doi: 10.17221/131/2016-PPS

Patterns of Resistance to AHAS Inhibitors in *Limnocharis flava* from Malaysia

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Abstract

Zakaria N., Ahmad-Hamdani M.S., Juraimi A.S.: Patterns of resistance to AHAS inhibitors in *Limnocharis flava* from Malaysia. Plant Protect. Sci.

Limnocharis flava (L.) Buchenau is among the most problematic rice weeds in Malaysia and is also reported to have developed multiple resistance to AHAS inhibitor bensulfuron-methyl and synthetic auxin 2,4-D. In this study, resistance across different AHAS inhibitors was characterised in a *L. flava* population infesting rice fields in Pulau Pinang, Malaysia. Dose-response experiments were conducted to determine the level of resistance to sulfonylureas, imidazolinone, triazolopyrimidine, and pyrimidinyl-thiobenzoate. Cross-resistance across different AHAS inhibitors was observed in the resistant *L. flava* population, exhibiting a high level of resistance to bensulfuron-methyl, while exhibiting a moderate level of resistance to metsulfuron-methyl and a low level of resistance to pyrazosulfuron-ethyl and pyribenzoxim. However, all resistant *L. flava* individuals were still sensitive to imazethapyr, penoxsulam, and bispyribac-sodium. Based on the results, it is likely that resistance to AHAS inhibitors in *L. flava* is conferred by target-site resistance mechanisms.

Keywords: acetohydroxyacid synthase; herbicide resistance; perennial weed; rice; Sawah flowering rush

Acetohydroxyacid synthase (AHAS, EC2.2.1.6), also known as acetolactate synthase (ALS), is the first enzyme involved in the biosynthetic pathway of synthesis of branched amino acids valine, leucine, and isoleucine. These amino acids are essential for plant growth and development, and their absence results in the rapid inhibition of root and shoot growth, eventually leading to plant death (Lamego *et al*. 2009). AHAS inhibitors include five classes of herbicides: sulfonylurea (SUs), imidazolinone (IMIs), triazolopyrimidinyl-thiobenzoates (PTBs), triazolopyrimidine (TPs), and sulfonylaminocarbonyl-triazolinones (SCTs) (Powles & Yu 2010).

AHAS inhibitors have high efficacy, possess broad-spectrum weed control, low mammalian toxicity while selectivity in major world crops made these herbicides favoured for global and intensive use in many different crops over wide regions (Tranel & Wright 2002; Powles & Yu 2010; Liu *et al*. 2013). It was suggested that the frequent occurrence of AHAS inhibitor resistant weeds can be attributed to the large number of AHAS inhibitor herbicides which is twice as many as herbicides in other groups and the way how the herbicides have been used, the exertion of strong selection pressure, soil residual activity, and resistance mechanism (Tranel & Wright 2002; Heap 2014). The first reported case of AHAS inhibitor resistance was SU herbicide (chlorsulfuron) in *Lolium rigidum* Gaud. in Australia (Heap & Knight 1982, 1986), followed by *Lactuca serriola* L. in the USA in 1987 (Mallory-Smith *et al*. 1990). Currently, resistance to AHAS inhibitors has been documented in 158 weed species from 44 countries. In fact, cases of weeds that have evolved resistance to this group of herbicides are faster than in any other group of herbicides (Heap 2016). In Malaysia,

Supported by the Ministry of Higher Education Malaysia, Fundamental Research Grant Scheme 07-01-04-1440FR.

doi: 10.17221/131/2016-PPS

15 species have been reported to develop herbicide resistance, from single to multiple herbicide modes of action (Heap 2016). Among all these resistant weed species, 7 species are common weeds in rice and *L. flava* is among the most problematic aquatic weed species in irrigated Malaysian rice fields.

Sawah flowering rush (*Limnocharis flava* (L.) Buchenau) is a monocotyledon weed in the family Limnocharitaceae/Alismataceae. *Limnocharis flava* is a common and competitive weed in irrigated and rainfed lowland rice fields in Malaysia (Juraimi *et al*. 2012; Weber & Brooks 2013). This broad-leaved weed is a perennial plant in humid climate regions but behaves as an annual herb in ephemeral water bodies and sites with definite dry seasons, moreover this tropical species prefers moist regions, ranging from saturated to full flooded conditions (Weber & Brooks 2013). *Limnocharis flava* has high seed production when 1 million seeds are potentially produced by a single plant per year (Weber & Brooks 2013), although *L. flava* is also capable to reproduce vegetatively through peduncles. The peduncle is assumed having a similar role to that of a stolon (Kotawala 1976). The flower is hermaphrodite and pollinating agents within Limnocharitaceae are poorly understood (Weber & Brooks 2013). In Malaysia, this weed first evolved multiple resistance to herbicides in the Groups B/2 and O/4 in 1998 in Pulau Pinang and nowadays is infesting many rice fields, predominantly during the main planting season. This particular population has been confirmed to develop resistance to 2,4-D and bensulfuron-methyl (Juraimi *et al*. 2012) and it is believed that it may already be cross-resistant to other herbicides in the Groups B/2 and O/4 (Heap 2016).

The present study aims to characterise resistance patterns across AHAS inhibitors in a *L. flava* population collected in Pulau Pinang, Malaysia. This information is crucial for quantifying the current resistance status towards AHAS inhibitors, further developing potential management strategies to control this resistant population.

MATERIAL AND METHODS

Seed collection and plant material. Seeds from a *Limnocharis flava* resistant population (hereinafter it will be regarded as R) were collected from commercial rice fields located in Malaysian Agriculture Research and Development Institute, Bertam, Seberang Perai, Pulau Pinang, Malaysia (5°32'37''N, 100°28'3''E). The previous study showed that this population is resistant to SU herbicide (bensulfuron-methyl) and synthetic auxin herbicide 2,4-D at various levels (Juraimi *et al*. 2012). Seeds of the herbicide-susceptible *L. flava* population (S) were collected in areas with no known herbicide exposure (2°59'16.76''N, 101°42'7.83''E). Seeds of both R and S populations were grown in the glasshouse for further seed increase under the same growing conditions to obtain enough seeds for subsequent herbicide evaluation. To avoid any chance of cross-pollination, individual plants were insulated in 350 × 450 mm micro-perforated plastic bags. All bulked seeds were air-dried and stored in air-tight plastic bags at 4°C until used in experiments.

