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Identification of SER-653-ASN mutation conferring resistance to imidazolinone in Malaysian weedy rice

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Abstract: Background: The prevalence of weedy rice infestation has been shown to cause a significant reduction in Malaysian rice yield. The introduction of the Clearfield© Production System has significantly reduce weedy rice infestation, but its mismanagement has led to an increase in imidazolinone-resistant weedy rice. **Objective:** This study aims to investigate the resistance levels and patterns of weedy rice and to identify possible causes of resistance. **Methods:** The putative imidazolinone-resistant and susceptible weedy rice were collected in two different rice fields in Kelantan, Malaysia. A petri dish seed bioassay and whole-plant dose-response studies were conducted using three imidazolinone herbicides at seven rates. Surviving plants underwent molecular DNA extraction, nucleotide sequencing, and *in vitro* acetohydroxy acid synthase (AHAS) inhibition assay studies. **Results:** The putative imidazolinone-resistant weedy rice was found to be resistant to all three imidazolinone herbicides at the recommended field rate. Further confirmation by DNA sequencing revealed a Ser-653-Asn mutation in all resistant populations. *In vitro* AHAS inhibition assays confirmed imidazolinone resistance at the enzymatic level. **Conclusion:** The weedy rice population on the eastern coast of Peninsular Malaysia is resistant to imidazolinone herbicides. The Ser-653-Asn mutation was detected and is known to be the primary cause of imidazolinone resistance in this population of weedy rice.

Keywords: AHAS; *Oryza sativa* f. *spontanea*; Herbicide Resistance; Sequencing.

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1. Introduction

Rice is a major food crop in many countries worldwide. (Deng et al., 2020). Following worries on food security, Malaysia aimed to increase rice production to meet the population's needs in order to reduce dependence on imported rice from Thailand and Vietnam (Dorairaj, Govender, 2023). Malaysia's annual self-sufficiency in rice production is around 67% and with rice import restrictions, there could be a risk for the nation's food supply. Therefore, increasing local rice production is imperative.

Modern rice production includes the implementation of herbicides on a large scale. Application of herbicides without proper stewardship could lead to herbicide resistance problem in rice fields (Ruzmi et al., 2021). Weedy rice (*Oryza sativa* f. *spontanea*) and cultivated rice (*O. sativa*) are similar species of *Oryza* with the former having weedy traits due to hybridisation events and gene flow (De Leon et al., 2019; Ponsen et al., 2023). Controlling weedy rice is difficult due to its shared morphology with cultivated rice varieties (Ziska et al., 2015). The 1980s marked the beginning of Malaysian weedy rice occurrences in major rice fields in the country (Sudianto et al., 2016), concurrent with the transition of traditional rice transplanting to the dry direct-seeded method. Without flood water to suppress weed growth, proliferation of weedy rice became inevitable. Over time, crossing between wild and cultivated rice has also led to the development of various weedy rice varieties (Nadir et al., 2017).

Strategic chemical control is necessary to curb the proliferation of weedy rice and prevent further damage to local rice production. Therefore, the Clearfield Production System (CPS) was introduced to address the issue (Sudianto et al., 2013). This system enables farmers to use IMI-resistant rice cultivars to chemically control weedy rice proliferation (Avila et al., 2021). These cultivars were developed in collaboration with BASF and MARDI (Dilipkumar et al., 2018), through mutation of the enzyme acetohydroxy acid synthase (AHAS, EC 2.2.1.6), making rice plants insensitive/resistant to AHAS-inhibiting herbicides, particularly imidazolinones (IMI). In AHAS-sensitive plants, IMI herbicides will bind to the enzyme, thus preventing the biosynthesis of essentials branched-chain amino acids (BCAAs) important for plant growth (Yu et al., 2010). The resulting lack of BCAA impedes the continued growth of the shoots and roots, leading to plant mortality.

The resistance to herbicide through chemical mutagenesis resulted in their survival amidst herbicide treatment. Weedy rice that has a morphology similar to that of cultivated rice but without the resistance is susceptible. However, repeated use of the same system could select resistance in weedy rice due to the evolution of resistance and the occurrence of gene flow from IMI-resistant rice to weedy rice. Consequently, resistance cases have increased due to repeated herbicide applications (Sudianto et al., 2013). The United States recorded the first herbicide resistance cases in weedy rice in 2002 for IMI, then followed by other countries such as Brazil in 2006, Costa Rica in 2010, Italy in 2010, Greece in 2013, Argentina in 2018, Colombia in 2018, Malaysia in 2018 and most recently Turkey in 2020 (Heap, 2024). At the present time, resistance to IMI has been confirmed in Malaysian weedy rice in the northern states of Kedah, Perlis and Penang (Dilipkumar et al., 2018; Ruzmi et al., 2020) where CPS has been used for more than eight consecutive planting seasons. Thus, this study aimed to 1) investigate the occurrence and patterns of resistance to IMI-herbicides in weedy rice population, and 2) identify the possible mechanisms conferring resistance to IMI-herbicides in the resistant weedy rice population.

