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Characterization of Acetyl CoA carboxylase resistance in *Phalaris minor* populations of North-Western states of India

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Abstract

Adoption of rice-wheat monoculture in North-Western states of India ushers the major weed flora shift. *Phalaris minor* is an important grass weed of wheat and it has developed resistance towards most of the prevailing herbicide groups due to many reasons. To understand the mechanism of the resistance in different populations of *P. Minor* from Punjab, molecular studies were carried out. *Phalaris minor* populations which escaped the herbicide spray, samples from them were collected from the fields of 66 farmers from fourteen different districts of Punjab alongwith known susceptible and resistant check populations grown in pots and sprayed with all the recommended herbicides for the control of *P. minor* at the rates of 0.5 to 4X of the recommended dose of herbicide. Screening of *P. minor* populations was done for resistance studies. The common T to A nucleotide transversion in the region of the ACCase (Acetyl CoA carboxylase) gene is primarily involved in resistance and this region has been used in the current study using PCR-restriction digestion and sequencing of ACCase gene. The PCR amplification and restriction digestion assay yielded 3 types of alleles viz. 13 accessions with homozygous resistant allele (404 bp band), 41 accessions had homozygous sensitive alleles (169 and 235 bp band) whereas 12 accessions possessed heterozygous resistant alleles (169, 235 and 404 bp band). Sequencing and data analysis revealed the presence of homozygous sensitive alleles in more than 50% of *P. minor* populations. So, to prevent or delay of resistance development, site-specific and ecological-based agronomic inventions are needed.

Keywords: Resistance; Herbicides; *Phalaris minor*; Populations; ACCase

Introduction

Rice-wheat is the one of the major resource exploiting cropping systems of Indo-Gangetic plains of India comprises of Punjab, Haryana and western Uttar Pradesh (Kumar et al., 2018). Weed menace is not uncommon under present input intensive cultivation of short stature high yielding wheat varieties under Rice-wheat cropping system (Nakka et al., 2019; Ali F and Afzal I, 2026). Weeds pose a major yield threat by competing with crops for CO₂, space, water and squeezing major portion of applied nutrients and making crop deprived of them (Ahlawat et al., 2023; Soni et al., 2023). Weeds are the most harmful pests of wheat and contribute up to twenty four percent yield reductions amongst various biotic stresses (Jabran et al., 2017). Yield losses of wheat due to *Phalaris minor* alone are estimated from 25-50 per cent (Gharde et al., 2018; Meena et al., 2019). Under very severe infestation, the losses may be up to 80 per cent and sometimes crop failures (Kaur et al., 2021). Adoption of rice-wheat monoculture in North-Western states of India ushers the major weed flora shift (Weiskopf et al., 2020). *Phalaris minor* is an important grass weed of wheat (Nandan et al., 2020; Weisberger et al., 2019) and it has developed resistance towards the most of the prevailing herbicide groups (Heap, 2026; Kaur et al., 2024) due to many reasons like repeated and continuous use of same group of herbicides, with same mode of action, lack of awareness regarding use of herbicides and proper spray technology among the farmers, use of either suboptimal or higher doses of herbicides (Puniya et al., 2023; Nath et al., 2024), usage of un-recommended herbicidal



tank mixtures and due to unavailability of background study for site specific herbicide recommendation, etc; has led to the problem of *P. minor* as notorious weed of wheat that causes huge yield reductions (Gharde et al., 2025). The acetyl coenzyme-A carboxylase (ACCase) inhibitors, acetolactate synthase (ALS) inhibitors and synthetic auxins are the prevailing group of herbicides used for weed control in wheat and throughout the world 21, 57 and 23 active ingredients are available, respectively (Heap 2016). The ALS inhibitors provided control of both grass and broad-leaved weeds, while the ACCase inhibitors only provide control for grass weeds whereas, the synthetic auxins only provided control for broadleaved weeds. Presently, *P. minor* has evolved multiple herbicide resistance across three modes of action, acetyl CoA carboxylase (ACCase), photosynthesis at the photosystem II site A, and acetolactate synthase (ALS) inhibitors (Heap, 2026; Kaur et al., 2022).

