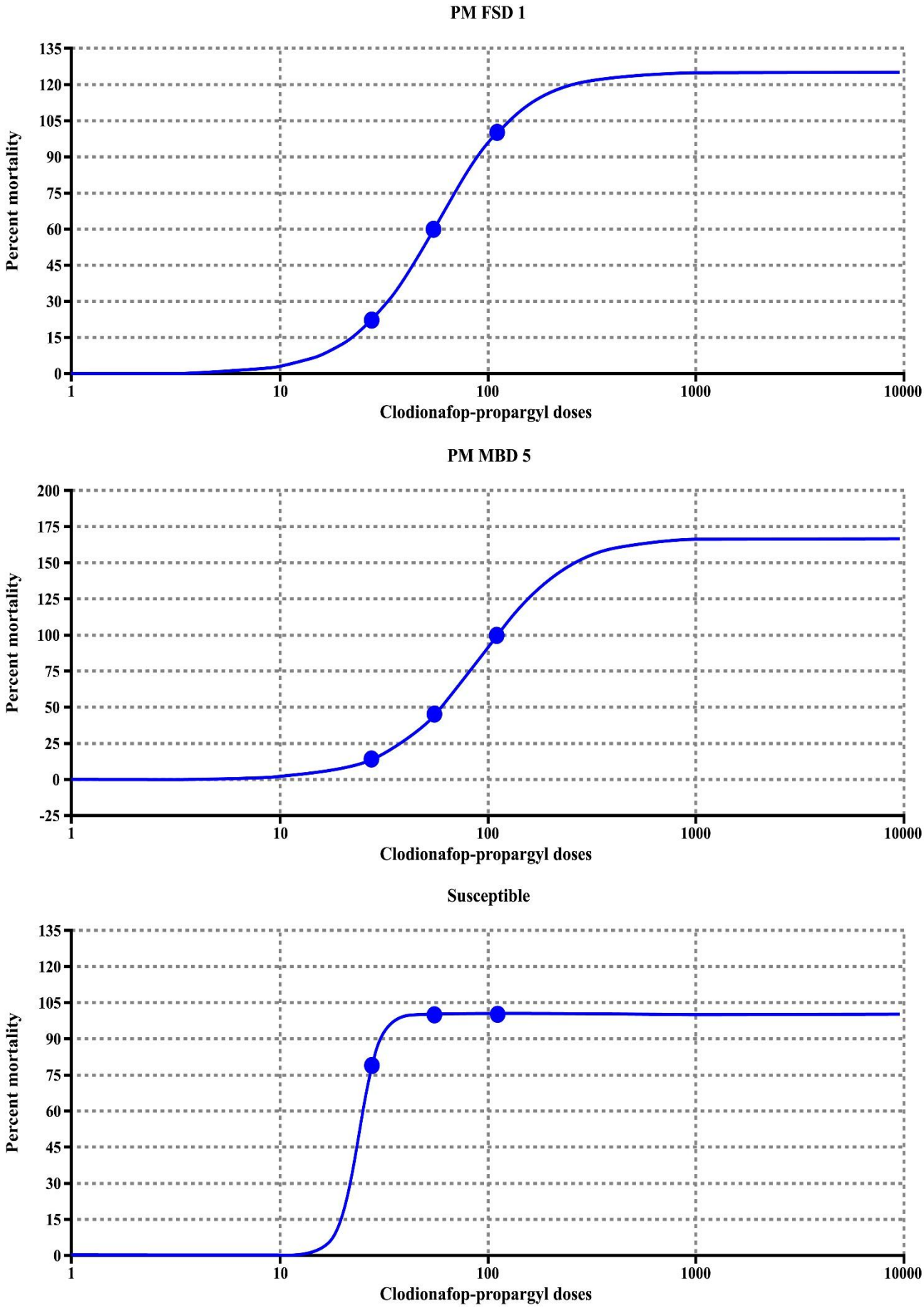

Fig. 1. Mortality (%) of fenoxaprop-p-ethyl resistant and susceptible *P. minor* biotypes, after application of different doses of clodinafop-propargyl, 18 days after spray.

