Research Article

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Target-site EPSPS Pro-106 mutations: sufficient to endow glyphosate resistance in polyploid *Echinochloa colona*?

Heping Han,[a] Qin Yu,[a*] Michael J Widderick[b] and Stephen B Powles[a]

Abstract

BACKGROUND: This study confirms and characterises glyphosate resistance in two polyploid *Echinochloa colona* populations from north-eastern Australia.

RESULTS: Glyphosate dose response revealed that the two resistant populations were marginally (up to twofold) resistant to glyphosate. Resistant plants did not differ in non-target-site foliar uptake and translocation of ^{14}C-glyphosate, but contained the known target-site 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) mutation Pro-106-Thr and/or Pro-106-Leu. Although plants carrying either a single or two EPSPS mutations were glyphosate resistant relative to the susceptible population, they were still controlled at the field rate of glyphosate (450 g a.e. ha^{-1}) when treated under warm conditions (25/20 °C). However, when treated in hot conditions (35/30 °C), most mutant resistant plants (68%) can survive the field rate, and an increase (2.5-fold) in glyphosate LD$_{50}$ was found for both the R and S populations.

CONCLUSIONS: This study shows that one or two EPSPS Pro-106 mutations are insufficient to confer field-rate glyphosate resistance in polyploidy *E. colona* at mild temperatures. However, control of these mutant plants at the glyphosate field rate is poor at high temperatures, probably owing to reduced glyphosate efficacy. Therefore, glyphosate should be applied during relatively mild (warm) temperature periods in the summer growing season to improve *E. colona* control.

Keywords: *Echinochloa colona*; EPSPS; glyphosate efficacy; glyphosate resistance; target-site mutation; temperature effect

1 INTRODUCTION

The globally important herbicide glyphosate inhibits the nuclear encoded enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19) which catalyses the reaction of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to form 5-enolpyruvylshikimate-3-phosphate, an important step in the biosynthesis of aromatic amino acids in plants. Initially as a non-selective herbicide and later as a selective herbicide with the wide adoption of glyphosate-tolerant transgenic crops, glyphosate has become the most widely used herbicide in the world.[1–3] Consequently, glyphosate overreliance for weed control over large cropping areas has resulted in the evolution of glyphosate resistance in populations of many weed species. Globally, glyphosate resistance has been documented in 32 weed species since the first identification in *Lolium rigidum*.[4–6] In Australia, glyphosate resistance has been reported in at least 700 populations of ten weed species.[7]

Echinochloa colona L. (Link), a tetraploid or hexaploid,[8,9] is a major annual grass weed species in summer crops and fallows in subtropical Australia.[10,11] Glyphosate has long been used for its control. Glyphosate-resistant *E. colona* was first confirmed in 2007 in northern New South Wales.[12] Since then, many more glyphosate-resistant populations have been identified in this region,[7] with one glyphosate-resistant population identified in Western Australia.[13] *E. colona* is now second only to *Lolium rigidum* in the number of glyphosate-resistant populations in Australia.[7] Glyphosate-resistant *E. colona* has also been reported in Argentina and the United States.[6,14,15]

A number of resistance mechanisms have been identified in glyphosate-resistant weed species. These mechanisms include EPSPS target-site alteration (mutations, gene amplification) and non-target-site-based reduced glyphosate uptake/translocation or enhanced vacuole sequestration.[16–20] Currently, four single amino acid substitutions at the target-site EPSPS Pro-106 (i.e. Pro-106-Ala, Pro-106-Ser, Pro-106-Thr, Pro-106-Leu) and a double amino acid substitution (Thr-102-Ile + Pro-106-Ser, referred to as TIPS) have been identified in field-evolved glyphosate-resistant weed species.[20,21] In glyphosate-resistant *E. colona* reported so far, mutation of the target-site EPSPS is the major mechanism associated with glyphosate resistance. The EPSPS Pro-106-Ser or Pro-106-Thr mutation has been identified in US glyphosate-resistant *E. colona*,[14,15] and Pro-106-Ser

* *Correspondence to: Qin Yu, Australian Herbicide Resistance Initiative, School of Plant Biology, University of Western Australia, Western Australia 6009, Australia, E-mail: qin.yu@uwa.edu.au*

a *Australian Herbicide Resistance Initiative, School of Plant Biology, University of Western Australia, Western Australia, Australia*

b *Queensland Department of Agriculture and Fisheries, Toowoomba, Australia*

or Pro-106-Leu mutation has been preliminarily reported in Australian glyphosate-resistant *E. colona*.[22,23] However, there are differing views on the significance of these mutations in conferring glyphosate resistance at recommended field rates. While the importance of the Pro-106-Ser mutation in endowing glyphosate resistance has been elucidated in the diploid weed species *Eleusine indica*,[24] little is known about the effects other EPSPS Pro-106 mutations have in conferring resistance in polyploid species such as *E. colona*.