At the global level, there are five hundred and nine unique cases (species × site of action) of herbicide-resistant weeds and many weed species already have evolved resistance to one or more herbicides. Currently, resistance to 168 different herbicides has been reported worldwide (Heap, 2026). Yadav *et al.* (2002) reported 1.2-1.7-fold resistance (low level) to clodinafop, sulfosulfuron, fenoxaprop, and tralkoxydim. Further Yadav and Malik (2005) reported that the amount of these herbicides required for reducing 50% of the growth (GR_{50}) increased with the duration of their use. At present, either acetolactate synthase or acetyl-CoA carboxylase inhibitor post emergent herbicides are being widely used in wheat (Kumar and Ladha, 2011), which are highly prone to evolution of resistance (Bhullar et al., 2017). Herbicide resistance is the result of the adaptive evolution of weed populations to the intensive selection pressure exerted by herbicides is now recognized widely (Raghav et al., 2016). Dhawan et al (2008) reported 10-90% polymorphism amongst populations of *Phalaris minor*. High level of polymorphism found among *P. minor* populations from various geographical locations in wheat growing areas of India indicate that the populations are heterogeneous which could be one major factor for differential response to the herbicides and multiple herbicide resistance. According to Heap 2016, herbicide resistant populations of *Phalaris minor* Retz. are common in India and Mexico. The understanding of herbicide resistance mechanisms in weeds can be important for the selection of alternative herbicides or herbicide modes of action for the management of resistant weeds. It is necessary to understand the resistance mechanisms of the resistant individuals at the target site and non-target site. The present investigation was carried out to identify and analyze the mechanism of resistance development in resistant populations of *Phalaris minor* from Punjab.

Materials and Methods

Collection of *P. Minor* and DNA isolation

Populations of *Phalaris minor* were collected from the different districts of Punjab. Collected samples were grown in pots and sprayed with all the recommended herbicides for the control of *P. minor* at the rate of 0.5 to 4X of the recommended dose of herbicide (Table 1). Screening of *P. minor* was done for resistance. DNA was isolated from the fresh leaves of different populations of *P. minor* 20 days after spray using CTAB method. The quality and quantity of DNA was estimated using Nano-drop (Thermo Fischer Scientific, USA). The concentration of isolated DNAs was initially normalized to 500ng/μl using TE buffer and further up to 100 ng/ μL using distilled water.

Table 1. Herbicides used and their doses along with field use rate

Herbicide	Dose (g ha ⁻¹)	Field use rate (g ha ⁻¹)	Time of application
Isoproturon	0, 234.5, 469, 938, 1876, 3752	938	Post-emergence
Clodinafop-propargyl	0, 15, 30, 60, 120, 240	60	Post-emergence
Pinoxaden	0, 12.5, 25, 50, 100, 200	50	Post-emergence
Sulfosulfuron	0, 6.25, 12.5, 25, 50, 100	25	Post-emergence
Mesosulfuron+ Iodosulfuron	0, 3.6, 7.2, 14.4, 28.8, 57.6	14.4	Post-emergence

Methodology used for herbicide resistance studies

Collection of populations of *P. minor* from 14 districts of Punjab



Collected samples first tested for germination in petri plates and samples having more than 90 % germination were used for growing in pots



P. minor populations were sprayed with the recommended herbicides for its control at the rate of 0.25 to 4 times the recommended dose of herbicide



Screening of *P. minor* populations for herbicide resistance

Methodology for Molecular Studies

Collection of fresh and young leaves of resistant populations along with susceptible population (Check)



DNA extraction from the different populations of *P. minor* using CTAB method



Previously published primers used to amplify the region



Comparative sequence analysis performed to check the presence of mutation in the conserved region of ACCase gene



Fig. 1. Over view of pot experiment (sowing of each population with three replicates for 0.25 X to 4 X dose of each herbicide application) before herbicide application

Amplification and restriction digestion of putative substitution region of ACCase in *P. Minor* populations

