Multiple resistance across glufosinate, glyphosate, paraquat and ACCase-inhibiting herbicides in an *Eleusine indica* population

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Summary

An *Eleusine indica* population was previously reported as the first global case of field-evolved glufosinate resistance. This study re-examines glufosinate resistance and investigates multiple resistance to other herbicides in the population. Dose–response experiments with glufosinate showed that the resistant population is 5-fold and 14-fold resistant relative to the susceptible population, based on GR_{50} and LD_{50} R/S ratio respectively. The selected glufosinate-resistant subpopulation also displayed a high-level resistance to glyphosate, with the respective GR_{50} and LD_{50} R/S ratios being 12- and 144-fold. In addition, the subpopulation also displayed a level of resistance to paraquat and ACCase-inhibiting herbicides fluazifop-P-butyl, haloxyfop-P-methyl and butroxydim. ACCase gene sequencing revealed that the Trp-2027-Cys mutation is likely responsible for resistance to the ACCase inhibitors examined. Here, we confirm glufosinate resistance and importantly, we find very high-level glyphosate resistance, as well as resistance to paraquat and ACCase-inhibiting herbicides. This is the first confirmed report of a weed species that evolved multiple resistance across all the three non-selective global herbicides, glufosinate, glyphosate and paraquat.

Keywords: herbicide resistance, Indian goosegrass, non-selective herbicides, fluazifop-P-butyl.

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Introduction

Eleusine indica (L.) Gaertn. (Indian goosegrass), one of the world's worst weeds (Holm *et al.*, 1977), is a very competitive and cosmopolitan species. *Eleusine indica* is fecund, found across a range of soils and temperatures (Nishimoto & McCarty, 1997) and infests a wide range of crops including cotton, maize, upland rice, sweet potatoes, sugarcane and many fruit and vegetable orchards (Holm *et al.*, 1977), causing major crop yield loss (Lourens *et al.*, 1989).

In tropical countries such as Malaysia, *E. indica* infestation occurs mostly in field crops areas, fruit and vegetable orchards, nurseries and young palm oil plantations. *Eleusine indica* has been shown to affect crop growth, cause yield loss and increase the incidence of plant disease such as *Phytophthora* spp. (Chee *et al.*, 1990; Teng & Teo, 1999). Control of *E. indica* is mainly with herbicides, but over-reliance on herbicides has resulted in resistance evolution in this species in at least eight countries (Heap, 2013). This includes resistance to dinitroaniline herbicides (Mudge *et al.*, 1984), acetyl coA carboxylase (ACCase)-inhibiting herbicides

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(Leach *et al.*, 1993; Osuna *et al.*, 2012), the acetolactate synthase (ALS)-inhibiting herbicide imazapyr (Valverde *et al.*, 1993), the glycine herbicide glyphosate (Lee & Ngim, 2000), the bipyridilium herbicide paraquat (Buker *et al.*, 2002), photosystem II inhibitors (Brosnan *et al.*, 2008), and most recently, the glutamine synthetase-inhibiting herbicide glufosinate (Chuah *et al.*, 2010; Jalaludin *et al.*, 2010).

Glyphosate and its alternative, glufosinate, are two of the most important herbicides globally. Glyphosate was initially used in Malaysia to control *E. indica* and other weeds in fallows, nurseries and to remove ground cover vegetation in plantations. Over-reliance on glyphosate was a strong selection pressure and glyphosate resistance in *E. indica* quickly evolved (Lee & Ngim, 2000). Now, many *E. indica* populations have been identified as glyphosate resistant (Ng *et al.*, 2003, 2004; Kaundun *et al.*, 2008). In response to glyphosate-resistant evolution in *E. indica*, high glufosinate usage has occurred. In 2010, the first case of glufosinate resistance was reported in a Malaysia *E. indica* population (Jalaludin *et al.*, 2010). Prior to glufosinate usage, this resistant population had a field history of paraquat, fluazifop-P-butyl and glyphosate treatment.

