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RESISTANCE MECHANISM OF Limnocharis flava (L.) BUCHENAU TO ACETOHYDROXY ACID SYNTHASE INHIBITORS

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By

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Limnocharis flava, a problematic weed of rice fields, is reported to have multiple resistance to 2,4-D (synthetic auxin), and bensulfuron-methyl (AHAS inhibitor) due to over-reliance on their use. The present study was conducted to appraise the level of resistance and cross-resistance patterns in *L. flava*, and to elucidate the mechanisms endowing its resistance. Morphological responses between susceptible (S) and resistant (R) populations were evaluated with a view of addressing the species resistance profile. The levels and patterns of resistance in the R population were determined by LD_{50} and GR_{50} values. Seven AHAS inhibitors (bensulfuron-methyl, metsulfuron-methyl, pyrazosulfuron-ethyl, pyribenzoxim, imazethapyr, penoxsulam, and bispyribac-sodium) were examined for their efficacy at different dosages against the S and R populations, respectively. It was found that the S population was successfully controlled by all AHAS inhibitors but the R population survived the applications of the first four from seven tested inhibitors, indicating high-level of resistance to bensulfuron-methyl, with various levels of cross-resistance to other AHAS inhibitors. To demonstrate the mechanisms endowing resistance in the R population, molecular investigation was carried out by AHAS amino acids sequence comparison on R plants that survived the inhibitors application. Evidently, the plants were endowed with similar AHAS gene mutation of single nucleotide polymorphism (SNP) (GAC to GAG). This resulted in Asp substitution by Glu at amino acid position 376, suggesting point mutation as the molecular basis of resistance. *In vitro* assays were conducted using standards for AHAS inhibitors consisting of bensulfuron-methyl (99.6%), metsulfuron-methyl (99.2%), pyrazosulfuron-ethyl (99.1%), and pyribenzoxim (99.8%) with seven concentrations (0, 0.001, 0.01, 0.1, 1, 10, and 100 µM). The I_{50} of *in vitro* AHAS activity showed that R population was >83333-, 398-, 172-, and 48-fold greater than S population for the respective AHAS inhibitors. This suggests stronger cross-resistance to sulfonylureas than to pyrimidinyl (thio) benzoate (pyribenzoxim). The result of non-target site mechanism experiments showed that S and R populations did not differ significantly in their ability to absorb, translocate, and metabolize bensulfuron-methyl. This indicates that non-target site resistance mechanisms of differential metabolism, absorption, and translocation did not confer resistance in the R population. The morphological and physiological characteristics of AHAS inhibitors on S and R populations were examined using progenies of AHAS herbicide-resistant (R) individuals that survived the application of bensulfuron-methyl (Progeny 1) and pyribenzoxim (Progeny 2). Both R and S plants were compared in terms of fresh weight, dry weight, height, epicuticular wax weight, stomata density, leaf area index, and leaf micromorphology for growth assessment. Net photosynthetic rate, stomatal conductance, intercellular carbon dioxide, and transpiration rate were monitored using Progeny 1 from R population with S plants as control. The results showed that R plants carrying AHAS Asp-376-Glu mutation did not differ in the morpho-physiological characteristics and were comparable to that of S plants. In conclusion, the basis of AHAS-inhibitors resistance in *L. flava* was identified to be due to an Asp-376-Glu mutation that reduced sensitivity of the target site to AHAS inhibitors. The current study presents the first report of resistance mechanism in *L. flava*.

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MEKANISASI RINTANGAN OLEH *Limnocharis flava* (L.) Buchenau TERHADAP PENGHALANG ACETOHYDROXY ACID SYNTHASE