Two putative glyphosate-resistant *E. colona* populations were collected from the north-eastern region of Australia. The objectives of this study were to confirm glyphosate resistance and to identify glyphosate resistance mechanisms in these two populations. This study highlights that one or two EPSPS target-site P106 mutation(s) in these two *E. colona* populations are not sufficient to endow field-rate glyphosate resistance at relatively mild (versus hot) summer temperatures, and suggests that early-summer glyphosate application at lower temperatures provides better control of both susceptible and resistant populations of this summer-growing polyploid weed species.

2 MATERIALS AND METHODS

2.1 Plant material

Seeds of the two putative glyphosate-resistant (R) *E. colona* populations (R1 and R2) were originally collected in 2009 near Millmerran, Queensland (approximately 27° 52′ 18.6″ S, 151° 15′ 03.3″ E), from a cotton/grain field that was in the fallow phase of a non-irrigated Roundup Ready® crop, and from near Bellata, New South Wales (approximately 29° 53′ 49.1″ S, 149° 44′ 47.1″ E), from a grain field in fallow following a wheat crop. Both fields had a history of >15 years of glyphosate use for weed control, with crops established using reduced-tillage systems. For this study, a known glyphosate-susceptible (S) *E. colona* population was collected from Dalby, Queensland (approximately 27° 32′ 21.0″ S, 151° 50′ 47.1″ E), from an agricultural research station with a conventional tillage system and little reliance on glyphosate. The two putative R populations were initially tested in a growth cabinet (28 °C) for glyphosate resistance at the early tillering stage with 225 g a.e. ha^{-1} of glyphosate. Survivors were isolated and grown to maturity, to produce glyphosate-resistant progeny for further study.

2.2 EPSPS gene sequencing

Genomic DNA was extracted from the leaf tissue of resistant (plants that survived 225 g a.e. ha^{-1} of glyphosate) and susceptible plants.[25] Total RNA was isolated using the Plant RNeasy Mini kit (Qiagen, Valencia, CA). Genomic DNA contamination was removed from the total RNA using the TURBO DNA-free kit (Ambion®; Life Technologies, Carlsbad, CA). For EPSPS DNA partial sequencing, a pair of published primers[26] were used to amplify a highly conserved region (^{95}LFLGNAGTAMRPL^{107}) in which point mutations conferring glyphosate resistance in plants have been reported.[20,27–30] The forward primer EleuEPSPSF (5′-GCGGTAGTTGTTGGCTGTGGTG-3′) and the reverse primer EleuEPSPSR (5′-TCAATCCGACAACCAAGTCGC-3′)[26] were used to amplify a 301 bp DNA fragment (includes 99 bp intron) flanking the potential mutation sites.

The PCR products (301 bp) from the plants with uncertain EPSPS mutations due to the double peak at the first two bases of Pro-106 codon were cloned into the pGEM-T vector (Promega, Madison, WI) and transformed into the competent *E. coli* cell (strain JM109). White colonies with putative inserts were used as templates for PCR reamplification of the fragment. Using the same primer pairs, a 202 bp cDNA fragment (without intron) was amplified from RNA samples of pregenotyped individuals from each resistant population. The PCR was conducted in a 25 μL volume that consisted of 1–2 μL of genomic DNA or cDNA, 0.5 μM of each primer and 12.5 μL of 2× GoTaq Green Master Mix® (Promega). 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 30 s; 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 the PCR Clean-up System (Promega) and sequenced by commercial services. All sequence chromatograms were visually checked for quality and consistency before sequences were assembled and aligned.

2.3 Generation of resistant subpopulations

Twenty-three plants that were identified as containing the single EPSPS Pro-106-Thr mutation from population R1 were isolated and bulked to produce progeny subpopulation R*1 (Pro-106-Thr). Eleven plants that contained the single Pro-106-Leu mutation from population R_2 were bulked to generate subpopulation R*2 (Pro-106-Leu). A single available plant containing the two mutations (Pro-106-Thr and Pro-106-Leu) from population R2 was selfed to produce subpopulation R*2-2 (Pro-106-Leu/Thr).