At the same time, another Malaysian *E. indica* population was reported to be resistant to glufosinate and paraquat (Chuah *et al.*, 2010). Subsequently, glufosinate resistance and multiple resistance to glufosinate and glyphosate have been reported in *Lolium perenne* L. populations in Oregon, USA (Avila-Garcia & Mallory-Smith, 2011; Avila-Garcia *et al.*, 2012). The objective of this study was to characterise the glufosinate-resistant population of *E. indica* following the preliminary study by Jalaludin *et al.* (2010) and evaluate for possible multiple resistance to a range of herbicides with different modes of action.

Materials and methods

Plant material

The glufosinate-resistant (*R*) *E. indica* population used in this study was preliminarily described (Jalaludin *et al.*, 2010). A glufosinate-susceptible population was originally provided by T S Chuah, and a subset of this population that was confirmed to be susceptible to all herbicides examined in this study was generated and used as the herbicide susceptible (*S*) population.

Glufosinate dose–response

Eleusine indica seeds were germinated on water-solidified 0.6% agar containing 0.2% potassium nitrate (KNO_3) (Ismail *et al.*, 2002). After 4–7 days, seedlings

were transplanted into pots (18 cm diameter with 15–20 seedlings per pot) and kept in a glasshouse during the normal summer growing months (January to March) with average temperatures of 30/20°C (day/night), and 15-h photoperiod under natural sunlight. At the 3–5 leaf stage, seedlings were treated at various rates of glufosinate (0, 20.6, 41.3, 82.5, 123.8, 247.5, 495, 1485, 1980, 3960 and 7920 g a.i. ha^{-1}) (Basta, 200 g a.i. L^{-1}, SC; Bayer CropScience), using a custom-built, dual nozzle cabinet sprayer delivering herbicide at 118 L ha^{-1} at 210 kPa, with a speed of 1 m s^{-1}. After herbicide treatment, plants were returned to the glasshouse. The pots were arranged in a completely randomised block design with at least three replicate pots per herbicide rate. Visual assessment for resistance (*R*) and susceptibility (*S*) was made 21 days after treatment. Plants were considered as *R* if they are actively growing or tillering, while *S* plants were dead. Above-ground shoots were harvested and dried in oven (65°C) for 3 days for dry-weight measurements.

Additionally, six individual plants surviving 1485 and 1980 g a.i. ha^{-1} of glufosinate were allowed to grow together to produce seeds (*E. indica* is a self-pollinated species), and the progeny was designated as selected glufosinate-resistant subpopulation (referred as *R**). This subpopulation was tested again for glufosinate resistance and used for subsequent experiments.

Glyphosate dose–response

Seed germination and seedling growth were the same as described above for glufosinate experiments. Glyphosate rates at 0, 33.8, 67.5, 100, 135, 170, 200, 540, 1080, 4320, 8640, 12 960, 17 280 and 25 920 g a.e. ha^{-1} (Roundup Attack with IQ inside, 570 g a.e. L^{-1}, SL; Nufarm Australia) were used.

Paraquat dose–response

Seed germination was carried out as described earlier. After transplanting into pots, the seedlings were grown in a controlled environment room with alternating temperatures of 30/25°C (day/night), 12-h photoperiod with light intensity of 400 µmol m^{-2} s^{-1} and 75% humidity. At the 3–4 leaf stage, the plants were treated with paraquat at 0, 47, 94, 188, 375, 750, 1500 and 3000 g a.i. ha^{-1} (Gramoxone, 250 g a.i. L^{-1}, SL; Syngenta Crop Protection).