A previously reported primer (F₅ 5'-CAGCCTGATTCCCATGAGC-3' R₄ 5'- CCATGCATTCTTGGAGTTCCT-3') covering putative substitution region in ACCase was used for amplification (Hochberg et al., 2009). The PCR reaction mixture was prepared by adding 2.5 µL of template DNA (100ng/µL), 1.8 µL of forward and reverse primers, 4.8 µL of Mastermix (Takara, Japan) and 1.1 µL of distilled water to make reaction volume of 12 µL (Table 2). The PCR was carried out in 96-welled micro-litre plate in Applied Biosystems master cycler with varying temperature conditions such as initial denaturation at 94° for 5 min followed 10 cycles of PCR at 94° C for 30 seconds, 60° C for 30 seconds, 72° C for 30 seconds, further 25 cycles of PCR at 94° C for 30 seconds, 55° C for 30 seconds, 72° C for 30 seconds and final extension at for 4 minutes. Amplified products were confirmed using agarose gel electrophoresis and utilized further for sequencing. Amplified PCR product was incubated with EcoR1 restriction enzyme for 16 hrs at 37° C. Incubated PCR products with EcoR1 restriction enzyme were run on 2.5 % agarose gel.

Table 2. Composition of Sequencing PCR

Component	Volume(µl)
Template DNA	2.0 µl
Ready reaction mix	1.0 µl
Dilution buffer	1.0µl
Primer (F/R)	0.5µl
MiliQ water	0.5µl
Total	5.0 µl



Fig. 2. Over view of pot experiment (survival view of each population with three replicates for 0.25 X to 4 X dose of each herbicide application) after herbicide application

Sequencing and data analysis

For each 1X sequencing reaction, the components of 5 µl reaction volume are as given in Table 2. DNA Baser was used to assemble the forward and the reverse sequences to form a consensus sequence. The mis-matches were compared with the electropherogram and were curated manually according to the quality of the base call peaks. The software was by default set to trim the poor base calls at the ends. Clustal X was used to form the multiple sequence alignment of the sequences of the different accessions with respect to the transcript obtained from the transcriptome database which was used as the reference and the trueness of the SNPs was verified with the electropherogram to check whether the peak is devoid of any overlaps.

Results and Discussion

Amplification and validation putative substitution in ACCase gene across *P. minor* populations *Phalaris minor* Retz. is a weed of grass family. It has developed resistance to many herbicides with different modes of action. Similar research was conducted to see the level of resistance in *P. minor* populations collected from Punjab. The amplification of specified region of ACCase gene was carried out using reported primer, yielded amplicon of 404 bp band across the populations of *P. minor*. The region consists of common mutation site (Ile₂₀₄₁ to Asn) associated with resistance towards herbicides. This mutation in genic region causes T-6278-A transition leads to loss of restriction site of EcoRI enzyme. Taking advantage of this, amplicon was subjected to restriction digestion with EcoRI enzyme for overnight. Restriction EcoRI enzyme digested alleles and three different types of fragments of variable lengths were obtained (Fig. 3). Sensitive alleles yielded 169 and 235 bp band fragments upon digestion whereas resistant alleles were remained non digested with 404 bp band fragment. This result was in agreements with studies of Deyle *et al* 2003; Zhang and Powell 2006; Liu *et al.*, 2007. However, heterogenous populations on digestion with EcoRI enzyme yielded three fragments of 169, 235 and 404 bp lengths in present study. The details analysis of gel results (Fig. 4) indicated that 19.7 % populations had homozygous resistant alleles (404 bp band), 62.21 populations had homozygous sensitive alleles (169 and 235 bp band) whereas 18.2 populations had heterozygous resistant alleles (169, 235 and 404 bp band).

Analysis of *P. minor* populations for presence of substitutions using sequencing and data analysis

Phalaris minor biotype acetyl CoA carboxylase gene sequence was retrieved from NCBI database with the accession number AY196481.1. Nucleotide sequence of *Phalaris minor* biotype acetyl CoA carboxylase and different populations of *P. minor* has been presented in Fig. 5 and 6.