Whole-plant herbicide dose response. All experiments were conducted in pots that were maintained in the glasshouse during the period from March to June 2015 at Universiti Putra Malaysia. In mid-March 2015, approximately 1200 seeds from each of the *L. flava* R and S populations were soaked in 0.2% potassium nitrate (KNO_3) (0.2 g KNO_3 in 100 ml H_2O) at room temperature (20–25°C) for 48 h until the seeds began to germinate. The seeds were then sown in 30-cm diameter pots containing commercial paddy soil at 2/3 full. Eight seedlings of 3 cm height from each population were transplanted at a depth of 1 cm in each pot. The pots were placed in the glasshouse maintained with a day/night temperature of 32/18°C. The plants were grown in flooded conditions (2–5 cm water depth) during the experimental period, and fertilisers were applied at N 170, P_2O_5 80, and K_2O 150 kg/ha. Seedlings at the 4–5 leaf stage (approximately 30 days old) were sprayed with selected commercial AHAS inhibitors, commonly used in rice fields to control rice weeds.

Commercial herbicide formulations were used in all studies. AHAS inhibitors bensulfuron-methyl (Buron 600, 60% w/w a.i.; Farmcochem Sdn. Bhd., Perak, Malaysia), metsulfuron-methyl (Nu-MSM 20WG, 20% w/w a.i.; Nufarm Malaysia Sdn. Bhd., Selangor, Malaysia), imazethapyr (Imaz 5.25 L, 5.2% w/w a.i.; Farmcochem Sdn. Bhd., Perak, Malaysia), bispyribac-sodium (Abimee 9.5 SC, 9.5 % w/w a.i.; Advansia Sdn. Bhd., Kuala Lumpur, Malaysia), pyrazosulfuron-ethyl (Alimin 10 BP, 10% w/w a.i.; Advansia Sdn. Bhd., Kuala Lumpur, Malaysia), penoxsulam (Rainbow, 2.67% w/w a.i.; Syngenta Crop Protection Sdn. Bhd., Selangor, Malaysia), and pyribenzoxim (Pyanchor 5EC, 5% w/w a.i.; Imaspro Resources Sdn. Bhd., Selangor, Malaysia) were evaluated for their efficacy against the resistant *L. flava* population. A compression type sprayer with

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an adjustable flat fan nozzle, delivering 200 l/ha at a spray pressure of 150 kPa, was used in this experiment. The herbicides were applied at seven rates, ranging from zero (untreated check) to eight times the recommended field rates. Herbicides were applied to both R and S populations at the following rates. The herbicides applied and their recommended field rates (indicated in parentheses) were: bensulfuron-methyl at 0, 10, 20, 40, 80, 160, 320 g a.i./ha (40 g a.i./ha), metsulfuron-methyl at 0, 3.8, 7.5, 15, 30, 60, 120 g a.i./ha (15 g a.i./ha), pyrazosulfuron-ethyl at 0, 3.5, 7, 14, 28, 56, and 112 g a.i./ha (14 g a.i./ha), imazethapyr at 0, 37.7, 75.4, 150.8, 301.6, 603.2, and 1206.4 g a.i./ha (150.8 g a.i./ha), penoxsulam at 0, 3.3, 6.7, 13.4, 26.7, 53.4, and 106.8 g a.i./ha (13.4 g a.i./ha), bispyribac-sodium at 0, 7.1, 14.3, 28.5, 57, 114, and 228 g a.i./ha (28.5 g a.i./ha), and pyribenzoxim at 0, 7.5, 15, 30, 60, 120, and 240 g a.i./ha (30 g a.i./ha).

All herbicides were sprayed in mid-April 2015. The average temperature during the experiment ranged from 25°C to 32°C, which is similar to the conditions in rice fields. Twenty-one days after herbicide treatments, survival of plants was assessed by inspecting the growing points. The plants were considered as resistant when they survived and produced new shoots/tillers following herbicide treatments and regarded as susceptible when they showed severe symptoms of stunted growth or no new active growth eventually leading to plant death, similar to the S population. Plants were harvested at 1 cm above the ground, dried at 65°C for 72 h, and weighed. The mean dry weight of all plants (dead and alive) was calculated for each population, and expressed as a percentage of the untreated controls for that population. The experiment was repeated twice.

Statistical analysis. The pots were laid out in a Randomized Complete Block Design (RCBD) with three replications per treatment. Plant survival rate and dry weight were expressed as a percentage of the untreated control value and was transformed. Both datasets were subjected to ANOVA using SAS software (Version 9.4; SAS Institute Inc., Cary, USA) to determine the level of significance. The interaction of plant populations and herbicide doses were significant at $P < 0.05$ (Table 3).

All analyses were conducted using Sigmaplot Version 11.0 (Systat Software Inc., GmbH, Erkrath, Germany). A dose-response curve was obtained by nonlinear regression using the logistic response equation (Eq. 1) proposed by Knezevic *et al.* (2007)commonly known as an effective dose (e.g., ED_{30}, ED_{50}, ED_{90}):

$$Y = c + |\,|(d - c)/|1 + \exp\{b[\log(x) - \log ED_{50}]\}\,|\,|\,| \quad (1)$$

where: c – lower limit; d – upper limit; b – slope; ED_{50} – dose required to give 50% effect

In the regression equation, the herbicide dose or concentration was the independent variable (x), and the growth response or plant survival (percentage of the control) was the dependent variable (y). The fitted equations were used to estimate the amount of herbicide causing a 50% reduction in plant dry weight (GR_{50} value) or a 50% reduction in plant survival (LD_{50} value).