2. Material and Methods

2.1 Sample collection, seed increase and resistance screening

Putative resistant (R) and susceptible (S) weedy rice seeds were collected from Kelantan, Malaysia (Location R: 6°04'03.5N 102°10'04.5"E; Location S: 6°03'11.9"N 102°05'16.8"E). The MR220CL2 and MR297 rice seeds were obtained from the Malaysian Agricultural Research and Development Institute (MARDI) and used as controls. MR220CL2 is resistant to the IMI-herbicide imazapic + imazapyr (On Duty©, 77% w/w, WG), herbicides used for CPS in Malaysian rice fields (Dilipkumar et al., 2018). Meanwhile, MR297 is a rice variety with no resistance to any herbicide. The weedy rice seeds (R and S) were cultivated in a glasshouse under optimal rice growing conditions to maximise seed output for both seed bioassay and whole-plant dose-response. To confirm the IMI-resistance in both weedy rice populations, 1-day old rice seedlings were screened with a commercial imazapic + imazapyr herbicide at the recommended rate of 154 g ai ha^{-1} (data not shown).

2.2 Seed bioassay

Seeds from resistant and susceptible weedy rice populations, as well as MR220CL2 and MR297 rice varieties were soaked in water for 24 hours, followed by 12-hour incubation at room temperature (24–26 °C) to induce germination. Twenty germinated seeds of each population were placed on a 9-mm petri dish lined with two sheets of filter paper. The concentrations of the treatments were 0, 0.25, 0.5, 0.75, 1, 2 and 4 times the recommended application rate of commercial IMI-herbicides. Aliquots of 5 mL aqueous imazapic + imazapyr (On Duty©, 77% w/w, WG), imazapic (Cadre©, 240 g ai L^{-1}, SC) and imazethapyr (Imaz 5.2SL©, 540 g ai L^{-1}, SC) solutions were applied at 0, 0.19, 0.29, 0.58, 0.77, 1.54, 2.08 mg L^{-1} (imazapic + imazapyr); 0, 0.06, 0.12, 0.18, 0.24, 0.48, 0.96 mg L^{-1} (imazapic); and 0, 0.14, 0.27, 0.41, 0.54, 1.08, 2.16 mg L^{-1} (imazethapyr).

Each treatment was arranged in a randomised complete block design (RCBD) and replicated four times for each population (n = 20 seeds in each replication). The petri dishes were placed on workbenches at standard ambient temperature (24–26 °C) and light for 14 days. The petri dish covers were unsealed to allow gas exchange and prevent anaerobic conditions. The germination rate was measured after 14 days (DAT).

2.3 Whole-plant herbicide dose-response

Germinated seeds from the R, S, MR220CL2 and MR297 populations were transferred to trays with 30 cm width x 40 cm length and a depth of 15 cm containing fine clay soil and maintained in a greenhouse. The seedlings were treated with three selected IMI-herbicides at the 1–2 leaf stage (3 days after germination). The doses of the treatments were 0, 0.25, 0.5, 0.75, 1, 2 and 4 times of the recommended application rate of commercial IMI-herbicides, namely imazapic + imazapyr, imazapyr (Chopper©, 14.9 g ai L^{-1}, SC) and imazethapyr. The doses of the respective herbicides were: 0, 38.5, 77, 115.5, 154, 308, 616 g ha^{-1} (imazapic + imazapyr); 0, 14.9, 29.8, 44.6, 59.5, 119, 238 g ha^{-1} (imazapyr); and 0, 61.1, 122.2, 183.3, 244.4, 488.8, 977.6 g ha^{-1} (imazethapyr).

All treatments were repeated four times (n = 10 plants in each replication) in RCBD. The plants were maintained for 21 days after treatment (DAT) and, the plant survival count recorded. The plants were then harvested 2 centimetres above the ground, dried for 72 hours at 65 °C, and weighed. Resistant weedy rice plants that survived the herbicide treatments at the recommended rate and above were allowed to regrow, self-pollinate and produce seeds for the AHAS molecular sequencing and *in vitro* inhibition assay.

2.4 Genomic DNA extraction and sequencing

Plants that had survived the recommended and above herbicide doses were subjected to genomic DNA extraction. Each sample of approximately 100 mg was collected by flash-freezing in liquid nitrogen and kept in a -80 °C freezer until further use. The leaf tissue was extracted using a modified version of the cetyltrimethylammonium bromide (CTAB) method by Doyle and Doyle (1987).

The acetohydroxy acid synthase (AHAS) gene fragment comprises six conserved regions with potential mutation sites for imidazolinone resistance and contains the domains C, A, D, F, and E (Merotto et al., 2009). The amplification of the AHAS gene was done using the forward primer

(5'-GTAAGAACCACCAGCGACACC-3') and a reverse primer (5'-GATGCATATGCCTACGGAAAAC-3') suggested by Kaloumenos et al. (2013).

The polymerase chain reaction (PCR) cocktail consisted of 7.5 µL REDiant II PCR Master Mix (First BASE, Singapore), 1.0 µL genomic DNA template (70 ng/µl), 1 µL each of forward and reverse primer (10 mM), and 4.5 µL nuclease-free water bringing a total volume of 15 µL. PCR was carried out using Thermal Cycler T100 (Bio-Rad, Hercules, CA, USA) based on the following cycle: 95 °C (5 min) initial denaturation, 30 cycles of 95 °C (30 s) denaturation, annealing at 58 °C (30 s), elongation at 72 °C (2 min), and followed by final elongation at 72 °C (10 min).