2.4 Glyphosate dose response

E. colona seeds were treated with concentrated sulphuric acid (98%) for 5 min and rinsed with water for 3 min to break seed dormancy and germinated on agar (0.6%) solidified water in a growth incubator at 25/20 °C. Germinating seedlings were transplanted to plastic pots (18 mm in diameter, 20 seedlings per pot) containing potting mix (50% composted fine pine bark, 30% cocopeat and 20% river sand) and grown in a glasshouse with day/night temperature fluctuations at 31–17 °C/26–17 °C in summer (January–February). For comparison of the temperature effect on resistance, transplanted seedlings were grown in controlled environmental rooms (CERs) with day/night temperatures of 25/20 °C, a photon flux density of 300–400 μmol m^{-2} s^{-1}, a photoperiod of 12 h and a relative humidity of 70–75%.

Glyphosate (Roundup Attack, 570 g a.e. L^{-1}; Nufarm Limited, Laverton, Victoria, Australia) was applied to 3–4-leaf-stage plants in 117 L ha^{-1} spray volume, delivered in two passes at 200 kPa with a cabinet sprayer equipped with two flat-fan nozzles. Glyphosate rates at 0, 56, 84, 112, 168, 225, 450 and 900 g a.e. ha^{-1} were used for the S population, and 0, 112, 225, 337, 450, 675, 900 and 1800 g a.e. ha^{-1} for R populations. There were four replicates per treatment. After herbicide spraying, plants were returned either to the glasshouse or to CERs with contrasting day/night temperatures of 25/20 °C and 35/30 °C under similar conditions to those specified above. Plant mortality (those plants having no new growth are considered to be dead) was determined 3 weeks after treatment.

2.5 ^{14}C-glyphosate uptake and translocation

The S and two R (R1 and R2) populations were used in this experiment. Seedlings at the 1–2-leaf stage were transplanted into plastic cups (60 × 60 × 100 mm, one seedling per cup) containing the potting mixture. Seedlings were maintained in a CER at alternating temperature of 25/20 °C and a 12 h day/night photoperiod with

a light intensity of 650 μmol m^{-2} s^{-1} and a relative air humidity of 75%.

When seedlings reached the 2–3-leaf stage, a single droplet (1 μL) of ^{14}C-labelled glyphosate solution (^{14}C-labelled in the phosphonomethylene, with a specific radioactivity of 55.18 mCi $mmol^{-1}$; PerkinElmer, Inc., Boston, MA) was applied with a micropipette to the midpoint of the adaxial surface of the second fully expanded leaf. The radiolabelled glyphosate treatment solution was a mixture of ^{14}C-glyphosate diluted in commercial glyphosate formulation (Roundup Attack herbicide as potassium salt, 570 g a.e. L^{-1}; Nufarm) and 0.25% (v/v) nonionic surfactant BS1000. The ^{14}C-radioactivity contained in a 1 μL droplet was 0.89 kBq, with a final glyphosate concentration of 5 mM (equivalent to the glyphosate rate of 123 g a.e. ha^{-1}, which is a discriminating rate for the glyphosate-resistant and susceptible plants).

Five treated plants from each R and S population were harvested 24, 48 and 72 h after treatment (HAT). At harvest, whole plants (above- and below-ground biomass) were carefully removed from the potting mixture. The treated leaf surface of each plant was rinsed in 20 mL of washing buffer containing 20% (v/v) methanol and 0.2% (v/v) Triton X-100 to remove unabsorbed ^{14}C-glyphosate. The radioactivity in the leaf wash was measured using a liquid scintillation counter (LSC) (Packard 1500, Tri-Carb®; PerkinElmer). Similarly, root tissue of each plant was rinsed in 50 mL of washing buffer, and the wash-off radioactivity was quantified. The plant samples were then blotted dry with paper towels, pressed and oven dried for 2 days at 60 °C. Translocation of ^{14}C-glyphosate from the application site to other parts of the plant was visualised by phosphor imaging (Personal Molecular Imager™; Bio-Rad Laboratories, Inc., Hercules, CA).