When it was not possible to fit a log-logistic model to the survival rate data and dry weight data, an exponential decay model was used. Datasets were analysed by ANOVA and *LSD* ($P = 0.05$) to determine significant differences between populations and herbicide doses:

$$y = y_0 + ae^{-bx} \quad (2)$$

where: y_o – lower limit; $a + y_0$ – upper limit; b – slope; x – dose causing 50% response

For the data that did not fit the above equations (where the survival or shoot dry weight was less than a 50% reduction), the indication symbol as greater (>) than the highest rate was used in LD_{50} and GR_{50} values for each herbicide. The resistance index (RI) was calculated by dividing the estimated LD_{50} or GR_{50} value of the R population by that of the LD_{50} or GR_{50} of the S population.

RESULTS

Dose response and patterns of resistance to AHAS inhibitors. For the overall analysis of variance, all herbicides showed significant interactions at $P < 0.05$ for survival rate except for herbicides imazethapyr and penoxsulam. While for dry weight, all herbicides showed significant interactions at $P < 0.05$ except for the herbicide imazethapyr (Table 3). The results showed that the R *L. flava* population survived the applications of four AHAS inhibitors, namely bensulfuron-methyl, metsulfuron-methyl, pyrazosulfuron-ethyl, and pyribenzoxim at different levels of herbicide dosage. Nonetheless, all R individuals showed severe symptoms, similar to the S population towards AHAS inhibitors imazethapyr, penoxsulam, and bispyribac-sodium. The S population was effectively controlled at the recommended dose by all AHAS inhibitors applied.

In general, the resistant *L. flava* population exhibited a high level of resistance to bensulfuron-

doi: 10.17221/131/2016-PPS

methyl, and a moderate and low level of resistance to metsulfuron-methyl and pyrazosulfuron-ethyl, respectively. The S population was 100% controlled using bensulfuron-methyl at the recommended rate of 40 g a.i./ha, while the R population survived even at the highest rate of 320 g a.i./ha resulting in a resistance index (RI) > 109-fold (Table 1 and Figure 1), which is the highest resistance index among AHAS inhibitors. On the contrary, a substantial reduction in shoot dry weight was observed in the R population (Figure 1), bensulfuron-methyl producing the GR_{50} RI value of only 9-fold (Table 2). This indicates that although all R plants survived bensulfuron-methyl, their growth was still hampered by the herbicide.

A moderate resistance to another SU herbicide, metsulfuron-methyl, was observed in the resistant *L. flava* population (Figure 1). The RI for LD_{50} was 9-fold (Table 1) greater than in the S population. Similarly, metsulfuron-methyl was found to moderately reduce the plant growth in the R population (Table 2 and Figure 1). The GR_{50} value for the R population was 11.7 times higher than for the S population. The resistant *L. flava* population exhibited a low level of resistance to the SU herbicide pyrazosulfuron-ethyl. The pyrazosulfuron-ethyl LD_{50} value for the R population was 5.8 times higher than for the S population (Table 1 and Figure 1). Meanwhile, pyrazosulfuron-ethyl was found to have a minimum impact on R population growth, where the shoot dry weight (GR_{50}) was only 1.4 times higher than in the S population (Table 2 and Figure 1).

Pyribenzoxim and bispyribac-sodium herbicides are classified in the AHAS chemical family of PTBs. For pyribenzoxim, all R individuals exhibited a low resistance level (Figure 2) having the LD_{50} value 4.6 times higher than the S population (Table 1). Similarly, a slight reduction was observed in the GR_{50} with the RI value of 4-fold (Table 2). Based on the results, bispyribac-sodium was found to successfully control both R and S populations at the recommended dose of 28.5 g a.i./ha. Surprisingly, based on the LD_{50} and GR_{50} values, a low level of resistance was recorded to bispyribac-sodium (Figure 2), exhibiting the LD_{50} value of 6-fold and GR_{50} value of 2.3-fold greater than the S population (Tables 1 and 2).

The growth response of the R *L. flava* population to the IMI herbicide imazethapyr is shown in Tables 1 and 2. Imazethapyr was found to successfully control both S and R plants at a rate of 75 g a.i./ha, which is half of the recommended rate (150.8 g a.i./ha) (Figure 2). The respective values of LD_{50} and GR_{50} were 0 and 1-fold greater than in the S population. A similar pattern was observed for the TPs herbicide penoxsulam where both R and S populations had 100% mortality at a rate of 5 g a.i./ha which is below the recommended rate (13.4 g a.i./ha) (Figure 2). The resistant *L. flava*

Table 1. Parameters of the log-logistic analysis of the AHAS inhibitor dose required to cause 50% mortality (LD_{50}) and the resistance index (RI) of susceptible (S) and resistant (R) *L. flava* populations (standard errors are in parentheses)

Chemical class	Population	Herbicide	*c*	*d*	*b*	LD_{50} (g a.i./ha)	r^2	*P*	RI (LR_{50} R/LR_{50} S)
Sulfonylurea	S	bensulfuron-methyl	−3.43	99.97	−0.89	2.93 (2.8)	0.99	0.0014	
	R		0	101.34	−0.88	> 320	0.98	0.0010	> 109
	S	metsulfuron-methyl	–	–	–	1.28[a]	0.99	< 0.0001	
	R		−2.22	100.3	−1.73	11.77 (1.55)	0.99	0.0004	9
	S	pyrazosulfuron-ethyl	−3.72	99.72	−1.3	3.1 (0.90)	0.99	0.0015	
	R		−0.06	98.68	−6.88	17.89 (0.59)	1.00	< 0.0001	5.4
Pyrimidinyl-thiobenzoates	S	pyribenzoxim	–	–	–	4.76[a]	0.98	< 0.0001	
	R		–	–	–	25.64[a]	0.92	< 0.0001	4.6
	S	bispyribac-sodium	–		–	1.8	1.00	< 0.0001	
	R		–	–	–	10.9[a]	0.87	< 0.0001	6
Imidazolinone	S	imazethapyr	−1	−100	−3	0.00	1.00	ns	
	R		−1	−100	−3	0.00	1.00	ns	–
Triazolopyrimidine	S	penoxsulam	−1	−100	−3	0.00	1.00	ns	
	R		−1	−100	−3	0.00	0.99	ns	