The PCR product was resolved in a 1.4% agarose gel stained with FloroSafe DNA Stain (First BASE, Singapore), and a 1 kb DNA ladder (Thermo Scientific, MA, USA) served as a standard. The successful PCR amplification product was forwarded to a commercial sequencing service (Apical Scientific Sdn. Bhd., Seri Kembangan, Selangor, Malaysia) for purification, gel extraction by general agarose, and DNA sequencing.

Sequencing was done with both forward and reverse primer from the 5' to 3' end for accuracy. BLAST (http://www.ncbi.nlm.nih.gov) was utilised to compare the nucleotide sequence to the previously described *Oryza* AHAS gene in the GenBank database. The nucleotide sequences were aligned and compared using the the AHAS gene chromatograms, which were visualised using the UGENE software version 46.0. The aligned sequences were converted to their corresponding amino acid sequences using BLASTX (http://www.ncbi.nlm.nih.gov). All amino acid sequences were normalised to *Arabidopsis thaliana* sequence (NM114714).

2.5 AHAS enzyme inhibition assay

Plants from dose-response experiments that survived the recommended and above herbicide application rates were allowed to regrow and produce seeds. The seeds were planted in a glasshouse until the 3–4 leaf stage and then harvested. The AHAS *in vitro* inhibition assay was performed by utilising the modified method of Yu et al. (2010). The protein content was measured using the Bradford method and immediately used in an inhibition experiment.

The certified analytical standards for IMI-herbicides used for AHAS enzyme inhibition were imazapic (99.3%) and imazapyr (99.6%). The reaction was terminated by adding 40 µL 6N H_2SO_4 after 60 minutes of incubation with the extracted enzyme. The solutions were incubated at 60 °C for 15 minutes to convert acetolactate to acetoin. Using a Multiskan GO Microplate Spectrophotometer (Thermo Scientific, MA, USA), the acetoin production from acetolactate was quantified colourimetrically four times per sample at 530 nm.

2.6 Statistical analysis

The R software (R Core Team, 2024) was used to test the significance of the data using a two-way analysis of variance (ANOVA). All analyses were performed using SigmaPlot version 14 (Grafiti, 2024) to obtain the $LD_{50}/GR_{50}/I_{50}$ values and the concentration/dose-response curve. The $LD_{50}/GR_{50}/I_{50}$ were obtained by non-linear regression using the log-logistic response equation proposed by Knezevic et al. (2007). The $LD_{50}/GR_{50}/I_{50}$ is the 50% reduction in mortality/growth rate/inhibition of the samples towards herbicide treatment. The resistance index (RI) was calculated by dividing the $LD_{50}/GR_{50}/I_{50}$ of the other population by the $LD_{50}/GR_{50}/I_{50}$ of the S population (Iwakami et al., 2014; Merotto et al., 2009).

3. Results and Discussion

3.1 Seed bioassay

In the seed bioassay, a resistance was observed in the resistant (R) and MR220CL2 populations across the three IMI-herbicides used (Table 1). None of the susceptible (S) and MR297 populations survived at the recommended rate for each IMI-herbicide. The R population had an LD_{50} value of 5.9-fold for imazapic + imazapyr, 4.9-fold in imazapic, and 3.6-fold in imazethapyr compared to the S population (Figure 1). Previous results of other weedy rice populations in Malaysia recorded a greater resistance (RI = 16.4) in one population on the West Coast (Ruzmi et al., 2020). Two other populations sampled in the same area had an RI of 14.9 and 1.3 for the seed bioassay treated with the premix imazapic + imazapyr herbicide (Dilipkumar et al., 2018).

Table 1 - LD_{50} (mg ai L^{-1}) and RI of susceptible, resistant, MR220CL2 and MR297 populations in petri dish seed bioassay. Standard errors are in parentheses. The means are significant at p<0.001

Herbicide	Population	LD_{50}	RI[a]
Imazapic + imazapyr	Susceptible	0.22	-
	Resistant	1.30 (0.04)	5.9
	MR220CL2	1.44 (0.06)	6.5
	MR297	0.18	0.8
Imazapic	Susceptible	0.08	-
	Resistant	0.39 (0.03)	4.9
	MR220CL2	0.43 (0.02)	5.4
	MR297	0.06	0.8
Imazethapyr	Susceptible	0.27 (0.20)	-
	Resistant	0.97 (0.02)	3.6
	MR220CL2	0.99 (0.01)	3.7
	MR297	0.10 (0.03)	0.4

Abbreviations: LD_{50}, dose required to cause 50% mortality; RI, resistant index; [a]RI was determined using the ratio between resistant LD_{50} and susceptible LD_{50}

The observation on the shoots and roots of the treated populations showed desiccation and browning. IMI-herbicides work by inhibiting the production of branched-chain amino acids (BCAAs) needed for early plant growth (Guo et al., 2024). Therefore, the severe injuries observed on the young germinating plants especially for the S and MR297 populations were the result of the lack of BCAAs due to IMI-herbicides inhibition of AHAS. This lack of BCAAs production resulted in plant mortality (Yean et al., 2021). The imazethapyr-treated R population recorded a lower resistance level than the other IMI-herbicide treatments used in the same population (Table 1). Imazethapyr, however, is not used in Malaysian rice fields for weed control, but mainly in oil palms, rubber and coconuts (Nordin et al., 2019).

