RESISTANCE TO IMIDAZOLINONE HERBICIDES IN WEEDY RICE IN MALAYSIA

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FP 2020 1

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

October 2019

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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October 2019

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The IMI-herbicides rice package is among the most effective methods for weedy rice control nowadays. However, the sole dependence and ignorance on the appropriate use on imidazolinone herbicide (IMI-herbicides) in the IMI-herbicides rice package by rice growers has caused weedy rice to develop resistance to the herbicides. Thus, this study was conducted to elucidate the occurrence, level, and mechanisms endowing resistance to IMI-herbicides on field-reported resistant (R) weedy rice populations collected from three local Malaysian IMI-rice fields (A, B, and C). The R weedy rice populations were compared with a susceptible weedy rice population (S), an imidazolinone-resistant rice cultivar (IMI-rice), and a susceptible local rice cultivar (MR219). Dose-response experiments were conducted to evaluate the sensitivity of all of the populations (A, B, C, S, IMI-rice, and MR219) to commercial IMI-herbicides (premix of imazapic and imazapyr) in seed bioassay (imazapic evaluation for pre-emergence) and whole-plant bioassay (imazapyr evaluation for post-emergence). Seeds (in seed bioassay) and plants (in whole-plant bioassay) of all populations were treated with seven different dosages of IMI-herbicides. Based on the Resistance Index (RI) quantification in seed bioassay, A and B possessed high and moderate level of resistance to IMI-herbicides, respectively. Population C, S, and MR219 were all sensitive to the herbicides (susceptible). Similar with A, IMI-rice were highly resistant to IMI-herbicides with RI index values of 50.5. In whole-plant bioassay, population A, B, and C were resistant at low level to the herbicides. Meanwhile, S and MR219 were both sensitive to IMI-herbicides. IMI-rice recorded a moderate level of resistance to IMI-herbicides in whole-plant assessment. Conclusively, population A and B were more resistant to imazapic than imazapyr, while conversely, population C was more resistant to imazapyr than imazapic. S and MR219 populations were equally sensitive to both herbicides. IMI-rice was observed to be more resistant to imazapic than imazapyr. Molecular investigation was conducted by comparing acetohydroxyacid synthase (AHAS) gene sequences between R weedy rice (A, B, and C), S, IMI-rice, and MR219. The sequence of the 1884 base pair AHAS gene fragment showed that S

and MR219 were 99% similar. Evidently, AHAS gene sequences of R weedy rice were identical to the IMI-rice, where the same amino acid substitution of Ser-653-Asn was revealed in both populations when compared to S. *In vitro* assays were conducted using standards for imidazolinone herbicides consisting of imazapic (99.3%) and imazapyr (99.6%) with seven concentrations (0, 0.001, 0.01, 0.1, 1, 10, and 100 µM). The results showed that the AHAS enzyme extracted from R populations and IMI-rice were less sensitive to IMI-herbicides in comparison to S and MR219. Cross-resistance to imazapic and imazapyr was also observed in R and IMI-rice populations in this assay. In conclusion, the basis of imidazolinone resistance in selected populations of Malaysian weedy rice was identified to be due to a Ser-653-Asn mutation that reduced sensitivity of the target site to IMI-herbicides. The current study presents the first report of resistance mechanism in weedy rice in Malaysian rice fields.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KERINTANGAN KEPADA RACUN HERBA IMIDAZOLINONE DALAM PADI ANGIN DI MALAYSIA

