Glyphosate resistance in barnyard grass (*Echinochloa colona*)

Hoan Nguyen Thai, Jenna Malone, Peter Boutsalis and Christopher Preston
School of Agriculture, Food and Wine, University of Adelaide, Urrbrae SA 5064, Australia
(hoan.nguyen@adelaide.edu.au)

Summary *Echinochloa colona* is an important summer-growing weed species in northern Australian cropping regions. As a result of the intensive use of glyphosate in summer fallows, glyphosate resistant populations of *E. colona* have evolved, with the number of resistant populations identified rapidly growing. This study identified glyphosate resistance in *E. colona* collected from different locations in Australia and investigated the mechanism of glyphosate resistance.

Pot trials conducted on populations of*E. colona* collected from northern Australia identified resistance to glyphosate in 11 populations of this weed species. The level of resistance varied among the populations from 2- to 11-fold. Sequencing of the target-site (EPSPS) identified a mutation at position 106 leading to a change from proline to serine in the most resistant population A533.1 only. With the range of resistance levels identified, it is expected that different mechanisms of resistance will be present among the rest of the resistant populations.

Keywords Barnyard grass, *Echinochloa colona*, glyphosate resistance, resistance mechanism, EPSPS.

INTRODUCTION

Barnyard grass (*Echinochloa colona*) is an important grass species for agriculture worldwide due to it being highly competitive with many major agricultural crops. In Australia, *E. colona* is recognised as a common weed species in summer fallows, as well as in crops (Walker *et al.* 2004). It is rated in the top three worst weeds in vegetable crops (Holm *et al.* 1977, Walker *et al.* 2004). Many methods of controlling *E. colona* have been used (Matsunaka 1983) and herbicides are the most widely used in most countries in the world. The intensive use of herbicides over a long period of time has resulted in the evolution of herbicide resistance in this grass species (Norsworthy *et al.* 1998).

Since the herbicide glyphosate was introduced to world agriculture in 1974 (Duke *et al.* 2003), it has become the world's most widely used herbicide, especially since the development of genetically modified crops with resistance to glyphosate. However, continued dependence on glyphosate over a large area ranging from field agricultural systems to inner-city landscapes has increased the number of weed species, including *E. colona*, that have evolved resistance to this herbicide (Duke and Powles 2008). Up to now glyphosate resistance in *E. colona* has been found in several regions in the world including New South Wales, Queensland, Western Australia (Australia), California (America) and Santa Fe (Argentina) (Heap 2012). For these reasons, an understanding of the evolution of glyphosate resistance in *E. colona* through studies of the mechanism of glyphosate resistance is important.

MATERIALS AND METHODS

Plant materials Seeds of *E. colona* were collected from sites in New South Wales and Queensland. The locations were recorded using a global positioning system (GPS). The seeds were treated with 95% H_2SO_4 for 30 min, rinsed under running water for 1 h and germinated on 0.6% (w/v) agar in an environmentally controlled cabinet at 12 h light and 12 h dark periods at 22°C with 30 μmol $m^{-2}s^{-1}$ during the light period. After 7 days, seedlings at the one leaf stage were transplanted into 10 cm square pots containing standard potting mix with a density of nine seedlings per pot and transferred to a growth room under 25/23°C day/night temperatures and a 12-h photoperiod at 553 μmol $m^{-2}s^{-1}$. At the 3 to 4 leaf stage, seedlings were sprayed with glyphosate herbicide at various rates depending on the experiment.

Glyphosate dose response Initially, samples from 22 populations were tested for resistance to glyphosate at the rate of 270 g a.i. ha^{-1} to determine which were resistant to glyphosate. Following this, a dose response experiment was conducted on 10 resistant and one susceptible population chosen from the testing along with a known susceptible and resistant population as controls. Glyphosate was applied at rates of 0, 270, 540, 1080 and 2160 g a.i. ha^{-1} with three replicates for each rate. The glyphosate application was carried out using a moving-boom laboratory twin nozzle sprayer placed 40 cm above the seedlings with a water volume of 109.6 L ha^{-1} at a pressure of 250 kPa and a boom speed of 1 ms^{-1}. Control plants were not treated with herbicide. At three weeks after glyphosate application, survival rate was recorded and plants with new green leaf tissue were considered as survivors.