LD_{50} value was calculated from parameters obtained from exponential decay (Eq. 2); ns – not significant

doi: 10.17221/131/2016-PPS

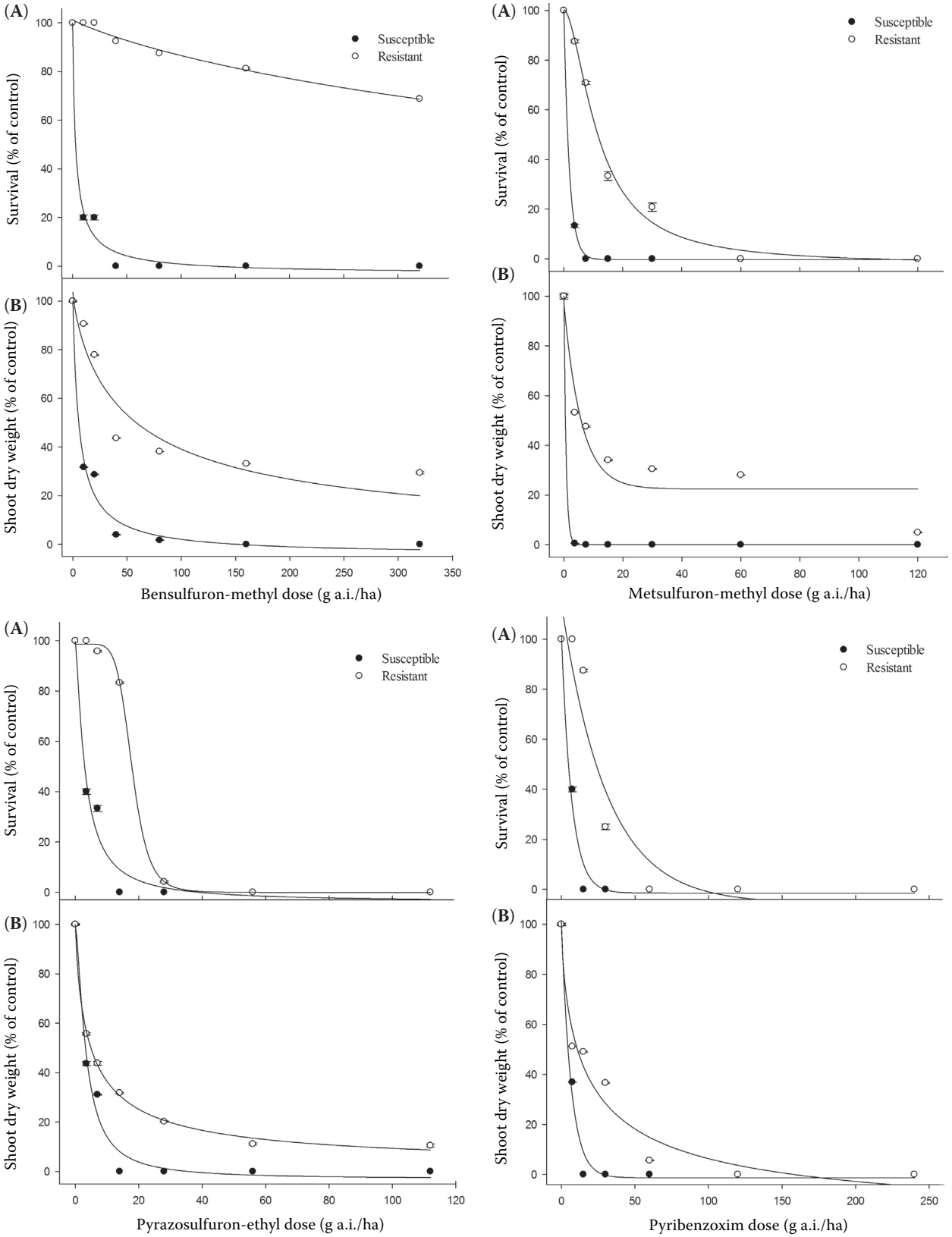

Figure 1. Survival (**A**) and shoot dry weight (**B**) of the susceptible and resistant populations of *Limnocharis flava* to bensulfuron-methyl, metsulfuron-methyl, pyrazosulfuron-ethyl, and pyribenzoxim 21 days after treatment (bars indicate the standard errors of the means of the three replicates)

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Table 3. Survival rate and dry weight of susceptible and resistant *Limocharis flava* treated with AHAS inhibitors with different herbicide rates