Herbicide single-rate test

In this experiment, germinating seedlings were transplanted to trays (50–60 seedlings per tray with two to

four trays per herbicide treatment) and kept in a glass-house with day/night temperature of 30/25°C under natural sunlight. Single discriminating or label rates of ACCase-inhibiting herbicides fluazifop-P-butyl, 210 g a.i. ha^{-1} (Fusilade Forte, 128 g a.i. L^{-1}, EC; Syngenta Crop Protection), haloxyfop-P-methyl, 60 g a.i. ha^{-1} (Verdict 520, 520 g a.i. L^{-1}, EC; Dow Agrosciences Australia), clethodim, 100 g a.i. ha^{-1} (Select, 240 g a.i. L^{-1}, EC; Sumitomo Chemical Australia), butroxy-dim, 100 g a.i. ha^{-1} (Falcon, 250 g a.i. kg^{-1}, WG, Nu-farm Australia) and sethoxydim, 230 g a.i. ha^{-1} (Sertin 186 EC, 186 g a.i. L^{-1}, EC; Bayer Crop-Science), and the ALS-inhibiting herbicide imazapyr, 50 g a.i. ha^{-1} (Arsenal, 250 g a.i. L^{-1}, SC; Nufarm Australia) were used for resistance screening.

Statistics

The herbicide rate causing 50% mortality (LD$_{50}$) or reduction in growth (GR$_{50}$) was estimated by non-linear regression analysis using Sigma Plot$^{®}$ software (version 12.0, SPSS, Chicago, IL, USA). The data were fitted to the three parameter logistic curve model:

$$y = \frac{a}{1 + (\frac{x}{ED_{50}})^b} \tag{1}$$

where a = upper limit, ED$_{50}$ = estimated dose causing 50% response (LD$_{50}$ or GR$_{50}$) and b = slope around ED$_{50}$. The LD$_{50}$ and GR$_{50}$ values of the susceptible and resistant biotypes were used to calculate the R/S ratio of the resistant population. There were several pilot trials prior to final herbicide dose–response experiments, which contained at least three replicate pots per herbicide rate. Each dose–response experiment was repeated at least twice with similar results, and there-fore, only results from a single experiment were pre-sented for each dose–response.

ACCase gene sequencing

Genomic DNA was extracted from the leaf tissue of surviving plants from $R*$ population and susceptible plants from S population according to Yu *et al.* (2008). Published primers (Osuna *et al.*, 2012) used to amplify two plastidic ACCase gene fragments in which point mutations known to confer ACCase herbicide resistance in plants have been identified (Délye & Michel, 2005; Powles & Yu, 2010; Beckie & Tardif, 2012). The PCR was conducted in a 25 μL volume that consisted of 1–2 μL containing 50–100 ng of genomic DNA, 0.5 μM of each primer and 12.5 μL of 2× GoTaq Green Master Mix$^{®}$ (Promega, Madison, WI, USA). The PCR was run with the following

profile: 94°C for 4 min; 40 cycles of 94°C for 30 s, 58°C (annealing temperature) for 30 s and 72°C for 1 min; followed by a final extension step of 7 min at 72°C. The PCR product was purified from agarose gel with Wizard$^{®}$ SV Gel and PCR Clean-up System (Pro-mega) and sequenced by commercial services. All sequence chromatograms were visually checked for quality and consistency before sequences were assem-bled and aligned.

Results

Glufosinate resistance

As expected, the S plants were well controlled with glufosinate (Fig. 1). In contrast, much higher rates of glufosinate were required to cause substantial mortality for resistant (R and $R*$) plants. The plants became dark grey from the middle of the leaves to the leaf tip, almost burnt-like, with slight wilting, 24 h after treat-ment. The damaged area then extended in the basipetal direction, developing necrosis over 14 days, turning wilted leaves from yellow into brown. While the S plants die, the R and $R*$ plants were observed to recover and grow again, 2 weeks after treatment. The glufosinate LD$_{50}$ for the R population was 820 g ha^{-1} as compared with 58 g ha^{-1} for the S population (Table 1), giving a LD$_{50}$ R/S ratio of 14. This is slightly higher than the previously reported LD$_{50}$ R/S ratio of 7.6 (Jalaludin *et al.*, 2010). The difference may be due to different susceptible populations and experi-mental conditions used in the two studies. The glufosi-nate GR$_{50}$ for R population was found to be 156 g ha^{-1}, which was about 5-fold greater than for
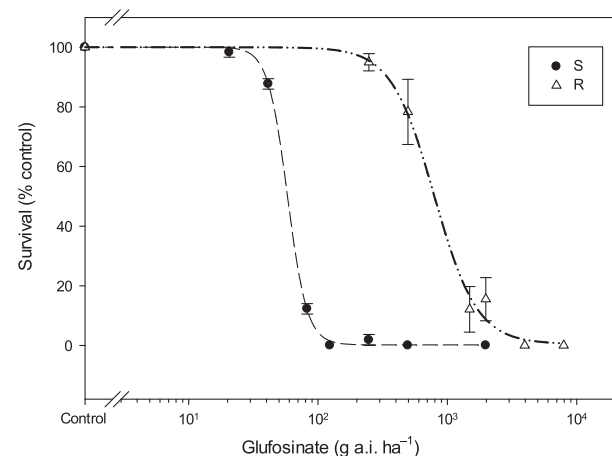