In order to evaluate the impact of different temperatures on glyphosate resistance, two temperature response experiments were conducted in controlled environment chambers with six resistant and one susceptible population. Glyphosate was applied at rates described above. One experiment was carried out at 20°C day/night and another one at 30°C day/night. Other growth conditions including light intensity and photoperiod were similar to that of the dose response experiment. The methods of growing plants, applying herbicide and recording data were as described above. All dose response data were analysed by probit analysis to compare the differences among the herbicide treatments.

Identifying target-site mutations New green leaf tissue of one individual from ten resistant and one susceptible population was collected for extracting DNA using the DNeasy Plant Mini Kit (Qiagen, Australia) in accordance with the manufacturer's instructions.

PCR reactions were conducted in 25 µl volumes containing 80 ng of ligated and diluted DNA, 1 × High Fidelity PCR Buffer [600mM Tris-SO_4 (pH 8.9), 180mM $(NH_4)_2SO_4$, 0.4mM of 10mM dNTP mixture, 4mM of 50mM $MgSO_4$, 0.4µM of each specific primer and 1 unit of Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, Australia). A forward (AW F: 5'-AACAGTGAGGAYGTYCACTACATGCT-3') and reverse (AW R: 5'-CGAACA GGAGGGCAMTCAGTGCCAAG-3') primer were used for amplification of an approximately 500 bp fragment of the EPSPS gene. An automated DNA thermal cycler (Eppendorf Mastercycler® Gradient, Germany) was used for amplification with the cycle parameters as follows: 3 min denaturing at 94°C; 39 cycles of 30 s denaturation at 94°C, 30 s annealing at 56°C and 1 min elongation at 68°C, and a final extension for 7 min at 68°C.

PCR products were examined on 1.5% agarose gels stained with 1 × of SYBR® Safe DNA gel stain. Samples were electrophoresed in 1× TAE Buffer [40mM Trizma base, 1mM Na_2EDTA (pH to 8) with glacial acetic acid) at 90 volts and photographed under UV light (λ302 nm). DNA fragment sizes were estimated *via* comparison to a DNA ladder with known size bands (Invitrogen, Australia). PCR products were sequenced at the Australian Genome Research Facility (AGRF) Ltd., Australia using the same primers used for amplification

DNA sequence data were assembled, compared and analysed using ContiExpress from the Vector-NTi Advance 11.5 programs (Invitrogen).

RESULTS AND DISCUSSION

Response of resistant populations to glyphosate Amongst the 22 *E. colona* population samples collected from northern Australia that were sprayed with glyphosate at 270 g a.i. ha^{-1}, eleven samples were identified as resistant (data not shown). Of the resistant populations, four populations were collected from Queensland and seven from New South Wales.

The results of the dose-response experiment with glyphosate rates from 0 to 2160 g a.i. ha^{-1} showed that the resistance level of populations varied from 2- to 11-fold compared to the susceptible population (Echi S). A533.1 was rated as the most resistant population with nearly 12-fold higher resistance. The glyphosate dose required to control 50% (ED_{50}) of this population was 1289 g a.i. ha^{-1}, far higher than the rate normally applied in the field. The next most resistant population was L594, having an ED_{50} value of 1069 g a.i. ha^{-1} or 9.7-fold resistant, followed by RL11 with an ED_{50} of 867 g a.i. ha^{-1} and a 7.8-fold resistance level (Table 1). These three populations were sampled from locations at Glenmorgan (Queensland), and Moree and North Star (New South Wales). This suggests that strongly resistant *E. colona* populations are widely distributed in two states of Australia. The remaining resistant populations had ED_{50} values that ranged from 2.1 to 5.7 times higher than that of standard susceptible population, Echi S.

The results of the temperature response experiments showed that glyphosate resistance was higher in *E. colona* populations at 30°C than that at 20°C. At the same time there was no difference in the susceptibility of Echi S to glyphosate between the two

Table 1. Glyphosate dose required to control 50% of susceptible and resistant *E. colona* populations (ED_{50}) and the resistance index (R/S) for the resistant populations.