Herbicide	Plant types	Concentration (g a.i./ha)	Survival rate	R^2	Dry weight	R^2
Bensulfuron-methyl	susceptible	0.0	10.02 ± 0^{a}	0.83	10.02 ± 0^{a}	0.97
		10.0	3.067 ± 2.4^{b}		5.71 ± 0.43^{a}	
		20.0	3.067 ± 2.4^{b}		5.42 ± 0.63^{b}	
		40.0	0.71 ± 0^{b}		1.73 ± 1.02^{b}	
		80.0	0.71 ± 0^{b}		1.32 ± 0.61^{c}	
		160.0	0.71 ± 0^{b}		0.71 ± 0^{c}	
		320.0	0.71 ± 0^{b}		0.71 ± 0^{c}	
	resistant	0.0	10.02 ± 0^{a}	0.84	10.02 ± 0^{a}	0.94
		10.0	10.02 ± 0^{a}		9.41 ± 0.18^{a}	
		20.0	10.02 ± 0.23^{a}		8.93 ± 0.69^{a}	
		40.0	9.5933 ± 0.21^{a}		6.65 ± 0.58^{b}	
		80.0	9.3633 ± 0.4^{ab}		6.28 ± 0.39^{b}	
		160.0	8.92 ± 0.2^{b}		5.86 ± 0.55^{b}	
		320.0	8.19 ± 0.25^{c}		4.33 ± 0.96^{c}	
Metsulfuron-methyl	susceptible	0.0	10.02 ± 0^{a}	0.92	10.02 ± 0^{a}	0.99
		3.8	2.6 ± 1.9^{b}		0.91 ± 0.2^{b}	
		7.5	0.71 ± 0^{b}		0.71 ± 0^{b}	
		15.0	0.71 ± 0^{b}		0.71 ± 0^{b}	
		30.0	0.71 ± 0^{b}		0.71 ± 0^{b}	
		60.0	0.71 ± 0^{b}		0.71 ± 0^{b}	
		120.0	0.71 ± 0^{b}		0.71 ± 0^{b}	
	resistant	0.0	10.02 ± 0^{a}	0.86	10.02 ± 0^{a}	0.99
		3.8	9.36 ± 0.39^{a}		7.33 ± 0.32^{b}	
		7.5	8.42 ± 0.48^{a}		6.93 ± 0^{b}	
		15.0	4.82 ± 2.3^{b}		5.86 ± 0.14^{c}	
		30.0	3.12 ± 0^{bc}		5.57 ± 0.22^{c}	
		60.0	0.71 ± 0^{c}		5.36 ± 0.17^{c}	
		120.0	0.71 ± 0^{c}		2.33 ± 0.24^{d}	
Pyrazosulfuron-ethyl	susceptible	0.0	10.02 ± 0^{a}	0.82	10.02 ± 0^{a}	0.98
		3.5	5.35 ± 2.43^{b}		7.12 ± 0.93^{b}	
		7.0	4.74 ± 2.39^{b}		5.6 ± 0.47^{c}	
		14.0	0.71 ± 0^{c}		0.71 ± 0^{d}	
		28.0	0.71 ± 0^{c}		0.71 ± 0^{d}	
		56.0	0.71 ± 0^{c}		0.71 ± 0^{d}	
		112.0	0.71 ± 0^{c}		0.71 ± 0^{d}	
	resistant	0.0	10.02 ± 0^{a}	0.99	10.02 ± 0^{a}	0.88
		3.5	10.02 ± 0^{a}		7.47 ± 0.23^{b}	
		7.0	9.81 ± 0.21^{a}		6.53 ± 0.88^{bc}	
		14.0	9.15 ± 0.23^{a}		5.66 ± 0.45^{bc}	
		28.0	1.68 ± 0.97^{b}		4.52 ± 0.48^{cd}	
		56.0	0.71 ± 0^{b}		3.14 ± 0.80^{d}	
		112.0	0.71 ± 0^{b}		2.57 ± 1.31^{d}	

doi: 10.17221/131/2016-PPS

Table 2 to be continued

Herbicide	Plant types	Concentration (g a.i./ha)	Survival rate	R^2	Dry weight	R^2
Pyribenzoxim	susceptible	0.0	10.02 ± 0[a]	0.9	10.02 ± 0[a]	0.99
		7.5	5.42 ± 2.4[b]		6.12 ± 0.06[b]	
		15.0	0.71 ± 0[c]		0.71 ± 0[c]	
		30.0	0.71 ± 0[c]		0.71 ± 0[c]	
		60.0	0.71 ± 0[c]		0.71 ± 0[c]	
		120.0	0.71 ± 0[c]		0.71 ± 0[c]	
		240.0	0.71 ± 0[c]		0.71 ± 0[c]	
	resistant	0.0	10.02 ± 0[a]	0.95	10.02 ± 0[a]	0.97
		7.5	10.02 ± 0[a]		7.23 ± 0.32[b]	
		15.0	9.36 ± 0.38[a]		7.08 ± 0.55[b]	
		30.0	4.3 ± 1.9[b]		6.15 ± 0.63[b]	
		60.0	0.71 ± 0[c]		2.21 ± 0.91[c]	
		120.0	0.71 ± 0[c]		0.71 ± 0[d]	
		240.0	0.71 ± 0[c]		0.71 ± 0[d]	
Bispyribac-sodium	susceptible	0.0	10.02 ± 0[a]	0.92	10.02 ± 0[a]	0.94
		7.1	1.98 ± 1.27[b]		2.17 ± 1.46[b]	
		14.3	1.98 ± 1.27[b]		1.27 ± 0.56[b]	
		28.5	0.71 ± 0[b]		0.71 ± 0[b]	
		57.0	0.71 ± 0[b]		0.71 ± 0[b]	
		114.0	0.71 ± 0[b]		0.71 ± 0[b]	
		228.0	0.71 ± 0[b]		0.71 ± 0[b]	
	resistant	0.0	10.02 ± 0[a]	0.92	10.02 ± 0[a]	0.96
		7.1	10.02 ± 0[a]		6.99 ± 0.47[b]	
		14.3	3.1 ± 2.41[b]		5.36 ± 0.34[c]	
		28.5	0.71 ± 0[b]		3.95 ± 0.65[d]	
		57.0	0.71 ± 0[b]		3.22 ± 0.74[d]	
		114.0	0.71 ± 0[b]		3.18 ± 0.43[d]	
Penoxsulam	susceptible	228.0	0.71 ± 0[b]		0.86 ± 0.15[e]	
		0.0	ns		10.02 ± 0[a]	0.98
		3.3	ns		1.73 ± 0.25[b]	
		6.7	ns		0.71 ± 0.46[b]	
		13.4	ns		0.71 ± 0.27[b]	
		26.7	ns		0.71 ± 0.77[b]	
		53.4	ns		0.71 ± 0[b]	
		106.8	ns		0.71 ± 0[b]	
	resistant	0.0	ns		10.02 ± 0[a]	0.98
		3.3	ns		4.41 ± 0.25[b]	
		6.7	ns		3.87 ± 0.46[b]	
		13.4	ns		2.47 ± 0.27[c]	
		26.7	ns		1.48 ± 0.77[d]	
		53.4	ns		0.71 ± 0[d]	
		106.8	ns		0.71 ± 0[d]	