3.2 Whole-plant dose-response

The R and MR220CL2 populations resisted the three IMI-herbicide applications at the recommended or greater rates (Table 2). The R population recorded LD_{50} values for survival of 22.3-fold in imazapic + imazapyr (RL = high), 19.6-fold in imazapyr (RL = high), and 11.9-fold in imazethapyr (RL = moderate) than the S population (Figure 2). The GR_{50} values were similar in pattern, with 18.9-fold in imazapic + imazapyr (RL = high), >100-fold in imazapyr (RL = high), and 5.9-fold in imazethapyr (RL = low) than the S population. The R population was less affected by imazethapyr than the other two IMI-herbicides used, probably due to lack of exposure to the former in Malaysian rice fields (Nordin et al., 2019). The S and MR297 populations were observed to be sensitive towards all the herbicide treatments.

A higher reduction in shoot dry weight was observed for imazapyr-treated populations. A reduction of >100 times higher than S for imazapyr was observed for the R and MR220CL2 populations, while the other two IMI-herbicides showed 5.4 to 21.5 times higher than the S population. This resistance could be attributed to the occurrences of weedy rice escapes and the

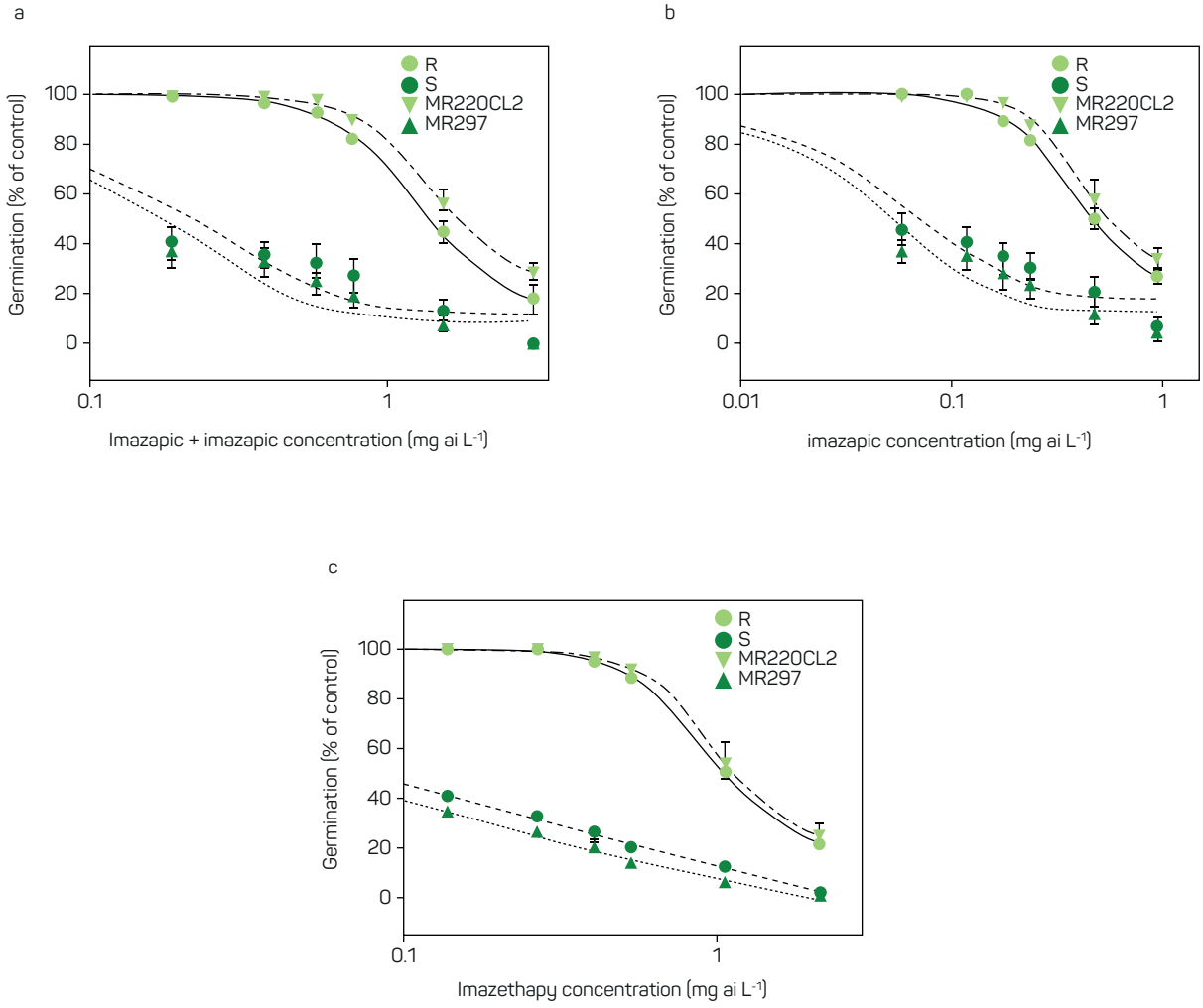

Figure 1 - The germination rate of resistant (R), susceptible (S), MR220CL2, and MR297 populations to imazapic + imazapyr (a), imazapic (b) and imazethapyr (c) 14 days after treatment. Bars indicate standard errors of the means of four replicates. The means are significant at p<0.001