Population	ED_{50} (g a.i./ha^{-1})	R/S
A533.1 (R)	1289	11.6
L594 (R)	1069	9.7
RL11 (R)	867	7.8
1352.1 (R)	635	5.7
1307.3 (R)	595	5.4
A516 (R)	508	4.6
A491 (R)	392	3.5
RL17 (R)	279	2.5
RL21 (R)	263	2.4
RL4 (R)	234	2.1
Echi S (S)	110	-

temperatures (Figure 1). This experiment suggests resistant populations became harder to control under warmer conditions that would occur normally during the summer in Queensland and New South Wales.

Target-site mutations To identify possible target-site mutations within EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) in populations, the EPSPS gene of glyphosate resistant individuals was sequenced. The predicted amino acid sequence for the part of the gene sequence around Pro 106 is shown in Table 2. The predicted amino acid sequence of the susceptible population Echi S was the same as the consensus sequence of other populations in the conserved region sequenced. The resistant populations, with the exception of A533.1, showed no differences to the susceptible sequence within the nucleotide sequence in this region. In population A553.1 a single nucleotide substitution of T for C at codon 106 was identified predicting a substitution of serine for proline at position 106. The result showed a target-site mutation for glyphosate resistance was present in this population.

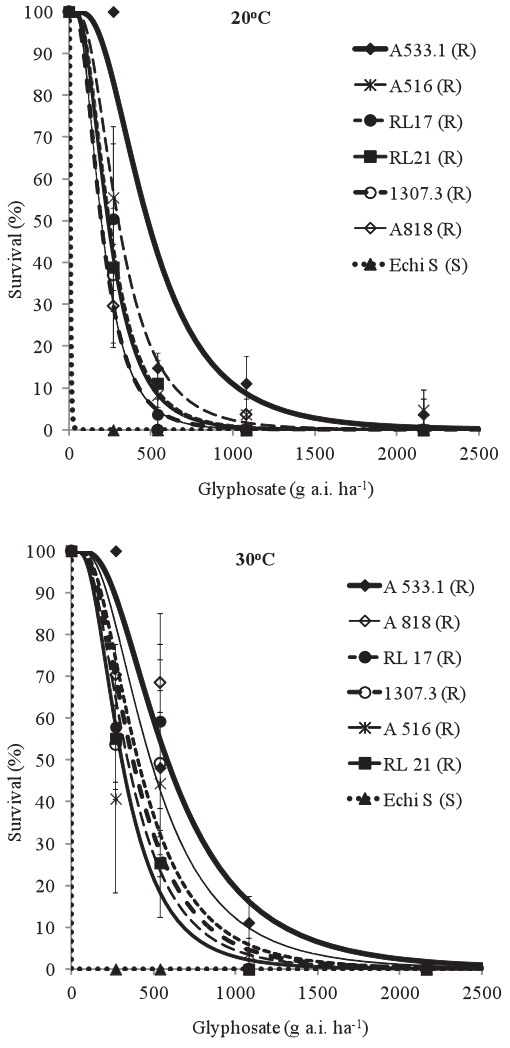

Figure 1. Response of *E. colona* populations to glyphosate at 20°C and 30°C. Lines are survival data back-transformed using the probit equations. Each data point represents the mean percentage survival from three replicates ± SE.

Four identified mechanisms of glyphosate resistance in plants have been determined so far including: (1) Target-site resistance: A mutation within the target-site prevents the herbicide from binding as effectively; (2) Non-target-site resistance is comprised of a decrease in translocation of the herbicide (Powles and Preston 2006); (3) Reduced foliar uptake from the abaxial leaf surface leads to the lower volume of herbicide reaching the target-sites (Michitte *et al.* 2007); and (4) Amplification of target-site gene: The genome of glyphosate resistant plants contained many more copies of the target-site gene than that of susceptible plants (Gaines *et al.* 2010). A mutation in the EPSPS gene was identified in this study in one population: A533.1. Glyphosate resistance in the remaining populations may be due to other mechanisms that need to be further studied.