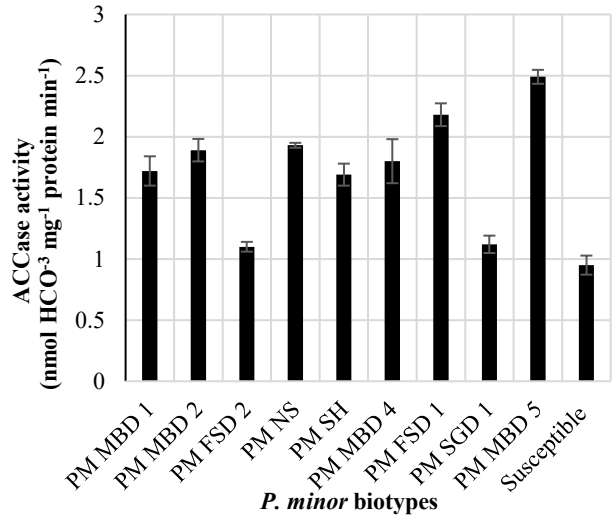

Fig. 2. ACCase activity of fenoxaprop-p-ethyl resistant and susceptible biotypes of *P. minor*. Vertical bars represent standard errors of four replicates.

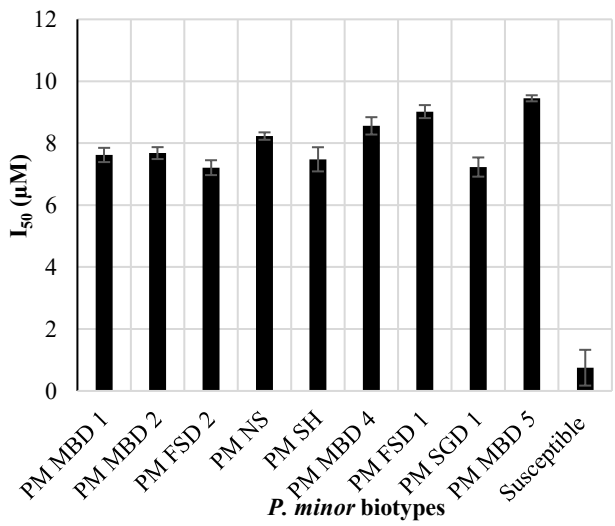

Fig. 3. I_{50} of fenoxaprop-p-ethyl resistant and susceptible biotypes of *P. minor.* Vertical bars represent standard errors of four replicates.

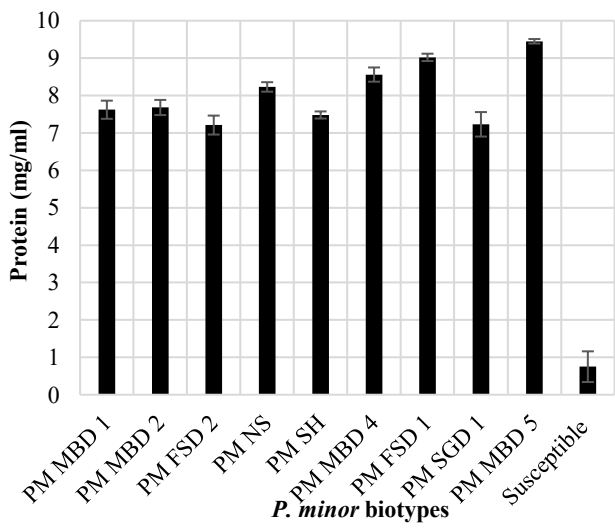

Fig. 4. Protein concentration in fenoxaprop-p-ethyl resistant and susceptible biotypes of *P. minor.* Vertical bars represent standard errors of four replicates.

Protein concentration of *P. minor* biotypes: Data pertaining to protein analysis of susceptible and resistant biotypes revealed that all the resistant biotypes had more protein concentration compared to susceptible (Fig. 4). Maximum protein concentration was observed in PM MBD 5 and PM FSD 1 (0.69 and 0.65 mg/ml, respectively) while minimum was noted in susceptible biotype (0.14 mg/ml). Whereas from resistant biotypes, minimum protein concentration was recorded for PM SH (0.21 mg/ml) followed by PM FSD 2 (0.31 mg/ml).