Oleh

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Limnocharis flava, spesies rumpai akuatik bermasalah di sawah padi, telah dilaporkan mempunyai kerintangan berbilang kepada racun rumpai yang berasaskan 2,4-D (auksin sintetik) dan bensulfuron-methyl (penghalang AHAS) oleh kerana pergantungan berlebihan terhadap penggunaannya. Kajian ini dijalankan untuk menilai pola kerintangan serta kerintangan bersilang pada *L. flava* dan untuk mengkaji mekanisma kerintangan tersebut. Reaksi morfologi antara populasi rentan (S) dan rintang (R) telah dikaji dengan tujuan penambahan maklumat dalam profil kerintangan pada spesies tersebut. Paras dan corak kerintangan dalam populasi R telah ditentukan dengan nilai LD_{50} dan GR_{50}. Tujuh penghalang AHAS (bensulfuron-methyl, metsulfuron-methyl, pyrazosulfuron-ethyl, pyribenzoxim, imazethapyr, penoxsulam, dan bispyribac-sodium) telah dikaji keberkesanannya pada dos yang berbeza terhadap populasi S dan R. Keputusan menunjukkan bahawa populasi S telah dapat dikawal keseluruhannya oleh kesemua penghalang AHAS tetapi populasi R terselamat daripada aplikasi empat penghalang pertama daripada tujuh penghalang AHAS yang diuji, menunjukkan daya ketahanan yang tinggi terhadap bensulfuron-methyl dengan beberapa paras kerintangan terhadap penghalang AHAS yang lain. Untuk mengkaji mekanisma kerintangan dalam populasi R, kajian molekular telah dijalankan dengan menggunakan kaedah perbandingan jujukan asid amino pada AHAS ke atas pokok R yang terselamat daripada aplikasi penghalang AHAS. Semua pokok R didapati mempunyai gen mutase yang serupa pada AHAS melalui polimorfisma nucleotid tunggal (SNP) (GAC to GAG). Ini menghasilkan penggantian Asp oleh Glu pada asid amino berkedudukan 376, sekaligus mentafsirkan mutasi tunggal sebagai asas mekanisma kerintangan. Ujian *in vitro* telah dijalankan dengan menggunakan penghalang AHAS standard yang mengandungi bensulfuron-methyl (99.6%), metsulfuron-methyl (99.2%), pyrazosulfuron-ethyl (99.1%), and pyribenzoxim (99.8%) dalam tujuh kepekatan (0, 0.001, 0.01, 0.1, 1, 10, and 100 µM). Aktiviti I_{50} bagi *in vitro* AHAS menunjukkan bahawa populasi R adalah >83333-, 398-, 172- dan

48-kali lebih tinggi ketahanannya daripada populasi S untuk setiap penghalang AHAS. Ini menunjukkan bahawa populasi R mempunyai kerintangan bersilang lebih tinggi terhadap sulfonylureas berbanding pyrimidinyl(thio)benzoate (pyribenzoxim). Keputusan eksperimen untuk mekanisma luar tapak-sasaran menunjukkan bahawa tiada perbezaan ketara antara populasi S dan R dalam keupayaan menyerap, mengangkut, dan memetabolisma bensulfuron-methyl. Ini membuktikan bahawa mekanisma luar tapak-sasaran iaitu perbezaan metabolisma, penyerapan, dan translokasi tidak menyumbang kepada kerintangan dalam populasi R. Sifat morfologi dan fisiologi penghalang AHAS ke atas populasi S dan R juga telah dikaji dengan menggunakan generasi rintang (R) daripada individuyang terselamat daripada rawatan bensulfuron-methyl (Progeny 1) dan pyribenzoxim (Progeny 2). Kedua-dua pokok R dan S telah dibandingkan dari segi berat segar, berat kering, ketinggian, berat lilin epikutikular, ketumpatan stomata, indeks keluasan daun, dan mikromorfologi daun bagi menilai tumbesaran. Kadar fotosintesis bersih, konduksian stomata, karbon dioksida antara sel, dan kadar transpirasi telah dipantau pada Progeny 1 dari populasi R dengan pokok S sebagai kawalan. Keputusan menunjukkan bahawa pokok R yang mempunyai mutasi AHAS Asp-376-Glu adalah tidak berbeza pada sifat morfo-fisiologi apabila dibandingkan dengan pokok S. Pada kesimpulan nya, asas kerintangan terhadap penghalang AHAS pada populasi R telah dikenalpasti disebabkan oleh mutasi Asp-376-Glu dalam gen AHAS yang mengurangkan kepekaan tapak-sasaran kepada penghalang AHAS. Kajian ini merupakan kajian pertama yang melaporkan mekanisma kerintangan kepada penghalang AHAS pada *L. flava*.