The presence of substitution at position 2041 in *P. minor* populations was verified using the reference genome. The similarity search conducted using the BLAST software with *Phalaris minor* populations acetyl CoA carboxylase gene

sequence (AY196481.1) as query. Results were correlated for any substitution and similarity with reference genome of *P. minor* (Fig. 5 and 6).

Target site mutation in wild-type and resistant codons at positions 2027 and 2041 of ACCase for resistance to clodinafop in *P. minor* has been reported in India (Raghav et al., 2016). Several scientists reported target site mutations in resistant populations of *P. minor*. The conformation of mutations occurred due to the binding pocket and the hydrogen bonding interactions on Trp2027 to Cys, Ile2041 to Asn, Asp2078 to Gly, and Gly2096 to Ala mutations as reported by different studies. In Iranian *P. minor* populations resistance to ACCase inhibitors occurred due to Trp2027 to Cys substitution (Gherekhloo et al., 2012). Hochberg et al (2009) reported Ile₂₀₄₁ to Asn mutation in awned canary-grass (*Phalaris paradoxa*) in Israel. The resistance to APPs (fenoxaprop, clodinafop, haloxyfop) conferred by the Trp2027Cys and Ile₂₀₄₁Asn mutations. Herbicide resistance Italian ryegrass also possessed the same Ile₂₀₄₁Asn mutations (Ghanizadeh et al., 2020).

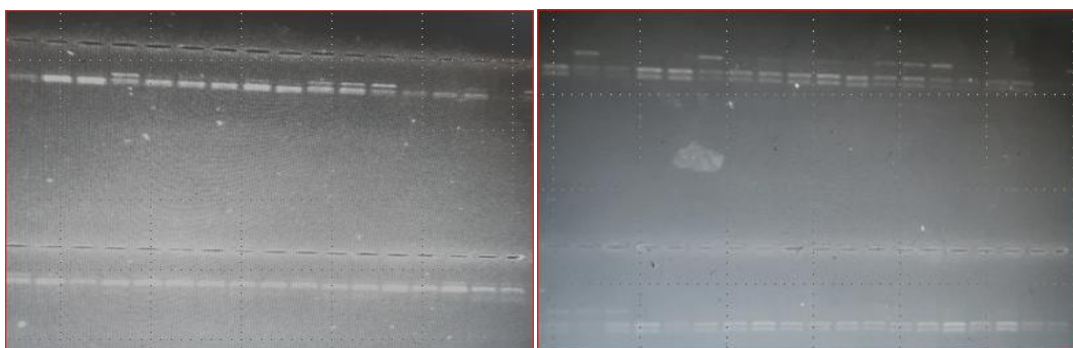


Fig. 3. Gel image showing populations with homozygous resistant alleles (1 band) homozygous sensitive alleles (2 bands) and heterozygous resistant alleles (3 bands)

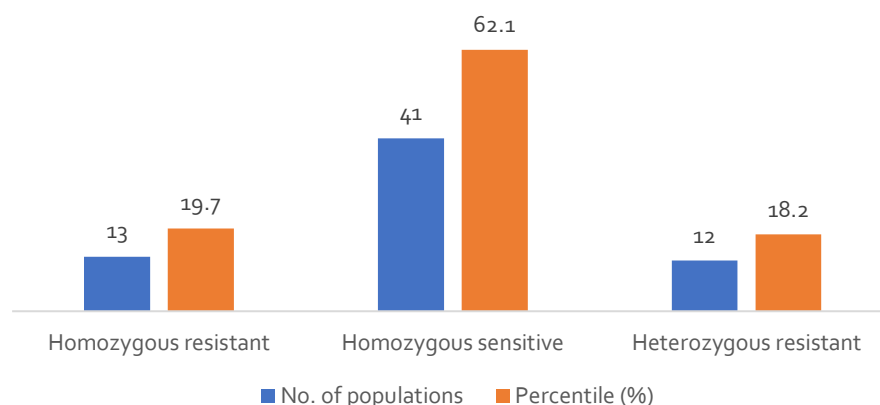


Fig. 4. Distribution of *P. minor* populations according to their level of resistance to ACCase