Oleh

RABIATULADAWIYAH BINTI RUZMI

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Pakej penanaman padi menggunakan racun rumpai IMI adalah di antara kaedah yang paling berkesan untuk mengawal padi angin pada masa kini. Walau bagaimanapun, kebergantungan tunggal dan pengabaian kepada penggunaan racun rumpai imidazolinone (IMI) yang sesuai dalam pakej tersebut oleh penanam padi telah menghasilkan padi angin yang rintang kepada racun rumpai tersebut. Oleh itu, kajian ini dijalankan untuk menjelaskan kejadian, paras, dan mekanisma kerintangan terhadap racun rumpai IMI pada populasi padi angin yang telah dilaporkan rintang (R) yang dikutip di tiga sawah padi IMI di Malaysia. Populasi padi angin R dibandingkan dengan populasi padi angin rentan (S), kultivar padi yang rintang kepada imidazolinone (padi IMI), dan kultivar padi tempatan yang rentan (MR219). Eksperimen tindak balas dos telah dijalankan untuk menilai sensitiviti semua populasi (A, B, C, S, padi IMI, dan MR219) terhadap racun herba IMI komersial (campuran imazapic dan imazapyr) dalam bioesei biji benih (penilaian imazapic sebagai pra-cambah) dan bioesei keseluruhan pokok (penilaian imazapyr sebagai lepas-cambah). Biji benih dan pokok untuk semua populasi telah dirawat dengan tujuh dos racun rumpai IMI yang berbeza. Berdasarkan kuantifikasi Indeks Rintang (IR) dalam bioesei biji benih, populasi A dan B masing-masing mempunyai paras kerintangan yang tinggi dan sederhana terhadap racun rumpai IMI. Populasi C, S, dan MR219 kesemuanya sensitif terhadap racun rumpai tersebut (rentan). Sama seperti populasi A, padi IMI mempunyai kerintangan yang tinggi terhadap racun rumpai IMI dengan nilai IR 50.5. Dalam bioesei keseluruhan pokok, populasi A, B, dan C mempunyai paras kerintangan yang rendah terhadap racun rumpai IMI. Manakala kedua-dua populasi S dan MR219 pula sensitif pada racun rumpai IMI. Padi IMI mencatatkan paras kerintangan yang sederhana terhadap racun rumpai IMI dalam penilaian keseluruhan pokok. Secara keseluruhannya, populasi A dan B adalah lebih rintang kepada imazapic berbanding imazapyr, sementara sebaliknya populasi C lebih rintang kepada imazapyr berbanding imazapic. Populasi S dan MR219 adalah sensitif kepada kedua-dua racun rumpai IMI tersebut. Padi IMI SPC didapati lebih rintang kepada imazapic berbanding imazapyr.

Pola kerintangan bersilang terhadap racun rumpai imazapic dan imazapyr pada populasi padi angin rintang (R) dan padi IMI juga jelas dikenalpasti dalam eksperimen ini. Kajian molekular telah dijalankan dengan membandingkan jujukan gen acetohydroxyacid synthase (AHAS) antara padi angin R (A, B, dan C), S, padi IMI, dan MR219. Jujukan 1884 pasangan bes serpihan gen AHAS menunjukkan bahawa S dan MR219 adalah 99% sama. Ternyata, jujukan gen AHAS dalam padi angin R didapati sama dengan jujukan gen AHAS dalam padi IMI di mana, penggantian asid amino yang sama di Ser-653-Asn dijumpai dalam kedua-dua populasi apabila dibandingkan dengan populasi rentan (S). Ujian *in vitro* telah dijalankan dengan menggunakan racun rumpai IMI standard yang terdiri daripada imazapic (99.3%) dan imazapyr (99.6%) dalam tujuh kepekatan (0, 0.001, 0.01, 0.1, 1, 10, dan 100 µM). Keputusan menunjukkan bahawa enzim AHAS yang diekstrak daripada populasi R dan padi IMI adalah kurang sensitif terhadap racun rumpai IMI berbanding S dan MR219. Kerintangan bersilang terhadap imazapic dan imazapyr juga dilihat dalam populasi padi angin R dan padi IMI dalam ujian ini. Kesimpulannya, asas kerintangan kepada racun rumpai IMI dalam populasi padi angin yang dikaji telah dikenalpasti disebabkan oleh mutasi Ser-653-Asn yang mengurangkan sensitiviti pada tapak-sasaran kepada racun rumpai IMI. Kajian ini merupakan kajian pertama yang melaporkan mekanisma kerintangan kepada racun rumpai IMI pada padi angin di Malaysia.

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious and the Most Merciful

Alhamdulillah, all praises to Allah for the strengths and His blessings in completing this thesis. Special appreciation goes to my supervisor, Assoc. Prof. Dr. Muhammad Saiful Ahmad Hamdani for his supervision and constant support. The door to his office was always open whenever I ran into a trouble spot or had a question about my research or writing. He consistently allowed this thesis to be my own work, but steered me in the right direction whenever he thought I needed it. Thank you for giving me the opportunity to attend conferences and meet so many interesting people.