This study has identified 11 glyphosate resistant *E. colona* populations from northern New South Wales and Queensland. The levels of glyphosate resistance compared to a known susceptible population ranged

Table 2. Nucleotide and predicted amino acid sequence of EPSPS DNA isolated from a susceptible and ten resistant populations of *E. colona.*

Amino acid number		104	105	106	107	108
Amino acid		Met	Arg	Pro	Leu	Thr
Consensus sequence		ATG	CGG	CCA	TTG	ACA
Echi S	(S)	-	-	-	-	-
1307.3	(R)	-	-	-	-	-
A533.1	(R)	-	-	Ser TCA	-	-
1352.1	(R)	-	-	-	-	-
A491	(R)	-	-	-	-	-
A516	(R)	-	-	-	-	-
A818	(R)	-	-	-	-	-
L594	(R)	-	-	-	-	-
RL4	(R)	-	-	-	-	-
RL11	(R)	-	-	-	-	-
RL17	(R)	-	-	-	-	-

from 2- to 12-fold. The most resistant population, A533.1, contained a mutation within the EPSPS target site. However, the other populations are likely to have other resistance mechanisms. The high levels of glyphosate resistance identified in some of the populations will make them impossible to control with glyphosate and other weed management practices will have to be used.

ACKNOWLEDGMENTS

The study presented in this paper was undertaken as part of a PhD study project which has been supported by The University of Adelaide, Australia. The authors are grateful to Ministry of Education and Training, Vietnam for financial support of this research.

REFERENCES

Duke, S.O., Baerson, S.R. and Rimando, A.M. (2003). 'Glyphosate'. Encyclopedia of agrochemicals. John Wiley & Sons, Inc.

Duke, S.O. and Powles, S.B. (2008). Glyphosate-resistant weeds and crops. *Pest Management Science* 64 (4), 317-318.

Gaines, T.A., Zhang, W.L., Wang, D.F., Bukun, B., Chisholm, S.T., Shaner, D.L., Nissen, S.J., Patzoldt, W.L., Tranel, P.J., Culpepper, A.S., Grey, T.L., Webster, T.M., Vencill, W.K., Sammons, R.D., Jiang, J.M., Preston, C., Leach, J.E. and Westra, P. (2010). Gene amplification confers glyphosate resistance in Amaranthus palmeri. Proceedings of the National Academy of Sciences of the United States of America 107 (3), 1029-1034.

Heap, I. (2012). 'The International Survey of Herbicide Resistant Weeds'. Viewed 10 May 2012, www.weedscience.com.

Holm, L.G., Plucknett, D.L., Pancho, J.V. and Herberger, J.P. (1977). 'The world's worst weeds: Distribution and biology'. The University Press of Hawaii, Honolulu, Hawaii.

Matsunaka, S. (1983). Evolution of rice weed control practices and research: World perspective. *In* Proceedings of the Conference on Weed Control in Rice, Los Baños, Laguna, Philippines, 31 August – 4 September 1981. International Rice Research Institute (IRRI), Los Banos, Philippines, 5-17.

Michitte, P., De Prado, R., Espinoza, N., Ruiz-Santaella, J.P. and Gauvrit, C. (2007). 'Mechanisms of resistance to glyphosate in a ryegrass (*Lolium multiflorum*) biotype from Chile', *Weed Science* 55 (5), 435-440.

Norsworthy, J.K., Talbert, R.E. and Hoagland, R.E. (1998). Chlorophyll fluorescence for rapid detection of propanil-resistant barnyard grass (*Echinochloa crus-galli*). *Weed Science* 46 (2), 163-169.

Powles, S.B. and Preston, C. (2006). Evolved glyphosate resistance in plants: Biochemical and genetic basis of resistance. *Weed Technology* 20 (2), 282-289.

Walker, S., Widderick, M., Storrie, A. and Osten, V. (2004). Preventing glyphosate resistance in weeds of the northern grain region. *In* Proceedings of the 14th Australian Weeds Conference – Weed management: balancing people, planet and profit, Wagga Wagga, New South Wales, 6–9 September 2004. Weed Society of New South Wales, Sydney, Australia, 428-431.