Data are expressed as means ± standard error; [a–e]means with the same letters in the column for each pot are not significantly different at $P < 0.05$; ns – not significant

doi: 10.17221/131/2016-PPS

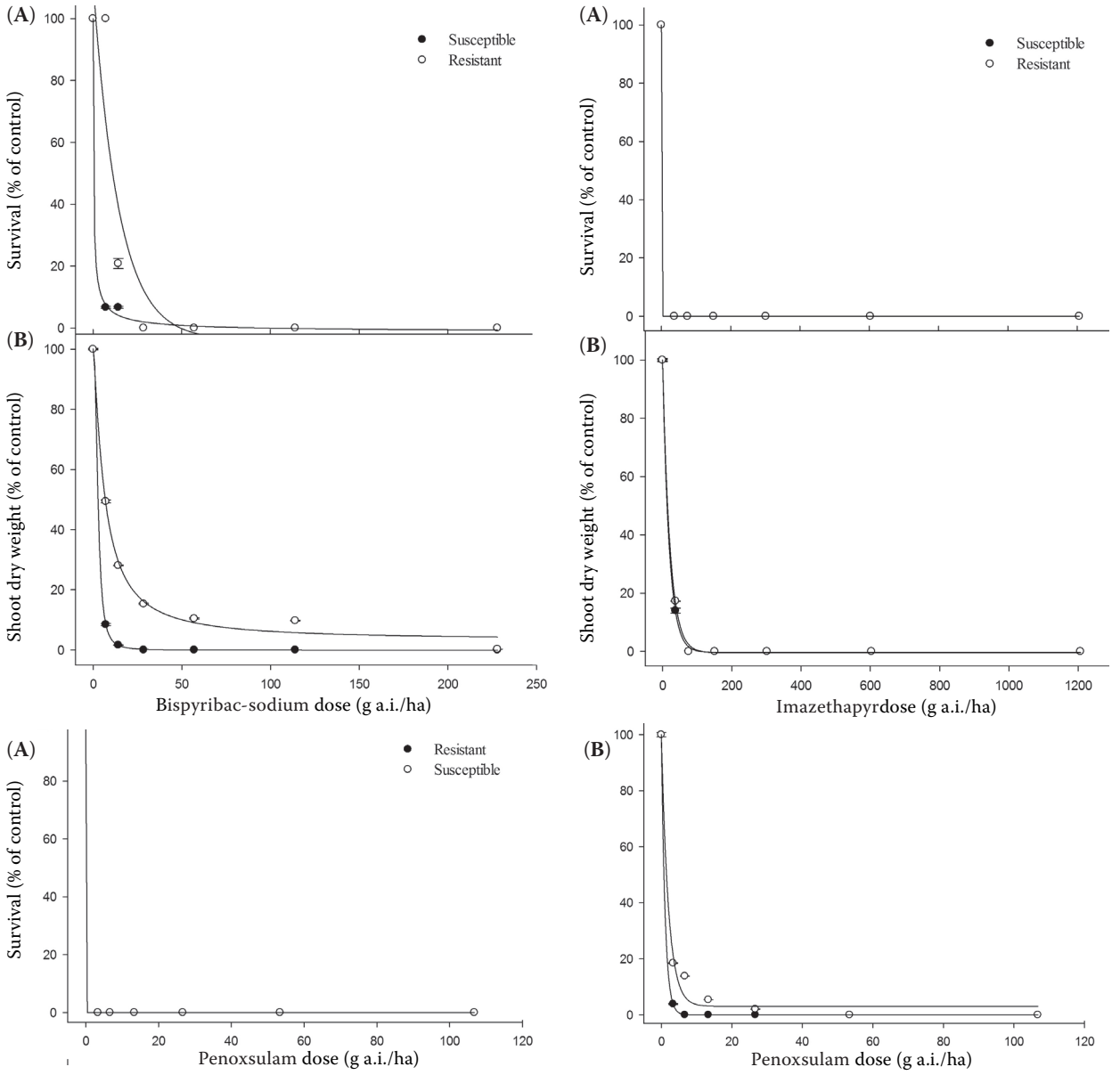

Figure 2. Survival (**A**) and shoot dry weight (**B**) of the susceptible and resistant populations of *Limnocharis flava* to bispyribac-sodium, imazethapyr, and penoxsulam 21 days after treatment (bars indicate the standard errors of the means of the three replicates)

plants showed similar detrimental symptoms like the S plants 21 days following the penoxsulam application, with resistance indices of 0 and 2 based on RI of LD_{50} and GR_{50}, respectively (Tables 1 and 2).

DISCUSSION

The dose response experiments were conducted to determine the resistance level of *L. flava*, a noxious rice field weed species, to different AHAS inhibitors. The resistance levels to sulfonylureas (SUs), pyrimidinyl-thiobenzoates (PTBs), imidazolinones (IMIs), and triazolopyrimidines (TPs) were quantified based on the RI index of herbicide rate resulting in 50% mortality (LD_{50}) and the herbicide rate required to reduce mean dry weight by 50% (GR_{50}). The resistance level to the SU, IMI, TP, and PTB herbicides for the R population has been classified as high (> 15), moderate (≥ 7 to 15), low (< 2 to < 7) and sensitive (≤ 2) in accordance with the resistance levels discussed by Iwakami *et al.* (2014) and Merotto *et al.* (2009).