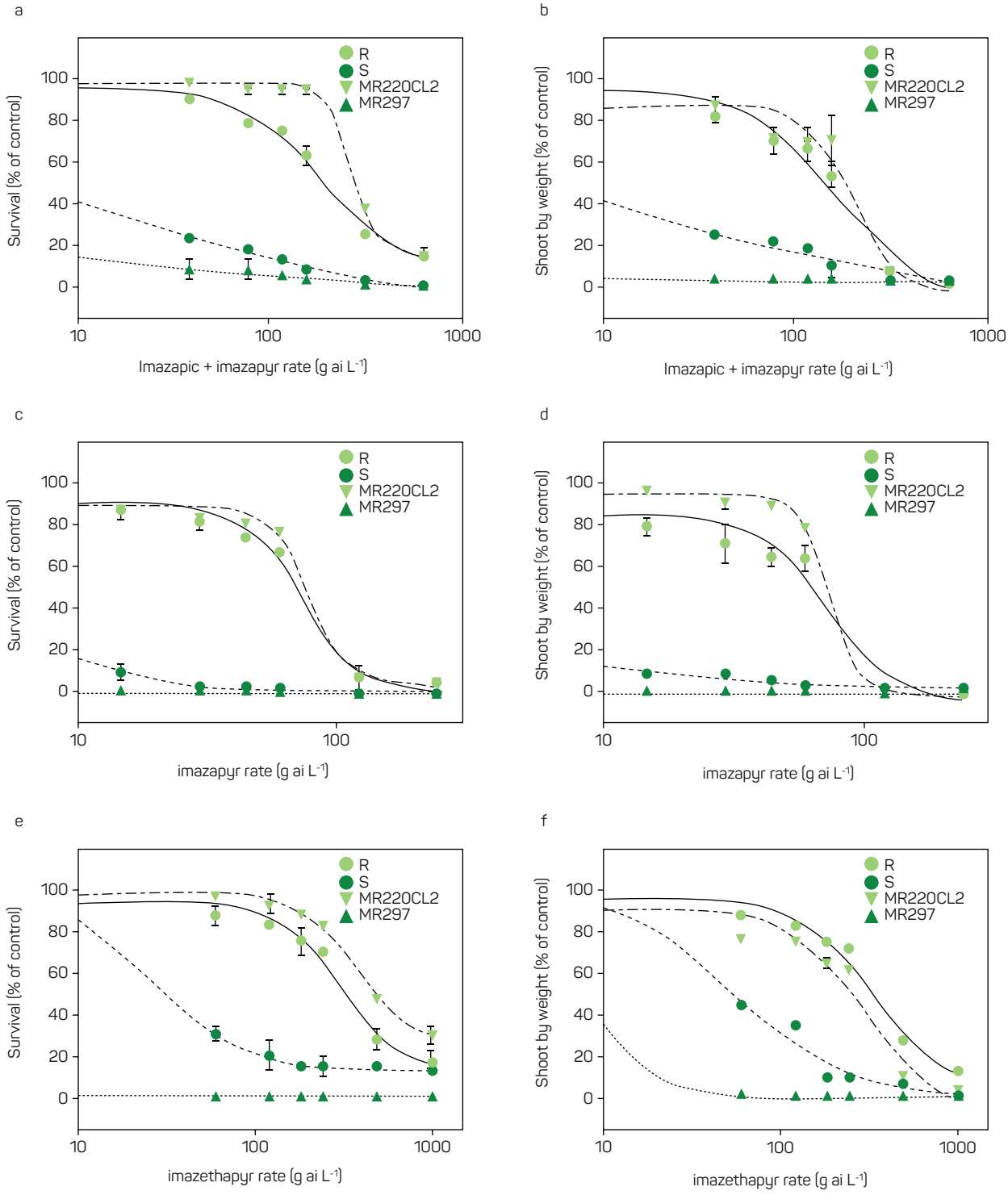

Figure 2 - The survival and shoot dry weight of resistant (R), susceptible (S), MR220CL2, and MR297 populations to imazapic + imazapyr (a & b), imazapyr (c & d), and imazethapyr (e & f) 21 days after treatment. Bars indicate standard errors of the means of four replicates. The means are significant at p<0.001

gene flow from cultivated rice (Avent et al., 2023; Ruzmi et al., 2021). Management involving systematic herbicide application and compliance are essential to prevent such occurrences.

Variable resistance levels were reported for other weedy rice populations on the West Coast of Malaysia for imazapic + imazapyr. Ruzmi et al. (2020) reported a high RI = 50 for their weedy rice population, however, Dilipkumar et al. (2018) observed an RI of 2.6-6.4 for weedy rice populations in their study. High resistance in weedy rice for the IMI group of herbicides was observed in other places such as

Italy (Scarabel et al., 2012), United States (Burgos et al., 2014), Greece (Kaloumenos et al., 2013) and Brazil (Merotto et al., 2016).