Fig. 1 Glufosinate dose–response for survival of the susceptible (S) population and resistant (R) populations of *Eleusine indica*. Data were collected at 21 DAT.

Table 1 Parameter estimates for logistic analysis of glufosinate, glyphosate and paraquat dose–response survival data for the susceptible (*S*) and resistant (*R*) *Eleusine indica* populations

Population	*a*	*b*	$x_0 = LD_{50}$ (g a.i. ha^{-1})	R^2 (coefficient)	*R*/*S* ratio of LD$_{50}$
Glufosinate dose–response					
S	100.00 (0)	5.71 (0.23)	58 (0.81)	0.99	
R	100.00 (0)	2.42 (0.37)	820 (85.6)	0.93	14
†*R**	100.00 (0)	2.3 (0.25)	1278 (63.9)	0.99	22
Glyphosate dose–response					
S	100.00 (0)	15.28 (1.71)	148 (1.81)	0.98	
†‡*R**	100.00 (0)	0.99 (0.1)	21 274 (1773)	0.98	144
Paraquat dose–response					
S	100.00 (0)	3.76 (0.66)	98 (23.6)	0.97	
†*R**	100.00 (0)	1.5 (0.2)	292 (27.9)	0.94	3

Standard errors are in parentheses.
†*R** refers to the selected glufosinate-resistant subpopulation.
‡*R** Glyphosate LD$_{50}$ is in g a.e. ha^{-1}.

the *S* population (Table 2). The selected *R** population (the progeny of plants surviving glufosinate rates of 1485 and 1980 g ha^{-1}) was only about 2-fold more resistant to glufosinate relative to the original *R* population (Fig. 2A, Tables 1 and 2), indicating the glufosinate-resistant subpopulation is still segregating.

Glyphosate resistance

As expected, the *S* population was susceptible to glyphosate, with 100% mortality at the glyphosate rate of 200 g ha^{-1} (Fig. 2B). However, the glufosinate-resistant subpopulation *R** was found to be highly resistant to glyphosate, requiring an extremely high rate (25 920 g ha^{-1}) to cause substantial mortality (Fig. 2B). Based on the LD$_{50}$ *R*/*S* ratio, the *R** population was more than 144-fold resistant to glyphosate (Table 1). While the *R** plants survived high glypho-

sate doses, their growth was affected. The GR$_{50}$ for the *R** and *S* populations were 481 and 41 g ha^{-1}, respectively, resulting in the *R** population being 12-fold more resistant than the *S* population (Table 2). Therefore, in addition to glufosinate resistance, this *R** population had a high level of glyphosate resistance.

Paraquat resistance

The *S* population was, as expected, well controlled by paraquat at 375 g ha^{-1}, whereas control of the *R* population required higher rates (Fig. 2C). Both *S* and *R** plants displayed rapid desiccation and necrosis following treatment. Similar to glufosinate-treated plants, the *R** plants recovered 2 weeks after treatment, while the *S* plants died. Based on the LD$_{50}$ or GR$_{50}$ *R*/*S* ratio (Tables 1 and 2), paraquat resistance in the purified glufosinate-resistant population was confirmed,