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

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LIST OF ABBREVIATIONS

%	Percent
^0C	Degree celsius
m^2	Square meter
cm	Centimeter
ha	Hectare
ml	Milliliter
nm	Nanometer
g	Gram
cm^2	Square centimeter
rpm	Revolution per minute
v/v	Volume per volume
m	Meter
ai	Active ingredient
g ai/ha	Gram a.i. per hectare
S	Susceptible
R	Resistant
TSR	Target site resistance
NTSR	Non-target site resistance
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA

LD_{50}	Lethal dose at 50%
GR_{50}	Growth reduction at 50%
I_{50}	Inhibition at 50%
RCBD	Randomized complete block design
HPLC	High Performance Liquid Chromatography
ANOVA	Analysis of variance
SE	Standard error
µM	Micromolar
LSD	Least significant differences
FAD	Flavin adenine dinucleotide
TPP	Thiamine pyrophosphate
Mg^{2+}	Magnesium
SU	Sulfonylurea
IMI	Imidazolinone
TP	Triazolopyrimidine
PTB	Pyrimidinyl(thio)benzoate
SCT	Sulfonyl-aminocarbonyl-triazolinone
HRAC	Herbicide Resistance Action Committee
WSSA	Weed Science Society of America
ng $^{g-1}$	Nanogram per gram
ppt	Parts per trillion
AHAS	Acetohydroxyacid synthase

MARDI	Malaysian Agriculture Research and Development Institute
DAS	Days after sowing
IC_{50}	Inhibitory Concentration of 50%
GST	Glutathione S-Transferase
MADA	Muda Agricultural Development Authority
KADA	Kerian Integrated Agriculture Development Authority
KETARA	North Terengganu Integrated Agriculture Development
Kg/ha	Kilogram per hectare
Ca	Calcium
Fe	Iron
PCR	Polymerase chain reaction
Kb	Kilobases
DTT	Dithiothreitol
PMSF	Phenylmethylsulphonyl fluoride
KH_2PO_4 / K_2HPO_4	Potassium phosphate
$(NH_4)_2SO_4$	Ammonium sulphate
ACCase	Acetyl coenzym-A carboxylase
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
PSII	Photosystem II
HAT	Hours after treatment
SEM	Scanning electron microscope

CHAPTER 1

INTRODUCTION

Agriculture is an important sector of Malaysia's economy. It has contributed significantly to the national Gross Domestic Product (GDP), providing employment for a good percentage of the population. Rubber and oil palm are the two main crops that have dominated the agricultural exports although the Malaysian share of the world's production of these crops has declined over the past years.

Rice is a staple food in the daily diet of Malaysians. It is a symbol of traditional Malaysian culture. Rice plays an important part in the country's agriculture although the overall production has yet to meet the country's needs. According to The National Agrofood Policy of Malaysia, 2011-2020, local rice production should be increased to ensure the country’s future demand. Rice production has been facing several constraints. Crop pests including weeds, pathogens, insects, rodents as well as birds have always been acknowledged as among the most devastating factors that significantly reduce production. Among other factors include overuse of pesticides, excessive nitrogen fertilizer applications and unpredictable weather conditions.

Weeds have always been a major pest in most rice production areas, be it in Malaysia or anywhere else in the world. Weeds are generally accepted as plants growing where they are not wanted. Most of the weeds co-evolved with crops and in some cases, they were ancestors of cultivated plant species. The common weeds in rice fields are frequently legacies of previous years’ crops which are seeds, rhizomes, tubers and bulb surviving in the soil. The general impact of weeds on rice fields includes increase in production costs as costs of weed control, act as secondary hosts for other insect pests, effect on harvesting and grain quality, and blockage of irrigation canals caused by invasive and persistent aquatic weed species.