The previous AHAS inhibitor dose-response study of the same resistant *L. flava* population conducted

doi: 10.17221/131/2016-PPS

Table 2. Parameters of the log-logistic analysis of the AHAS inhibitor dose required to reduce shoot dry weight (GR_{50}) and the resistance index (RI) of susceptible and resistant *L. flava* populations (standard errors are in parentheses)

Chemical class	Population	Herbicide	*c*	*d*	*b*	GR_{50} (g a.i./ha)	r^2	*P*	RI (GR_{50} R/ GR_{50} S)
Sulfonylurea	S	bensulfuron-methyl	–4.35	99.90	–0.96	5.93 (2.31)	0.98	< 0.0001	
	R		0	103.63	–0.80	53.61	0.95	< 0.0001	9
	S	metsulfuron-methyl	–	–	–	0.5[a]	1.00	< 0.0001	
	R		–	–	5.86[a]	0.90	< 0.0001	11.7	
	S	pyrazosulfuron- ethyl	–3.00	99.71	–1.48	3.35 (0.7)	0.98	< 0.0001	
	R		1.35	99.93	–0.79	4.75 (0.45)	1.00	0.0002	1.4
Pyrimidinyl-thiobenzoates	S	pyribenzoxim	–	–	–	4.59[a]	0.99	< 0.0001	
	R		–21.23	99.41	–0.71	18.19	0.97	< 0.0001	4
	S	bispyribac-sodium	–0.07	100	–2.49	2.74 (0.16)	1.00	< 0.0001	
	R		3.24	100.07	–1.26	6.45 (0.83)	0.99	< 0.0001	2.3
Imidazolinone	S	imazethapyr	–	–	–	13.80[a]	1.00	ns	
	R		–	–	–	13.80[a]	1.00	ns	1.0
Triazolopyrimidine	S	penoxsulam	–	–	–	0.71[a]	1.00	< 0.0001	
	R		–	–	–	1.44[a]	0.99	< 0.0001	2

LD_{50} value was calculated from parameters obtained from exponential decay (Equation 2); S – susceptible; R – resistant

by Juraimi *et al.* (2012) found that this resistant weed species survived bensulfuron-methyl at 0.16 kg a.i./ha, which is equal to a 4 times higher rate than the recommended one (0.04 kg a.i./ha). Through years, the resistant *L. flava* individuals have increased their resistance when in this study the same population could survive more than 8 times the recommended dose. On the contrary, a reduction in shoot dry weight was observed in the R population treated with bensulfuron-methyl, indicating that probably there is a fitness cost in plant growth associated with resistance to AHAS inhibitors in the resistant *L. flava* population. Fitness consequences have been observed for the Pro-197 to histidine (His) substitution (Guttieri *et al.* 1992) in AHAS inhibitor-resistant *L. serriola* (Alcocer-Ruthling *et al.* 1992a, b; reviewed by Vila-Aiub *et al.* 2009). Resistant *L. serriola* showed a significant 15% decline in vegetative growth as compared with susceptible *L. serriola* individuals when grown under competitive conditions. Similarly, strong morphological and physiological pleiotropic effects, leading to a fitness penalty, have been reported in AHAS inhibitor-resistant *Amaranthus powellii* S. Wats. populations carrying the Trp-574-Leu AHAS mutation (Tardif *et al.* 2006). The mutation caused all resistant *A. powellii* plants to produce thinner roots and stems, and a severe reduction in leaf area and seed production, where as high as 67% resistance cost in aboveground vegetative biomass was observed. Similarly, in a study conducted on AHAS inhibitor-resistant rice weed species, *Fimbristylis miliacea* (L.) Vahl, it was found that resistant individuals were less competitive with rice than the S plants under the absence of AHAS inhibitors (Schaedler *et al.* 2015). However, it is worth knowing that not all AHAS inhibitor-resistant weeds have conferred fitness disadvantage. There is a case where AHAS inhibitor-resistant plants were equally competitive with the S plants in *Cyperus difformis* L. (Dal Magro *et al.* 2011). Future studies on resistant *L. flava* will be carried out for quantification of resistance mechanisms and possible fitness cost associated with AHAS-resistance alleles.

In this study, the RI of metsulfuron-methyl was lower than that of bensulfuron-methyl, which is in agreement with the report on *Schoenoplectus juncoides* (Roxb.) Palla having RI ranging from 3 to 16 in all the accessions which were lower than those of imazosulfuron and bensulfuron-methyl (Sada *et al.* 2013). Pyrazosulfuron-ethyl was the most recent SU herbicide released in Malaysia for the control of aquatic weed species in rice fields (Ahmad-Hamdani, pers. comm.), thus as expected, the resistance level to this AHAS inhibitor is lower than in bensulfuron- and metsulfuron-methyl. In this study, we found that resistant *L. flava* showed a low level of resistance to this herbicide as well as a

doi: 10.17221/131/2016-PPS

minimum impact on plant growth. On the contrary, a resistant biotype of *Monochoria vaginalis* (Burm.f.) C. Presl ex Kunth in Korea (another common aquatic rice weed in Malaysia) showed varied levels of cross-resistance to other sulfonylurea herbicides, imazosulfuron, cyclosulfamuron, bensulfuron-methyl, and pyrazosulfuron-ethyl (Kuk *et al.* 2003a).

The results of pyribenzoxim and bispyribac-sodium herbicides indicate that it is likely for the resistant *L. flava* population, which has already evolved resistance to pyribenzoxim, to develop resistance at a sublethal dose to bispyribac-sodium. Evidently, resistance at sublethal doses was extensively quantified by Busi and Powles (2009) in a *L. rigidum* population to the EPSPS inhibitor glyphosate when selected progenies of the initially susceptible population shifted towards glyphosate resistance from one generation to another following the continuous selection pressure by glyphosate. Evidently, Fischer *et al.* (2000) reported a metabolic resistance to bispyribac-sodium in an *Echinochloa phyllopogon* (Stapf) Koss biotype resistant to bensulfuron-methyl and cross-resistant to bispyribac-sodium. The cytochrome P-450 inhibitors piperonyl butoxide and malathion, which were used for detection of herbicide degradation by cytochrome P-450 monooxygenation, strongly enhanced herbicide phytotoxicity to resistant plants, suggesting that metabolic degradation of bispyribac-sodium contributed significantly to the resistance in *E. phyllopogon*. This warrants further investigations on resistance build-up to bispyribac-sodium in the resistant *L. flava* population.