Applying similar herbicides with the same mode of action (MOA) repeatedly on the same weed population creates a selective pressure that may lead to herbicide resistance (Gaines et al., 2020). The timing for pre-emergence herbicide application is crucial as it can affect the growth of target weeds (Dilipkumar et al., 2021). For weedy rice, the herbicide imazapic + imazapyr should be applied at the 1-2 leaf stage or 0-5 days after sowing. The occurrence of gene flow involving the transfer of AHAS resistance gene mutation from IMI-rice to weedy rice was plausible by the high level of resistance recorded for imazapic + imazapyr (Table 2). The emergence of IMI-resistance in weedy rice within CPS rice fields, which mirrors the AHAS gene mutation found in cultivated rice, suggests that gene flow may be the main resistance mechanism in weedy rice (Yean et al., 2021). The IMI-resistance in weedy rice is linked to selection pressure as well as gene flow in countries that adopted CPS rice technology, and the risk is higher for tropical countries that practices multiple rice planting in one or two years (Ruzmi et al., 2021). IMI-resistance in weedy rice via gene flow was observed by Goulart et al. (2012) and Engku et al. (2016) in their studies. Nonetheless, insights from population genetics research are necessary to eliminate the likelihood of de novo mutation selection (Ruzmi et al., 2021).

Table 2 - LD_{50} (g ai ha^{-1}), GR_{50} (g ai ha^{-1}), and RI of susceptible, resistant, MR220CL2 and MR297 populations in whole-plant dose-response. Standard errors are indicated in parentheses. The means are significant at p<0.001

Herbicide	Population	LD_{50}	RI[a]	GR_{50}	RI[a]
Imazapic + imazapyr	S	8.34 (2.27)	-	9.32 (8.41)	-
	R	186.04 (37.69)	22.3	176.40 (50.71)	18.9
	MR220CL2	264.94 (11.17)	31.8	199.93 (46.70)	21.5
	MR297	0.0003 (0.02)	0	0.001 (0.02)	0
Imazapyr	S	3.75 (1.67)	-	0.001 (0.02)	-
	R	73.33 (9.77)	19.6	72.68 (18.29)	>100
	MR220CL2	76.92 (11.38)	20.5	73.62 (7.63)	>100
	MR297	0.003	0	0.003 (0.02)	3
Imazethapyr	S	26.27 (4.72)	-	56.04 (13.89)	-
	R	312.75 (59.09)	11.9	332.87 (63.13)	5.9
	MR220CL2	372.18 (26.89)	14.2	301.56 (94.08)	5.4
	MR297	0.006	0	7.89 (2.61)	0.1

Abbreviations: LD_{50}, the dose required to cause 50% mortality; RI, resistant index; GR_{50}, the dose required to cause 50% reduction in shoot dry weight; [a]RI was determined using the ratio between the resistant LD_{50} and susceptible LD_{50}

Table 3 - Nucleotide polymorphisms and amino acid substitutions in the AHAS sequences of *Arabidopsis thaliana*, susceptible, resistant, MR220CL2 and MR297 populations

Population	Amino acid position	
	377	653
Arabidopsis thaliana	CGT (R)	AGT (S)
Resistant	CGG (R)	AAT (N)
Susceptible	CGT (R)	AGT (S)
MR220CL2	CGT (R)	AAT (N)
MR297	CGT (R)	AGT (S)

Abbreviations: R, arginine; S, serine; N, asparagine

3.3 AHAS gene sequencing

A full-length of 2015 bp AHAS gene was amplified by the primer pairs in weedy rice and rice samples. The samples compared in BLAST have resulted in a 99% similarity to the previous samples of weedy rice AHAS gene in the GenBank database. All resistant weedy rice samples shared a single mutation (Ser-653-Asn substitution) in Domain E of the AHAS gene (Table 3, Figure 3). Substitution of G for A at position 653 of the AHAS gene in the R and MR220CL2 populations resulted in a transition from serine (AGT) to asparagine (AAT). Another single nucleotide polymorphism (SNP), Arg-377 was also observed in Domain F of the R population, where the third base of T was substituted with G in the resistant population. However, this substitution did not result in an amino acid change and was considered a silent mutation.

It has been postulated that the mutation in Ser-653 confers IMI-resistance to weedy rice through gene flow from IMI-rice (Yean et al., 2021). Different weedy rice populations in Kedah and Perlis CPS rice fields were also found to have similar Ser-653-Asn mutation (Ruzmi et al., 2020). This mutation is also highly conserved in the E region of the AHAS gene and was particularly interesting in many herbicide-resistant plants as it is the most widely found mutation in plants for resistance to AHAS (Merotto et al., 2009). Concomitantly, the same Ser-653-Asn mutation was observed in MR220CL2 individuals, indicating that the Ser-653 position might be vulnerable to mutation. This mutation is responsible for the CPS IMI-rice varieties resistant to IMI-herbicide (Ruzmi et al., 2021). The Ser-653 mutation has been linked to IMI-resistance weedy rice populations in the US (Wedger et al., 2022; Yean et al., 2021), Italy (Scarabel et al., 2012), Brazil (Roso et al., 2010), and Greece (Kaloumenos et al., 2013). This mutation decreases the sensitivity of the AHAS enzyme to IMI-herbicides (Yu, Powles, 2014) by reducing the binding affinity of IMI-herbicide to the AHAS enzyme (Gaines et al., 2020).