I would also like to thank my associate supervisors Prof. Dr. Abdul Shukor Juraimi and Assoc. Prof. Dr. Norida Mazlan for their encouragement, suggestions, and insightful comments in the completion of this project and thesis.

I would like to acknowledge the Department of Crop Science, Faculty of Agriculture, UPM, especially members and staffs of those laboratories; Weed Science, Breeding, Agro-gene Bank, Seed Technology, and Plant Physiology for the laboratory facilities and their kind assistance. Special appreciation also goes to the staff of Department of Plant Protection, Institute of Tropical Agriculture and Food Security (ITAFoS), and Muda Agricultural Development Authority (MADA) for the assistance and cooperation.

I am grateful for the funding source that allowed me to pursue my study, the Ministry of Higher Education for providing financial assistance in the form of MyMaster to pursue my study at UPM. My sincere thanks to UPM for the Graduate Research Fellowship (GRF), Special Graduate Research Allowance (SGRA), and Putra Grant – Putra Graduate Initiative (IPS) for the financial assistance and research funding.

Special thanks goes to my groupmate and postgraduate friends especially Anis, Dr. Azua, Ilya, Dr. Anisah, Huda, Farahin, Arina, Iffah, Anna, Eastiharah, Aimi, Zarina, Dila, Shahara, Syafiq, Wani, and Zul for their kindness and moral support during my study. Thanks for the friendship and memories.

Finally, I must express my very profound gratitude to my late parents especially my late mother, losing you in the middle of this journey is a difficult thing, but the piece of spirit you have left for me will always be alive. I would like to also thank my siblings, my step-parent, and my family in laws for their support and encouragement.

I owe thanks to a very special person, my husband Sayed Azrin for his continued and unfailing love, support and understanding during my study that made the completion of this thesis possible. You were always around at times I thought that it is impossible to continue. I appreciate my baby, my little girl Sharifah Dahlia for abiding my ignorance and the patience she showed during my thesis writing. I also place on record, my sense of gratitude to everyone, who directly and indirectly has lent his or her hand in this venture. Thank you very much.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

3D	Three-dimensional
AHAS	Acetohydroxyacid synthase
AHB	2-aceto-2-hydroxybutyrate
AL	2-acetolactate
ALS	Acetolactate synthase
ANOVA	Analysis of Variance
*At*AHAS	*Arabidopsis thaliana* AHAS
BCAA	Branched-chain amino acids
BSA	Bovine serum albumin
CPS	Clearfield Production System
CTAB	Cetyltrimethylammonium bromide
DAT	Day after treatment
DNA	Dioxyribonucleic acid
DOA	Department of Agriculture
DTT	Dithiothreitol
EMS	Ethyl methanesulfonate
FAD	Flavin adenine dinucleotide
FAO	Food and Agriculture Organization
GR_{50}	Growth reduction at 50%
I_{50}	Inhibition at 50%
IMI	Imidazolinone
IMI-herbicides	Imidazolinone-herbicides
IMI-rice	Imidazolinone-rice
IMI-TR	Imidazolinone-tolerant
LD_{50}	Lethal dose at 50%
LSU	Lousiana State University
MARDI	Malaysian Agriculturw Research and Development Institute

PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulphonyl fluoride
PTB	Pyrimydinyl-thiobenzoate
R	Resistant
RI	Resistance Index
S	Susceptible
SAS	Statistical Analysis Software
*Sc*AHAS	*Saccharomyces cerevisiae* AHAS
SCT	Sulfonyl-aminocarbonyl-triazolinone
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat
SU	Sulfonyl-urea
ThDP	Thiamine diphosphate
TP	Triazolopyrimidine
TPP	Thiamine pyrophosphate

CHAPTER 1

INTRODUCTION

Rice (*Oryza sativa* L.) has been consumed as a staple food for more than half of world population. In Malaysia, approximately 10% (689,700 ha) of the total arable lands are allocated for rice cultivation (DOA, 2018). Malaysia is currently producing about 2.51 million tons of rice at an average of 4.4 t/ha (DOA, 2018; FAO, 2017), which equals to only 73% of the national sufficiency level. Several constraints to high rice production in Malaysia have been recognised, ranging from water scarcity, unfavorable soil condition, poor crop management practices, pests, diseases, and weeds. Weeds have been known as among major contributors to reduction in rice yield, and in direct-seeded rice fields, weedy rice infestation is the most problematic weed identified (Burhanuddin Al-Helmy et al., 2015; Baki et al., 2012; Baki, 2006).