Table 2 Parameter estimates for logistic analysis of glufosinate, glyphosate and paraquat dose–response biomass data for the susceptible (*S*) and resistant (*R*) *Eleusine indica* populations

Population	*a*	*b*	$x_0 = GR_{50}$ (g a.i. ha^{-1})	R^2 (coefficient)	*R*/*S* ratio of GR$_{50}$
Glufosinate dose–response					
S	100.00 (0)	2.23 (0.36)	31 (2.3)	0.94	
R	100.00 (0)	1.36 (0.17)	156 (17.4)	0.98	5
†*R**	100.00 (0)	1.25 (0.25)	325 (37.1)	0.98	11
Glyphosate dose–response					
S	100.00 (0)	1.7 (0.22)	41 (3.6)	0.92	
†‡*R**	100.00 (0)	0.88 (0.09)	481 (55.6)	0.95	11.8
Paraquat dose–response					
S	100.00 (0)	3.22 (0.72)	52 (3.1)	0.95	
†*R**	100.00 (0)	1.84 (0.29)	105 (8.4)	0.96	2

Standard errors are in parentheses.
†*R** refers to the selected glufosinate-resistant subpopulation.
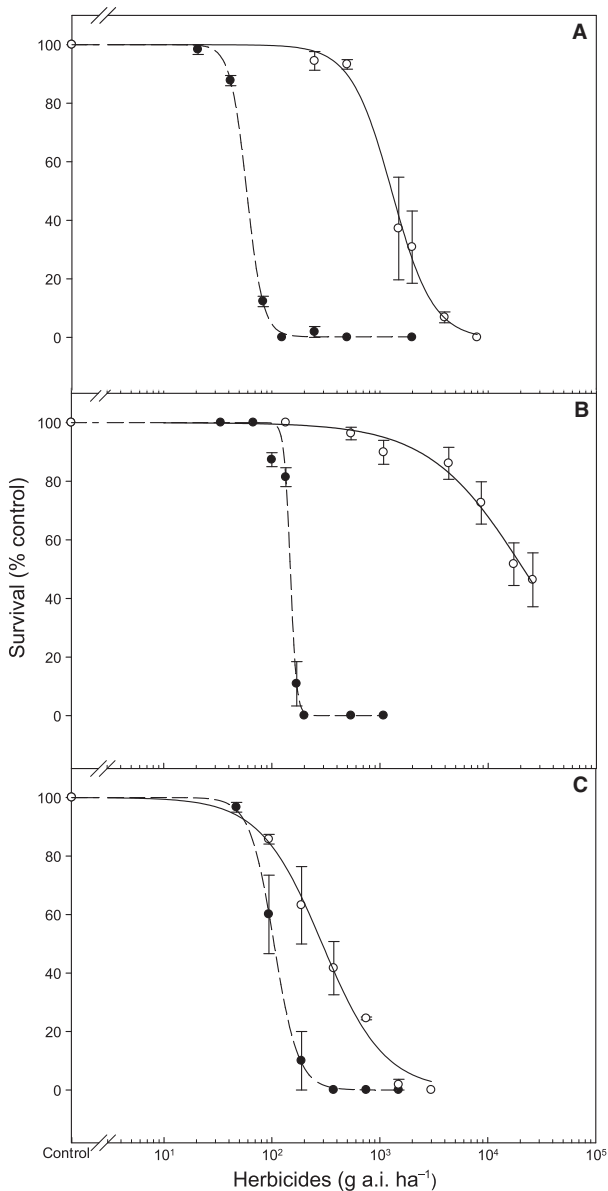‡*R** Glyphosate GR$_{50}$ is in g a.e. ha^{-1}.

Fig. 2 Survival response of the susceptible (closed circle; ●) and selected glufosinate-resistant (opened circle;○) R* subpopulations of *Eleusine indica* to glufosinate (A), glyphosate (B) and paraquat (C) treatment. Data were collected at 21 DAT. Glyphosate rates are in g a.e. ha⁻¹.

albeit at a low level (2- to 3-fold in relation to the used rate).