SDS-PAGE: The results indicating that a significant amount of ACCase is present in the samples. Which can be seen at 70 Kda (Killo Dalton) molecular weight. As 70 Kda is the molecular weight of ACCase (Fig. 5).

Studying molecular mechanism of ACCase resistance in *P. minor*: Translation of the DNA sequence to the amino acid sequence showed that there was mutation in resistant biotypes compared to susceptible biotype (Fig. 6). All the resistant biotypes showed changes or mutation in DNA sequence at two locations. All the (9) resistant biotypes had same mutation in their DNA sequence. If we see the DNA in figure 6, susceptible biotype had tryptophan (Trp, W) amino acid while resistant biotypes had cysteine (Cys, C) amino acid. Cysteine amino acid gave the resistance against ACCase-inhibiting herbicides.

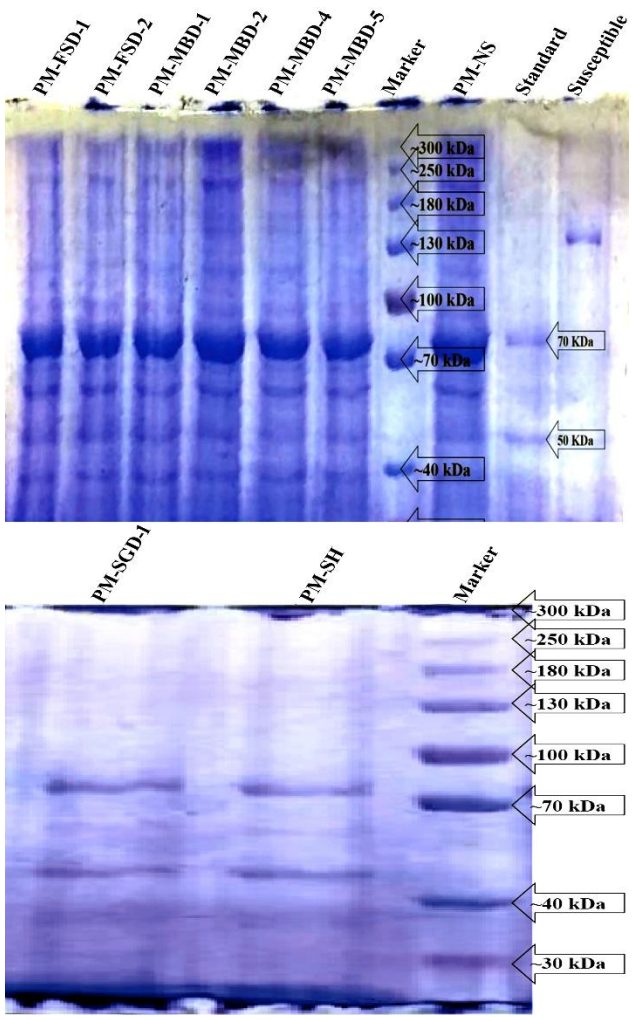

Fig. 5. SDS-PAGE image indicating a significant amount of ACCase is present in the samples. It can be seen at 70 Kda (Killo Dalton) molecular weight which is the molecular weight of ACCase.