To overcome weed problem in rice fields, farmers generally depend on herbicides for their control. Before the introduction and use of modern herbicides, rice farmers depended on hand weeding, ploughing or tillage, crop rotations as the main methods of weed control. However, these were not sustainable in vast fields because of high labour requirements and time-consuming issues. The introduction of herbicides for weed control has a huge relief to farmers all over the world. Among the modern herbicides, synthetic auxin, 2,4-D was developed for the control of broadleaf weeds. Following this introduction, the use of herbicides has been the most rapidly adopted strategy by farmers for the management of weeds in rice as well as in other crops production entities. Without doubt, it has become the most dependable and reliable weed control method (Heap, 2014). In Malaysian rice fields, herbicides have been extensively used for the control of broad-spectrum weed species.

There are numerous herbicide modes of action that have been used to control weeds. One of these most widely used herbicides is acetohydroxyacid synthase (AHAS) herbicides/inhibitors. Currently, there are 26 different herbicide sites of action being documented (Heap, 2017); including having various residual properties, selectivity, application times, and crop-type application suitability. Although herbicides are highly preferred by farmers because of their high efficacy in weed management, over-dependent on and overuse of a particular herbicide or groups of herbicides having similar mode of action is conceivable to result in weed species developing resistance towards the herbicides used. It has been reported that there are 485 cases of weeds resistant to herbicides with 252 weed species reported to have developed resistance to 163 herbicides in 92 crops over 69 countries (Heap, 2017). Among the herbicide groups, AHAS inhibitors top the list with the highest herbicide resistant cases of 159 weed species worldwide, including Malaysia (Heap, 2017).

Demand for herbicides for efficient weed control has always been high in Malaysian rice fields. Unfortunately, continuous dependence on herbicides has caused some weed species to develop resistance to the herbicides used. A total of 16 species have been recorded as herbicide resistant weeds, predominantly in vegetable farms, plantations, and rice fields (Heap, 2017). Of these, 9 species are weeds commonly found in rice field, namely (in the alphabetical order) *Bacopa rotundifolia* (Michx.) Wettst, *Echinochloa crus-galli* (L.) P. Beauv., *Fimbristylis quinquangularis* (Vahl) Kunth, *Leptochloa chinensis* (L.) Nees, *Limnocharis flava* (L.) Buchenau, *Limnophila erecta* Benth, *Oryza sativa* L. complex, *Sagittaria guayanensis* Kunth, and *Sphenoclea zeylanica* Gaertn. (Ruzmi et al., 2017; Dilipkumar et al., 2017). These weed species have been reported either resistant to single herbicide, or cross-resistant to several herbicides having similar mode of action, or multiple resistant to several herbicide modes of action. Among these herbicide-resistant weed species, *Limnocharis flava* has been recognized as one of the most common in Malaysian rice fields (Heap, 2017). This species was reported to have developed the resistance in 1998, and is the first weed species that evolved multiple resistance to both bensulfuron-methyl (AHAS inhibitors) and 2,4-D (synthetic auxins) (Heap, 2017). The species has become invasive in many rice fields, causing farmers to cost thousands of ringgit in terms of control and yield loss through competition, as well as blocking irrigation canals. To date, there has been no record on the resistance in terms of patterns and mechanisms for this aquatic weed species, resulting in lack of information on effective and efficient management of the species.

The present study specifically focused on the resistance evolution across AHAS inhibitors and aimed at defining the mechanisms conferring resistance to these herbicides in *L. flava* populations in Malaysian rice fields. The specific objectives were:

1. To quantify and characterize the level of resistance and cross-resistance patterns to AHAS inhibitors in a resistant *L. flava* population infesting rice fields;
2. To elucidate target-site AHAS gene mutation endowing resistance to AHAS inhibitors at the molecular and enzyme levels of the resistant *L. flava* population;
3. To identify the co-existence of possible non-target site resistance mechanism(s) from resistant *L. flava* individuals carrying no known AHAS gene mutation(s) by comparing the uptake, translocation, and metabolism of AHAS inhibitors with that of a susceptible population;
4. To determine and compare the morphological and physiological responses between susceptible and resistant *L. flava* populations in the presence and absence of AHAS inhibitor.

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