Resistance to ACCase-inhibiting herbicides

All ACCase herbicides examined (Table 3) caused 100% mortality in the *S* population at the respective rates used. However, there was about 50% of the *R** population surviving haloxyfop-P-methyl, fluazifop-P-butyl or butroxydim. In contrast, the *R** population remained susceptible to sethoxydim, clethodim and imazapyr (Table 3).

Table 3 Percentage survival of the susceptible (*S*) and selected glufosinate-resistant (*R**) subpopulations of *Eleusine indica* 21 days after treatment with various herbicides

Herbicide	Mean % survival	
	S	*R**
ACCase inhibitor		
Fluazifop-P-butyl (210 g a.i. ha⁻¹)	0	47
Haloxyfop-P-methyl (60 g a.i. ha⁻¹)	0	51
Sethoxydim (230 g a.i. ha⁻¹)	0	0
Clethodim (100 g a.i. ha⁻¹)	0	0
Butroxydim (100 g a.i. ha⁻¹)	0	49
ALS inhibitor		
Imazapyr (50 g a.i. ha⁻¹)	0	0

ACCase gene sequencing

The plastidic ACCase gene sequences from a total of nine individual plants surviving fluazifop-P-butyl or butroxydim were analysed in comparison with those of the susceptible plants. The primer pair ELEIN1781F/ELEIN1781R (Osuna *et al.*, 2012) amplified a 600 bp DNA fragment covering the known mutation site 1781, and the primer pair ELEIN2027f/ELEIN2027r amplified an 832 bp fragment with the known mutation sites 1999, 2027, 2041, 2078, 2088 and 2096. Sequence alignment revealed an amino acid substitution of Trp-2027-Cys in *R* individuals, resulting from a G to T change at the third position of the Trp codon (TGG). The same mutation was also recently found in several other fluazifop-resistant *E. indica* populations in Malaysia (Cha *et al.*, 2014). Generally, this mutation has been known to confer resistance to ACCase-inhibiting aryloxyphenoxypropionate herbicides (e.g. diclofop-methyl, fluazifop-P-butyl, haloxyfop-P-methyl) (Délye, 2005; Powles & Yu, 2010). However, it also confers resistance to ACCase-inhibiting cyclohexanedione herbicides, for example tralkoxydim in wild oats (Liu *et al.*, 2007). As the frequency of resistance to haloxyfop-P-methyl, fluazifop-P-butyl and butroxydim is close to each other (around 50%, Table 3), it is very likely that the Trp-2027-Cys mutation confers resistance to these three herbicides. Thus, this is the first case associating the Trp-2027-Cys mutation with butroxydim resistance at the rate used.

Discussion

In this study, we confirmed the preliminary report on the evolution of resistance to glufosinate in a Malaysian *E. indica* population (Jalaludin *et al.*, 2010). The level of glufosinate resistance determined for this population was modest (5- and 14-fold, based on GR$_{50}$ and LD$_{50}$, respectively), which is similar to the

glufosinate-resistant *E. indica* population reported by Chuah *et al.* (2010) (GR$_{50}$ *R/S* ratio 3.4), and slightly higher than glufosinate-resistant *Lolium perenne* populations in Oregon, USA (GR$_{50}$ *R/S* ratios between 2.2–2.8) (Avila-Garcia & Mallory-Smith, 2011; Avila-Garcia *et al.*, 2012). The level of paraquat resistance in this population was also similar to that observed in a glufosinate- and paraquat-resistant Malaysian *E. indica* population (Chuah *et al.*, 2010). It is worth noting that usually GR$_{50}$ *R/S* ratios are more variable than LD$_{50}$ ratios, due to variations in growth conditions and especially, the length of experiments. In this sense, LD$_{50}$ *R/S* ratios would be the better option for comparing results across research groups.