(DNA Regions in Primer B)	
PM-FSD-1	ACTTCAACCGTGAAGGTTTACCTCTGTTCATCCTTGCTAAC**TGC**AGAGGC
PM-FSD-2	ACTTCAACCGTGAAGGTTTACCTCTGTTCATCCTTGCTAAC**TGC**AGAGGC
PM-MBD-1	ACTTCAACCGTGAAGGATTACCTCTGTTCATCCTTGCTAAC**TGC**AGAGGC
PM-MBD-2	ACTTCAACCGTGAAGGTTTACCTCTGTTCATCCTTGCTAAC**TGC**AGAGGC
PM-MBD-4	ACTTCAACCGTGAAGGGTTACCTCTGTTCATCCTTGCTAAC**TGC**AGAGGC
PM-MBD-5	ACTTCAACCGTGAAGGATTACCTCTGTTCATCCTTGCTAAC**TGC**AGAGGC
PM-NS	ACTTCAACCGTGAAGGTTTACCTCTGTTCATCCTTGCTAAC**TGC**AGAGGC
PM-SGD-1	ACTTCAACCGTGAAGGGTTACCTCTGTTCATCCTTGCTAAC**TGC**AGAGGC
PM-SH	ACTTCAACCGTGAAGGATTACCTCTGTTCATCCTTGCTAAC**TGC**AGAGGC
Susceptible	ACTTCAACCGTGAAGGTTTACCTCTGTTCATCCTTGCTAAC**TGG**AGAGGC
(Protein for primer B)	
PM-FSD-1	GQVWFPDSATKTAQAMLDFNREGLPLFILAN**C**RGFSGGQRDLFEGILQAG
PM-FSD-2	GQVWFPDSATKTAQAMLDFNREGLPLFILAN**C**RGFSGGQRDLFEGILQAG
PM-MBD-1	GQVWFPDSATKTAQAMLDFNREGLPLFILAN**C**RGFSGGQRDLFEGILQAG
PM-MBD-2	GQVWFPDSATKTAQAMLDFNREGLPLFILAN**C**RGFSGGQRDLFEGILQAG
PM-MBD-4	GQVWFPDSATKTAQAMLDFNREGLPLFILAN**C**RGFSGGQRDLFEGILQAG
PM-MBD-5	GQVWFPDSATKTAQAMLDFNREGLPLFILAN**C**RGFSGGQRDLFEGILQAG
PM-NS	GQVWFPDSATKTAQAMLDFNREGLPLFILAN**C**RGFSGGQRDLFEGILQAG
PM-SGD-1	GQVWFPDSATKTAQAMLDFNREGLPLFILAN**C**RGFSGGQRDLFEGILQAG
PM-SH	GQVWFPDSATKTAQAMLDFNREGLPLFILAN**C**RGFSGGQRDLFEGILQAG
Susceptible	GQVWFPDSATKTAQAMLDFNREGLPLFILAN**W**RGFSGGQRDLFEGILQAG
(DNA Regions in Primer I)	
PM-FSD-1	AC**TGC**AGAGGCTTCTCTGGTGGGCAAAGAGATCTTTTTGAAGGAATTCTG
PM-FSD-2	AC**TGC**AGAGGCTTCTCTGGTGGGCAAAGAGATCTTTTTGAAGGAATTCTG
PM-MBD-1	AC**TGC**AGAGGCTTCTCTGGTGGGCAAAGAGACCTTTTTGAAGGAATTCTG
PM-MBD-2	AC**TGC**AGAGGCTTCTCTGGTGGGCAAAGAGACCTTTTTGAAGGAATTCTG
PM-MBD-4	AC**TGC**AGAGGCTTCTCTGGTGGGCAAAGAGACCTTTTTGAAGGAATTCTG
PM-MBD-5	AC**TGC**AGAGGCTTCTCTGGTGGGCAAAGAGACCTTTTTGAAGGAATTCTG
PM-NS	AC**TGC**AGAGGCTTCTCTGGTGGGCAAAGAGACCTTTTTGAAGGAATTCTG
PM-SGD-1	AC**TGC**AGAGGCTTCTCTGGTGGGCAAAGAGACCTTTTTGAAGGAATTCTG
PM-SH	AC**TGC**AGAGGCTTCTCTGGTGGGCAAAGAGACCTTTTTGAAGGAATTCTG
Susceptible	AC**TGG**AGAGGCTTCTCTGGTGGGCAAAGAGACCTTTTTGAAGGAATTCTG
(Protein for primer I)	
PM-FSD-1	**C**RGFSGGQRDLFEGILQAGSTIVENLRTYNQPAFVYIPKAAELRGG
PM-FSD-2	**C**RGFSGGQRDLFEGILQAGSTIVENLRTYNQPAFVYIPKAAELRGG
PM-MBD-1	**C**RGFSGGQRDLFEGILQAGSTIVENLRTYNQPAFVYIPKAAELRGG
PM-MBD-2	**C**RGFSGGQRDLFEGILQAGSTIVENLRTYNQPAFVYIPKAAELRGG
PM-MBD-4	**C**RGFSGGQRDLFEGILQAGSTIVENLRTYNQPAFVYIPKAAELRGG
PM-MBD-5	**C**RGFSGGQRDLFEGILQAGSTIVENLRTYNQPAFVYIPKAAELRGG
PM-NS	**C**RGFSGGQRDLFEGILQAGSTIVENLRTYNQPAFVYIPKAAELRGG
PM-SGD-1	**C**RGFSGGQRDLFEGILQAGSTIVENLRTYNQPAFVYIPKAAELRGG
PM-SH	**C**RGFSGGQRDLFEGILQAGSTIVENLRTYNQPAFVYIPKAAELRGG
Susceptible	**W**RGFSGGQRDLFEGILQAGSTIVENLRTYNQPAFVYIPKAAELRGG