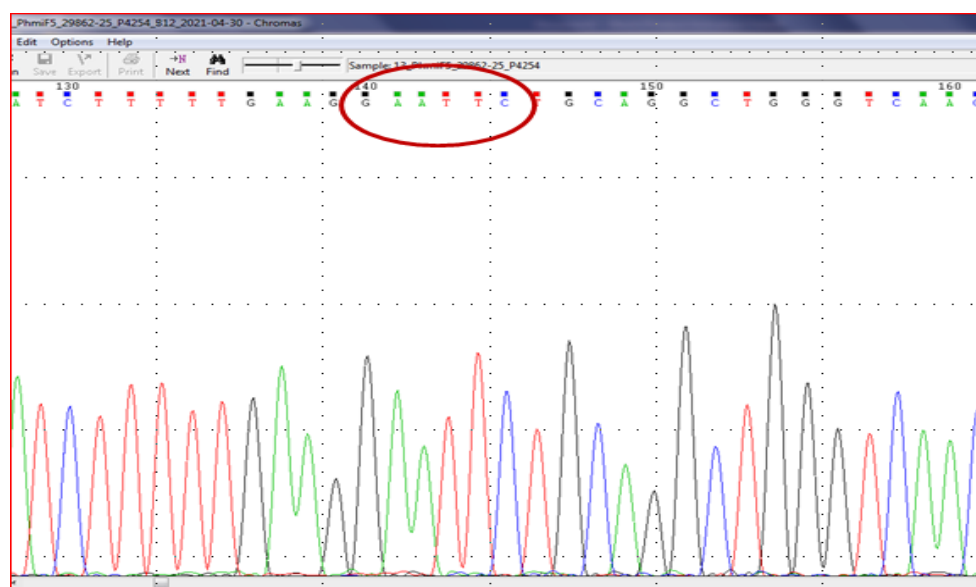


Fig. 5. Electropherogram showing the SNP (highlighted) in the multiple sequence alignment of susceptible population of *Phalaris minor*



Fig. 6. Electropherogram showing the SNP (highlighted) in the multiple sequence alignment of resistant population of *Phalaris minor*