The result of imazethapyr is in agreement with Calha *et al.* (2007), who observed a high sensitivity in two ALS inhibitor-resistant *Alisma plantago-aquatica* L. biotypes to imazethapyr, producing low LD_{50} and GR_{50} RI values of 0.7 and 1.3, respectively. Merotto *et al.* (2009) also found that bensulfuron-resistant *C. difformis* and *Schoenoplectus mucronatus* (L.) Palla were successfully controlled by penoxsulam, a new AHAS inhibitor that has been introduced for broad-spectrum weed control in rice fields (Busi *et al.* 2006).

From whole-plant dose-response experiments, the results show that the resistnat *L. flava* population in this study exhibited a high-level of resistance to bensulfuron-methyl, with various levels of cross-resistance to other SU and PTB herbicides. There was an increase in the resistance level to bensulfuron-methyl (320 g a.i./ha) as compared to the results obtained three years ago (160 g a.i./ha) (Juraimi *et al.* 2012), probably due to the continuous use of this AHAS inhibitor by rice growers to control this weed. Different patterns of resistance to AHAS inhibitors have been reported previously in other resistant weed species in rice (*Cyperus difformis, Schoenoplectus mucronatus, Alisma plantago-aquatica, Echinochloa phyllopogon, Monochoria vaginalis,* and *Cyperus iria* L.) showing different patterns of cross-resistance associated with different point mutations (Osuna *et al*. 2002; Kuk *et al.* 2003a, b; Busi *et al.* 2006; Calha *et al.* 2007; Merotto *et al.* 2009; Riar *et al.* 2015).

The finding from this study is crucial to elucidate the underlying resistance mechanism that conferred resistance to AHAS inhibitors in *L. flava*. There are two prominent mechanisms involved in AHAS inhibitors, which are target-site resistance (TSR) endowed by alterations in the gene encoding the herbicide target protein, and non-target-site resistance (NTSR) endowed by any mechanism reducing the herbicide concentration reaching the target site (e.g. reduced herbicide uptake or translocation, increased herbicide sequestration or enhanced herbicide metabolism) (reviewed by Tranel & Wright 2002; Délye 2013; Yu & Powles 2014; Yang *et al.* 2016). Naturally occurring amino acid substitutions in the AHAS enzyme that have conferred resistance to AHAS inhibitors in weed species are Ala_{122} to Thr, Tyr or Val; Pro_{197} to Ala, Arg, Asn, Gln, His, Ile, Leu, Ser, Thr, Glu or Tyr; Ala_{205} to Val or Phe; Asp_{376} to Glu; Arg_{377} to His; Trp_{574} to Leu, Gly or Met; Ser_{653} to Asn, Ile or Thr; and Gly_{654} to Glu or Asp (Tranel *et al.* 2017). Variable patterns of cross-resistance between AHAS inhibitor classes occur depending on the amino acid position affected and the specific substitution. As reviewed by Tranel and Wright (2002), particular patterns of cross-resistance across AHAS inhibitors are endowed by specific mutations. According to Heap (2014) the substitution of Pro_{197} has caused resistance to AHAS inhibitors in 30 of the 129 documented resistance cases. As reported by many researchers, the amino acid substitutions at Pro_{197} by Ser, His, Leu, Ala or Thr have been observed to result in resistance to SU herbicides (Heap 2017). Some of the mutations have a potential to confer broad-spectrum resistance to all five herbicide inhibitor classes SU, IMI, TP, PTB and SCT which are Asp-376-Glu, Ala-205-Val, and Trp-574-Leu (Ashigh *et al.* 2009; Powles & Yu 2010).

In this study, the resistant population of *L. flava* exhibited a high level of resistance to bensulfuron-methyl, while at the same time it was cross-resistant

doi: 10.17221/131/2016-PPS

to other SU and PTB herbicides at varied levels. Thus, the resistance is likely endowed by target site mutation(s). The other mechanism that is important is NSTR endowing resistance to AHAS inhibitors. This mechanism is most likely the major type of resistance of grass weeds to the world's second most important herbicide mode of action inhibitors (AHAS inhibitors; group B). As compared to TSR, NTSR to AHAS inhibitors is less investigated in broadleaf weeds and remains poorly understood due to its complexity and diversity. NTSR is contrary to TSR, which only confers resistance to herbicides targeting the protein concerned, while NTSR can cause weeds to evolve unpredictable resistance to herbicides with various modes of action, including non-marketed herbicides (Délye 2013; Yang *et al*. 2016)

The combination of knowledge concerning the geographic distribution of cross-resistance patterns along with the identification of target site mutation will offer an understanding of the factors that indicate the selection pressure on AHAS inhibitors, and can also give a vision of the future use of these herbicides such as herbicide tank-mixtures and rotation of different herbicide modes of action to better manage herbicide-resistant weed species. However, weed management in rice as well as in other crops should not solely rely on herbicides. Continuously repeating the same single herbicide or herbicides with similar mode of action will increase the risk of resistance evolution (Gressel 2009). The lack of herbicide rotation as well as the residual activity in a long term that drives selection pressure might lead to the fast occurrence of resistance (Lamego *et al*. 2009). It is crucial to diversify weed control tactics to lengthen the efficacy and efficient use of herbicide tools (Harker *et al*. 2012). Thus, the practice of integrated management strategies which utilise non-herbicide control practices such as mechanical, cultural, and biological control needs to be considered to minimise the risk of resistance evolution in weed species.

Acknowledgements. The authors gratefully acknowledge all persons involved in conducting this research.

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Received: 2016–09–09
Accepted after corrections: 2017–03–08
Published online: 2017–05–24