Fig. 6. Multiple alignments of different biotypes using T-Coffee.
Bold letters show amino acids having mutations linked with ACCase herbicide resistance
The protein (amino acid) sequence is given because it confirms the meaningful mutation. As the DNA have many mutations but all were not meaningful. As a meaningful change that change the amino acid at the given position is that when nitrogeneous base is changed at the 3rd place of a codon

Discussion

Results confirm the cross resistance in fenoxaprop-p-ethyl resistant *P. minor* biotypes to clodinafop-propargyl. After confirmation of fenoxaprop-p-ethyl resistance in *P. minor* in 2015 (Abbas *et al*., 2016a), farmers started using clodinafop-propargyl in wheat filed to control *P. minor*. Continued use of same herbicide for long time imposes the herbicide selection pressure which leads to development of resistance (Owen *et al*., 2007). Wheat-rice cropping system is a signal dominant cropping system in most of the wheat growing areas in Pakistan. Absence of crop rotation and dependency on same herbicide for several years, cause herbicide resistance (Travlos & Chachalis, 2010; Abbas *et al*., 2016a). *Phalaris minor* has higher ability of seed producing, mature earlier than wheat and seed viability favored by anaerobic rice conditions that were also contributing factors to enhance resistant seed proportion in soil-seed bank to enhance the supremacy of resistant *P. minor* (Yasin & Iqbal, 2011). The variation in resistance levels among collected biotypes may stem from distinct evolutionary pressures exerted by herbicides. This differentiation is likely influenced by the diverse origins of different biotypes of *P. minor*, each potentially having a

unique history of exposure to both wheat cultivation and herbicide use. Travlos *et al*., (2011) noted that fields from various locations necessitated varying approaches to weed control, encompassing both herbicidal and non-herbicidal methods. Additionally, the disparity in resistance levels could also be attributed to the presence of various resistance mechanisms within *P. minor* biotypes and other weed species. These mechanisms may include alterations in target sites, enhance metabolic processes, the presence of modified ACCase, compartmentalization, or increased expression of the target protein (Gherekhloo *et al*., 2011; Travlos *et al*., 2011; Gherekhloo *et al*., 2021).

Data on ACCase activity, I_{50} and protein concentration (Figs. 2, 3 and 4) revealed that on target enzyme level, the resistant biotypes of *P. minor* illustrated a higher degree of resistance to fenoxaprop-p-ethyl. This resistance might be because of less sensitivity to ACCase to this herbicide and some changes in amino acids at carboxyl transferase (CT) domain. Several studies showed that resistance in grassy weeds against herbicides was linked with decreased sensitivity of their target enzyme. A change in single amino acid in CT domain develops the target site resistance, which decreases or inactive the binding site of enzyme to herbicides. There is no place of binding between herbicides and ACCase enzyme due to continuous application of ACCase-inhibiters (Takano *et al*., 2020). It is detected in grassy weeds that they have seven different mutations which are responsible for resistance. Out of seven, two Ile-1781-Leu and Asp-2078-Gly substitutions were detected in resistant biotypes of *P. minor*. These substitutions can cause resistance against CHD and APP herbicides (Gherekhloo *et al*., 2012; Takano *et al*., 2020). Gherekhloo *et al*., (2011) depicted that modification in ACCase enzyme was liable for resistance. Continuous application of ACCase inhibiting herbicides imposed strong selection pressure which lead to enhanced ACCase specific activity. This increased protein expression continues the production of fatty acids even at recommended lethal dose of herbicide and plants survive (Gherekhloo *et al*., 2020). Previously, higher ACCase specific activity in fenoxaprop-P-ethyl resistant *Leptochloa chinensis* has been reported as mechanism of resistance development in *L. chinensis* biotypes in Thailand (Pornprom *et al*., 2006). Powles & Holtum (1994) demonstrated that some resistant biotypes of wild oat (*Avena sterilis* L.), green foxtail (*Setaria viridis* L.), *Lolium* species (ryegrass) and *Sorghum halepense* (L.) had modification in ACCase. De Prado *et al*., (2005) confirmed changes in isoform of ACCase in *Lolium rigidum* population in Spain. Cha *et al*., (2014) reported similar basis for resistance development in *Eleusine indica* to ACCase-inhibiting herbicide from Malaysia.