3.4 AHAS in vitro inhibition assay

The R and MR220CL2 populations exhibited high resistance to imazapic and imazapyr (Figure 4). The I_{50} values of population R (Table 4) for imazapic was 118- and for imazapyr was 82-fold more than the S population.

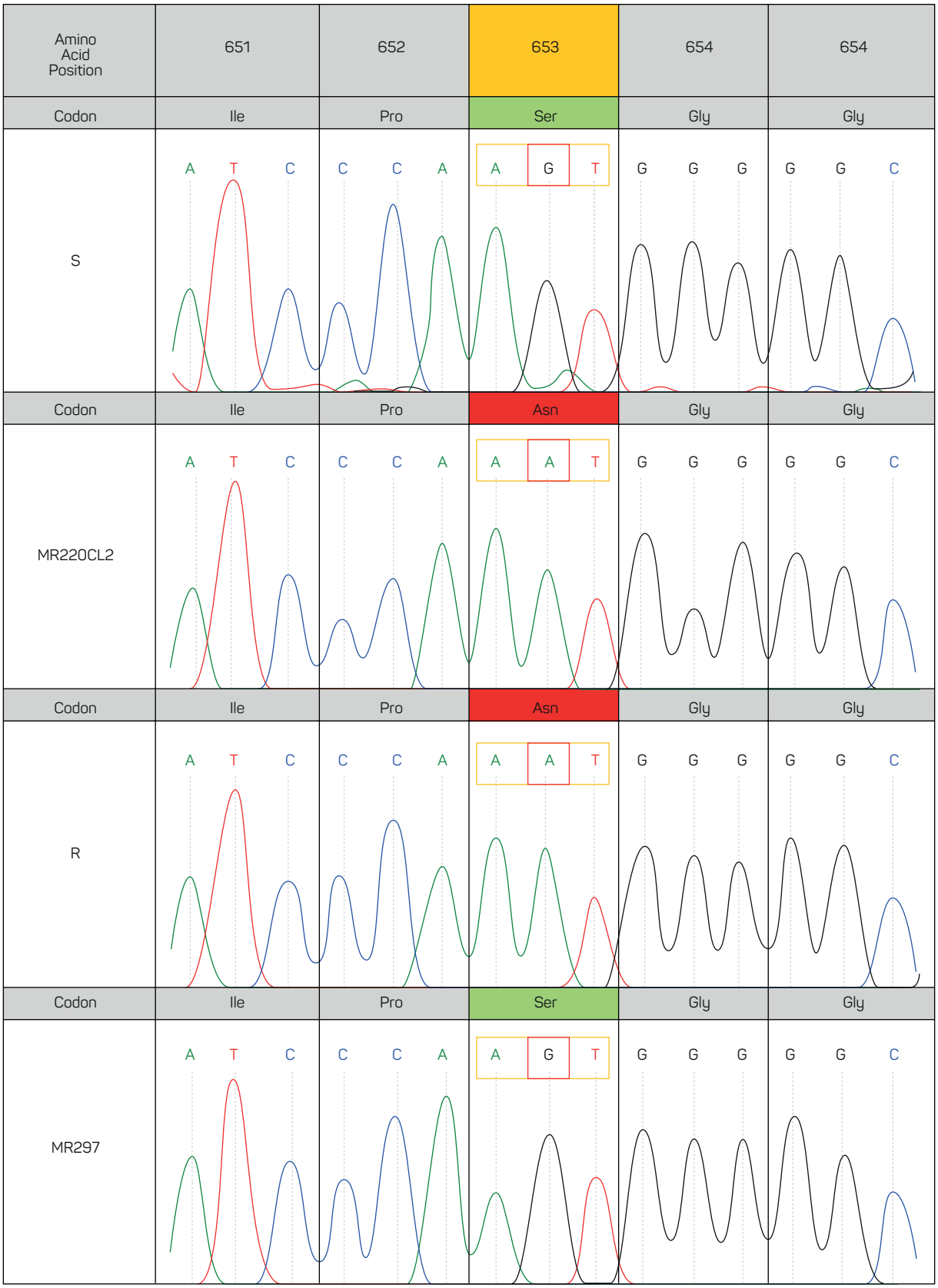

Figure 3 - The DNA sequencing results show the AGT codon in susceptible (S), AAT codon in MR220CL2, AAT in resistant (R), and AGT in MR297 of Ser-653 in homozygous plants. Coloured lines represent amino acids: black for guanine (G), green for thymine (T), red for adenine (A), and blue for cytosine (C); codon Ile is isoleucine, Pro is proline, Ser is serine, Asn is asparagine, and Gly is glycine