Following imaging, the plant samples were sectioned into treated leaf, untreated shoot and leaf and roots. The plant sections were then combusted in a biological oxidiser (RJ Harvey Instrument Corporation, Hillsdale, NJ), and the radioactivity in each section was quantified using the LSC. The average recovery of applied ^{14}C-glyphosate (leaf, root wash and combustion) across the three time points in R and S plants was 91 ± 1.1%. Glyphosate leaf uptake was calculated from the difference between the radioactivity applied and that in the leaf wash solution, and was expressed as percentage of total ^{14}C applied. Radioactivity distribution (and translocation) in plant parts was expressed as percentage of total ^{14}C absorbed.

2.6 Statistics

The LD_{50} (herbicide rate causing 50% plant mortality) and GR_{50} (herbicide rate required to reduce shoot weight by 50%) was estimated using the four-parameter logistic equation

$$y = C + (D - C) / \left[1 + \left(x/I_{50}\right)^{b}\right]$$

where C is the lower limit at the indefinitely large doses, D is the upper limit close to untreated controls and b is the slope of the best-fitting curve through the I_{50} (LD_{50}, GR_{50}). The estimates were obtained using Sigmaplot software (v.12.3; Systat Software, Inc., Evaston, IL), and Tukey's test ($\alpha = 0.05$) was used to test the significance of the regression parameters. There were several pilot trials prior to final herbicide dose response experiments. Each dose response experiment was conducted at least twice with similar results. Only results from a single experiment are presented in the figures.

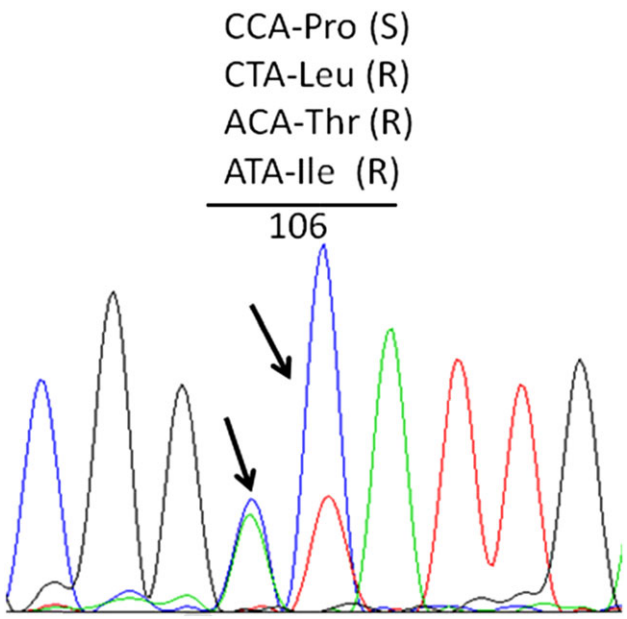

Figure 1. DNA sequencing results of the single plant from R2, showing the possible combinations of the first two bases at Pro-106 coding for different amino acids.

3 RESULTS

3.1 EPSPS DNA and cDNA sequencing

DNA samples of 23 glyphosate survivors (225 g a.e. ha^{-1}) from the R1 population and 11 survivors from the R2 population were analysed for EPSPS resistance-endowing mutations. In nearly all survivors analysed, the known glyphosate resistance EPSPS mutations Pro-106-Thr and Pro-106-Leu were identified from populations R1 and R2 respectively. It was noted that the resistant individuals possessing an EPSPS Pro-106 mutation always displayed double peaks (both mutant and wild-type nucleotides) at the mutation site in the sequence chromatograms (data not shown). The presence of both mutant and wild-type alleles in polyploid plants may be because the plant is a true allelic heterozygote within a genome, or a homoeoalleic heterozygote (homozygous resistant within a genome but heterozygous across genomes). The sequencing primers used in this experiment were for an unrelated diploid species[26] and not genome or gene-copy specific, and hence unable accurately to determine the genotype in polyploid plants. In addition, one individual (out of 11) from the R2 was found to display two double nucleotide peaks at the first and second nucleotides of the 106 code, indicating the presence of possible multiple mutations (e.g. Pro-106-Leu, Pro-106-Thr and Pro-106-Ile) in a single plant (Fig. 1). Therefore, EPSPS DNA sequencing was conducted for the progeny plants derived from self-pollination of the multiple mutation plant. Among 14 progeny plants analysed, four individuals were clearly identified to have the Pro-106-Leu mutation alone, while the exact mutation(s) from the other ten individuals could not be explicitly identified by direct sequencing. Thus, the 301 bp DNA PCR products from three of these individuals were cloned, with 23 clones sequenced in total, and only the wild-type (3) and the 106-Thr (20) sequences were found. Therefore, it is clear that population R1 contained resistant individuals possessing the Pro-106-Thr mutation, and population R2 contained resistant individuals that mostly possessed the Pro-106-Leu mutation, with some individuals (9%) containing both mutations.