Currently, documented glufosinate resistance evolution is confined to a few *E. indica* (Chuah *et al.*, 2010; Jalaludin *et al.*, 2010) and *L. perenne* populations (Avila-Garcia & Mallory-Smith, 2011; Avila-Garcia *et al.*, 2012), and all exhibit low to moderate levels of glufosinate resistance. Few resistance mechanisms studies have been undertaken. In resistant *L. perenne* populations, the resistance mechanism in one population was non-target-site based (Avila-Garcia & Mallory-Smith, 2011), while in another population it was due to a target-site mutation in the glutamine synthetase gene (Avila-Garcia *et al.*, 2012). We have commenced glufosinate resistance mechanism studies with this population.

Importantly, in addition to glufosinate resistance, individuals in this *E. indica* population were also highly resistant to glyphosate (Fig. 2B; Tables 1 and 2). Resistant plants survived very high glyphosate rates but suffered growth reduction, resulting in an *R/S* LD$_{50}$ ratio (144) much higher than the *R/S* GR$_{50}$ ratio (12). The *R/S* ratios based on survival and plant biomass were both higher than any previously reported evolved glyphosate resistance in any weed species (Lee & Ngim, 2000; Baerson *et al.*, 2002; Culpepper *et al.*, 2006; Mueller *et al.*, 2011; Gaines *et al.*, 2012). As is discussed above, we consider the LD$_{50}$ value is more accurate and meaningful in describing resistance levels, because it is less affected by experimental conditionals (e.g. harvest time, growth competition) as compared with the GR$_{50}$ value. Nevertheless, the large difference in the *R/S* LD$_{50}$ and GR$_{50}$ ratio obtained for glyphosate response in this *E. indica* population indicates that the potential glyphosate resistance mechanism(s) may incur fitness cost in the presence of herbicide. This unusually high-level glyphosate resistance needs investigation. A few possible mechanism(s) are (i) a new target-site EPSPS mutation, (ii) multiple EPSPS mutations and (iii) accumulation of several known glyphosate resistance mechanisms (e.g. EPSPS gene mutation or amplification, reduced glyphosate translocation or enhanced sequestration). We have initiated

studies to reveal the mechanistic basis of this very high level of glyphosate resistance.

Multiple resistance in *E. indica* has been reported previously. These multiple resistance cases encompass at most, two different herbicide groups at any one time, for example fluazifop-P-butyl and glyphosate (Heap, 2013) or glufosinate and paraquat (Chuah *et al.*, 2010). However, the current study is the first case where multiple resistance across four dissimilar herbicide groups, glufosinate, glyphosate, paraquat and ACCase inhibitor herbicides, is present in a single *E. indica* population. This is likely related to the herbicide selection history of this population (involving application of at least paraquat, fluazifop-P-butyl, glyphosate and up to 12 glufosinate applications per year). As resistance to glyphosate, paraquat and ACCase-inhibiting herbicides was detected from a purified glufosinate-resistant subpopulation, it is very likely (although not examined) that multiple resistance is also displayed at the individual level. Multiple resistance to glyphosate, paraquat and ACCase-inhibiting herbicides in individual plants has been documented in *Lolium rigidum* L. due to accumulation of multiple resistance mechanisms (Yu *et al.*, 2007). This is the first global report of a weed species with evolved resistance across all three of the world's non-selective herbicides (glufosinate, glyphosate and paraquat). It is an unavoidable consequence of the selection pressures resulting from over-reliance on herbicides for weed control. Herbicides should be used wisely (e.g. in rotation or mixture) and in combination with other non-chemical control options.

In summary, we have confirmed in an *E. indica* population the first case of multiple resistance across the three non-selective herbicides, glufosinate, glyphosate and paraquat. The same population also showed target-site resistance to ACCase-inhibiting herbicides, likely due to the Trp-2027-Cys mutation. The evolution of multiple resistance to herbicides across four different modes of action in this resistance-prone species is worrying, as it threatens the world's most important herbicide (glyphosate) and its alternatives (glufosinate, paraquat) and results in greatly reduced herbicide control options for the grower. Although other AC-Case- or ALS-inhibiting herbicides (e.g. sethoxydim, clethodim, imazapyr) still provide effective short-term control options, in the long run, additional diversity in weed control must be added, to limit seed set of resistant *E. indica* plants.

Acknowledgement

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