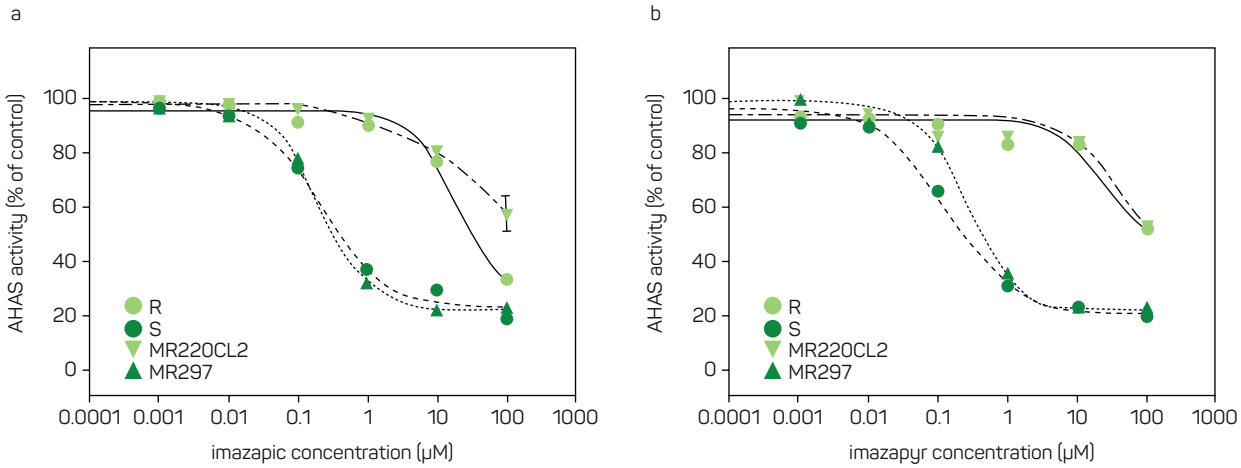

Figure 4 - AHAS activity of resistant (R), susceptible (S), MR220CL2 and MR297 populations toward (a) imazapic and (b) imazapyr. Bars indicate the standard error of the means of four replicates

The MR220CL2 population was 267- and 174-fold than the S population in imazapic and imazapyr, and recorded higher I_{50} values compared to the rest of the population in both herbicide treatments. Sensitivity to the IMI-herbicides was observed in both the S and MR297 populations (Figure 4), which had almost similar values in imazapic and more sensitivity in MR297 for imazapyr. The concentration-response curve also showed that AHAS activity decreased as the concentration of the analytical IMI-herbicides increased, showing a significant inhibitory effect on the enzyme.

A higher inhibitory effect was observed in imazapic, as shown by the higher RI value than imazapyr (Table 4). The imazapic + imazapyr formulation used in Malaysian CPS has a ratio of 3:1, with pre-emergence imazapic being considerably more effective than the latter (Bahm, Barnes, 2011; Dilipkumar et al., 2018). Although they belong to the same family, AHAS inhibitors have distinct binding orientations to an enzyme's target site (Yu, Powles, 2014). This further influences the variability of the resistance in the weedy rice populations to imazapic and imazapyr despite having a similar IMI-resistance mutation. Mutations in the AHAS gene can increase extractable AHAS activity, while others decrease or remain unaffected (Yu et al., 2010). The overexpression of the AHAS gene is unlikely to be the cause, and the increased activity may be due to greater AHAS stability (Guo et al., 2024).

Table 4 - AHAS activity (nmol acetoin formed mg^{-1} protein (min^{-1}), I_{50} (µM) and RI of susceptible, resistant, MR220CL2 and MR297 populations

IMI-herbicides	Populations	AHAS activity	I_{50}	RI[a]
Imazapic	Susceptible	3.46	0.18	-
	Resistant	4.51	21.38	118.8
	MR220CL2	4.78	48.06	267.0
	MR297	3.16	0.15	0.8
Imazapyr	Susceptible	3.34	0.34	-
	Resistant	4.99	28.05	82.5
	MR220CL2	5.00	59.31	174.4
	MR297	2.46	0.14	0.4

Abbreviations: IMI, imidazolinone; AHAS, acetohydroxyacid synthase; I_{50}, dose required to exhibit 50% inhibition of AHAS activity; RI, resistance index;; [a]RI was determined using the ratio between resistant I_{50} and susceptible I_{50}

4. Conclusions

The cross-resistance of imazapic and imazapyr has been verified in the weedy rice population on the Peninsular Malaysia East Coast, with the Ser-653-Asn mutation being the leading cause of resistance. Although not used in Malaysian rice fields, low to moderate resistance levels have also been observed in imazethapyr. A high probability of resistance evolution of weedy rice could occur with imazethapyr should it be incorporated for future CPS or general and integrated weed control in rice fields. Further studies should consider exploring the mechanisms of resistance based on herbicide metabolism and the fitness cost of IMI-resistance towards weedy rice growth and as a point of reference to develop new methods to control and prevent herbicide-resistant weedy rice.

Author's contributions

All authors read and agreed to the published version of the manuscript. MSAH and NZ: conceptualisation of the manuscript and development of the methodology. MAHJ: data collection and curation. MAHJ: data analysis. MSAH, NZ, and MAHJ: data interpretation. MSAH: funding acquisition and resources. MSAH: project administration. MSAH and NZ: supervision. MAHJ: writing the original draft of the manuscript. MAHJ and MSAH: writing, review, and editing.

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