Tryptophan (Trp, W) amino acid was detected in susceptible biotype while cysteine (Cys, C) amino acid was found in the resistant biotypes. This substitution might be responsible to confer resistance against ACCase-inhibiter herbicides. The outcomes are in line with those of Délye *et al*., (2005) who shared their initial report that changes in amino acid i.e. Trp-2027 to Cys substitution showed resistance in *Alopecurus myosuroides* (L.). According several other researchers revealed that this substitution is liable to initiate resistance against ACCase-inhibiters herbicides in different weeds (Délye *et al*., 2005; Zhu *et al*., 2009; Gherekhloo *et al*., 2012). Mutation in the sequence of DNA also modifies the target site against specific herbicide. The alteration in genes caused over expression of target enzyme (Powles & Yu, 2010; Saleem *et al*., 2025). Target-site mutation developed resistance in weeds against herbicides, which affected microtubule assembly, photosystem II (PSII) and some enzymes like ACCase, acetolactate synthase (ALS). Another possible reason for herbicide resistance is point mutation which results from alteration in single nucleotide in DNA sequence that encodes specific amino acid. This means that there is no availability of binding site and plants will have resistance against specific type of herbicides. There is a possibility that different biotypes are resistant to specific herbicide but their resistance is not by same mutation (Saleem *et al*., 2024). In current study, it seems this substitution might be related to fenoxaprop-p-ethyl resistance in *P. minor*. Powels & Yu (2010) and Beckie & Tardif (2012) designed a pair of primers on the basis of similar region of CT domain in ACCase sequence of *A. fatua*, *A. japonicus* and *A. myosuroides* to intensify 291 bp conserved in region, where 2 point mutations (Trp2027 to Cys and Ile2041 to Asn) conformed ACCase inhibitor resistance.

As the development of herbicides with novel mode of action is very rare, understanding resistance and the factors that contribute to its development are essential. Use of herbicide mixtures (Abbas *et al*., 2016b), narrow wheat row spacing (Abbas *et al*., 2018a) and allelopathic crop mulches (Abbas *et al*., 2018b) have been explored as effective strategies to control *P. minor* in wheat with reduced herbicide selection pressure. Further, enhanced efficacy of herbicide against *P. minor* with addition of adjuvant (Rizwan *et al*., 2018) and precise dose estimation based of leaf water content measurements (Abbas *et al*., 2025) can also be used to control resistant *P. minor* and to reduce herbicide selection pressure to delay resistance development in long run. Hence, integration of different chemical and non-chemical strategies is essential to control *P. minor* a troublesome weed for sustainable production of wheat and other winter crops.

Conclusion

Fenoxaprop-p-ethyl resistant *P. minor* biotypes have developed cross resistance with resistance level 2.22 and 3.52 to clodinafop-propargyl. Increased ACCase specific activity noted in the current study, might be possible reason for cross resistance to ACCase herbicides. Further, mutation in DNA sequence, susceptible biotypes had tryptophan (Trp, W) amino acid while resistant biotypes had cysteine (Cys, C) amino acid. Cysteine amino acid gave the resistance against ACCase-inhibiting herbicides.

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