Weedy rice (*Oryza sativa* L. complex) has continuously become a long unsettled weed problem in the Malaysian rice granaries since the establishment of direct seeding method in the early 1980s (Azmi and Abdullah, 1998). It is closely related to cultivated rice, making the use of selective herbicides to control weedy rice is limited (Chauhan, 2013). The use of herbicide-resistant rice cultivars is another strategy advocated by researchers for selective control of weedy rice in cultivated rice. Imidazolinone (IMI)-resistant rice (IMI-rice) is the only herbicide-resistant rice that has been commercialized hitherto.

The imidazolinone group of herbicides is an acetohydroxyacid synthase (AHAS) inhibitor, has broad-spectrum weed control perspective, applicable as pre- and post-emergence, possesses residual and soil activity, and has a favourable environmental profile. To date, Malaysia is the only country in Asia that commercialized IMI-rice variety. This herbicide resistant rice system was launched in late 2010, as a result of collaborative project between BASF Malaysia and MARDI, and known as Clearfield® Rice Production System (CPS). The CPS practice consists of the combination of IMI-rice resistant varieties (MR 220CL1 and MR 220CL2) with combination of acetohydroxyacid synthase (AHAS) IMI-herbicides imazapyr and imazapic (OnDuty™ WG), and Clearfield® Stewardship guide (Azmi et al., 2012a).

Despite of the advantages of herbicide-resistant rice technology at providing opportunities for selective weedy rice control, the continuous use of the same herbicide in the cultivation system and risk of gene transfer from herbicide resistant rice to weedy rice possess a serious threat for the long-term utility of this technology, which is the possibility for weedy rice and other weed species to develop resistance to the herbicide used. Since the first report in 1957, herbicide resistance phenomenon has increased dramatically, where currently 256 weed species are reported to evolve resistance to various herbicides that originally were effective (Heap, 2019). Malaysia is among countries facing herbicide resistant problem, predominantly in the plantation, vegetable farm, and rice fields. Since 1989, 16 weed species have been

recorded developed resistance to various herbicides, where 8 species are rice field weeds, and most of the resistance reported cases are to synthetic auxin and AHAS herbicides (Heap, 2019).

Acetohydroxyacid synthase (AHAS, EC 2.2.1.6) is the first enzyme that catalyzes the biosynthetic pathway for the branched-chain amino acids valine, leucine, and isoleucine, crucial for protein synthesis. The AHAS enzyme is the target site for a large number of AHAS herbicides such as sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine, pyrimidinyl-thiobenzoates, and sulfonyl-aminocarbonyl-triazolinone (Powles and Yu, 2010). In Malaysian rice fields, AHAS herbicides bensulfuron-methyl, metsulfuron-methyl, and imidazolinones (imazapyr and imazapic) have been widely used for effective weed control, mainly as pre-emergent and early post-emergent herbicides. AHAS herbicides have been recognised as the most important and widely used herbicides in the world (Liu et al., 2013; Powles and Yu, 2010; Tranel and Wright, 2002). Consequently, AHAS herbicides have also recorded the highest resistance cases, with 161 species have evolved resistance to this herbicide worldwide (Heap, 2019). Hence, an initiative was taken to investigate the occurrence and mechanisms endowing resistance to IMI-herbicide in weedy rice populations. The research focuses on the evolution and molecular basis of resistance to IMI-herbicides in weedy rice populations in IMI-rice fields.

Specific objectives of the research were to:

1. Determine the occurrence and level of resistance to AHAS IMI-herbicides in weedy rice populations collected from IMI-rice fields in Kampung Simpang Sanglang, Perlis, Kampung Behor Mentalon, Perlis and Kampung Sungai Kering, Kedah.
2. Investigate the AHAS gene mutations conferring target site resistance mechanism to AHAS IMI- herbicides in the confirmed-resistant weedy rice populations.
3. Elucidate the existence of possible non-target site resistance mechanisms in the resistant weedy rice individuals by comparing AHAS enzyme activity and inhibition by IMI-herbicides.

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