Fig. 5a. Nucleotide sequence of susceptible population of *Phalaris minor*

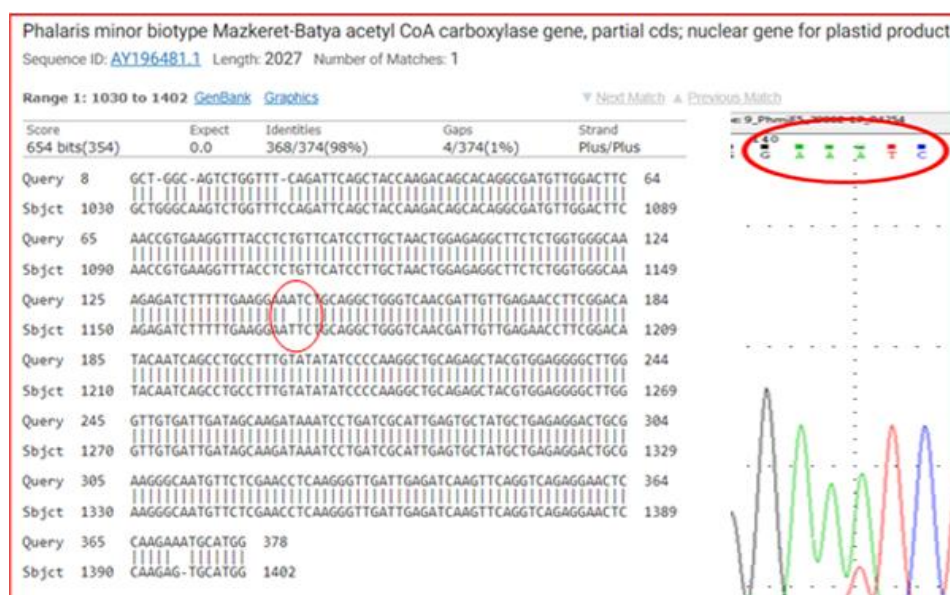


Fig. 6a. Nucleotide sequence of resistant population of *Phalaris minor*

Multiple sequence alignment using CLUSTALW

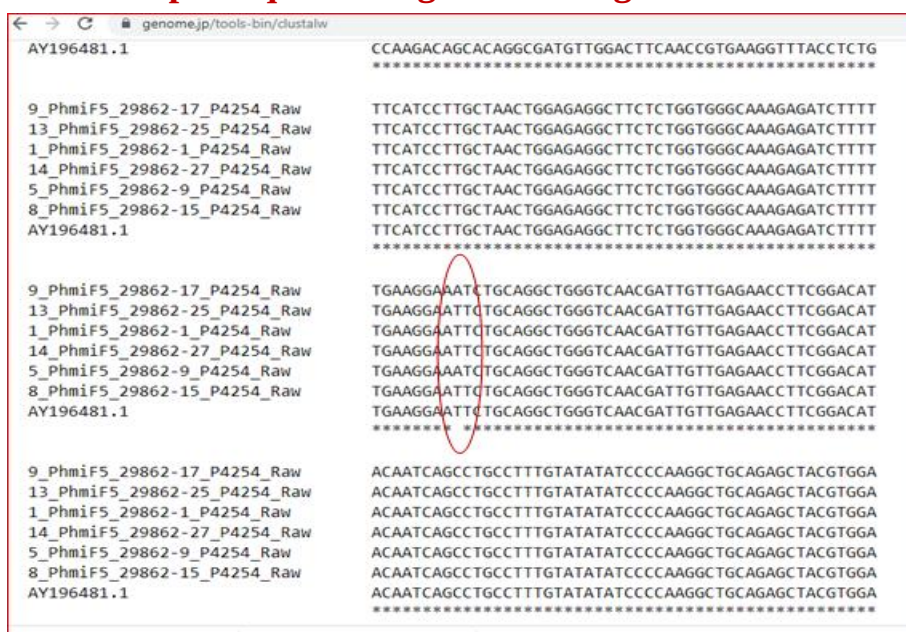


Fig. 7. Multiple sequence alignment using CLUSTALW of different populations of *Phalaris minor*

The multiple sequence alignment of ACCase sequence of *P. minor* populations was carried out (Fig. 7). The reference and under study transcripts were aligned and found that transcripts were perfectly aligned with predicted gene resulting in 99% identity. Only one substitution was recorded. It demonstrated that populations were highly resistant to ACCase inhibiting herbicides with transversion mutation of T to A (Fig. 6). The trueness of SNP was verified with the electropherogram to check whether the peak is devoid of any overlaps. Electropherogram results indicated the presence of clear peaks and advocated for good quality results of nucleotide sequencing. *P. minor* populations genomes were analysed with reference genome (from NCBI) using CLUSTAL software and have been presented in Fig. 5 and 6 with highlighted SNP.

Conclusion

A total of 13 populations had homozygous resistant alleles (404 bp band), 41 populations had homozygous sensitive alleles (169 and 235 bp band) whereas 12 populations had heterozygous resistant alleles (169, 235 and 404 bp band). T to A nucleotide transversion was observed in amplified region of ACC gene which is responsible for resistance to Acetyl CoA Carboxylase enzyme. This substitution (caused by T to A transversion) has been shown to endow resistance to ACCase inhibitors in *Phalaris minor* and other grass weeds. More than 50% *P. minor* populations still had homozygous sensitive alleles. Further investigation on different mutation causing herbicide resistance in *P. minor* is needed and site specific as well as ecological based agronomic inventions are needed to prevent or delay resistance development.

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Author Contributions

GK, PS, TK and MSB conceived the concept, wrote and approved the manuscript and DB edited the manuscript.

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Ethics approval

Not applicable.



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