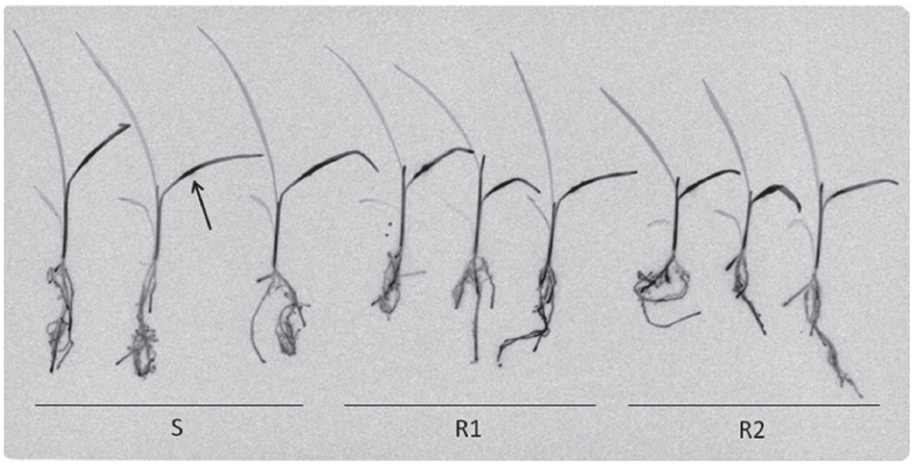

Figure 2. Translocation of ^{14}C-glyphosate from the application leaf (arrowed) to untreated parts of plants of glyphosate-susceptible (S) and glyphosate-resistant (R1, R2) *Echinochloa colona* populations at 48 h after treatment.

3.2 ^{14}C-glyphosate leaf uptake and translocation

^{14}C-glyphosate leaf uptake and translocation were determined in the S and the two resistant populations R1 and R2. No difference was found in foliar uptake of ^{14}C-glyphosate in R and S plants, with 60% of the applied ^{14}C being absorbed into leaf tissue by 24 HAT and remaining consistent up to 72 HAT (Table 1). Similarly, the R and S plants did not differ in glyphosate translocation, with 60–70% of ^{14}C-glyphosate being translocated from the treated leaf to other untreated plant parts by 24 HAT and remaining the same thereafter (Table 1, Fig. 2). Therefore, it is evident that glyphosate resistance in the R populations is not due to alteration in glyphosate leaf uptake or translocation.

3.3 Glyphosate dose response, comparing resistant populations (R1, R2) and resistant subpopulations (R*1, R*2, R*2-2)

The S population was controlled at ≥168 g a.e. glyphosate ha^{-1} (Fig. 3a), with an LD_{50} value of 115 g a.e. ha^{-1} (Table 2). All plants from the two resistant populations (R1 and R2) survived 112 g a.e. glyphosate ha^{-1} but were controlled at ≥337 g a.e. ha^{-1}, with an LD_{50} value of 240 and 229 g a.e. h^{-1} respectively. Based on the R/S LD_{50} ratios, the two resistant populations were clearly but only marginally (up to twofold) resistant to glyphosate. The recommended glyphosate field rate (450 g a.e. ha^{-1}) remained effective in controlling the resistant populations in the fluctuating temperature range used (31–17 °C/26–17 °C). Glyphosate dose response based on plant above-ground dry mass (GR_{50}) displayed similar resistant levels (Table 2).

Glyphosate dose response experiments were also conducted under the above conditions simultaneously using resistant subpopulations R*1, R*2 and R*2-2 composed of plants possessing the Pro-106-Thr, Pro-106-Leu and Pro-106-Thr/Leu mutation(s) respectively. However, the resistant subpopulations displayed a similar level of glyphosate resistance to that of resistant populations R1 and R2 (Table 2, Fig. 3b), based on the LD_{50} or GR_{50} ratios, with very few individuals surviving the glyphosate field rate (450 g a.e. ha^{-1}). These results show that there is no phenotypic resistance segregation in progeny R* populations at the discriminating glyphosate rate of 112 g a.e. glyphosate ha^{-1}, and indicate that plants possessing the single EPSPS mutation are likely to be true allelic homozygotes, and the parent plant carrying the two mutations

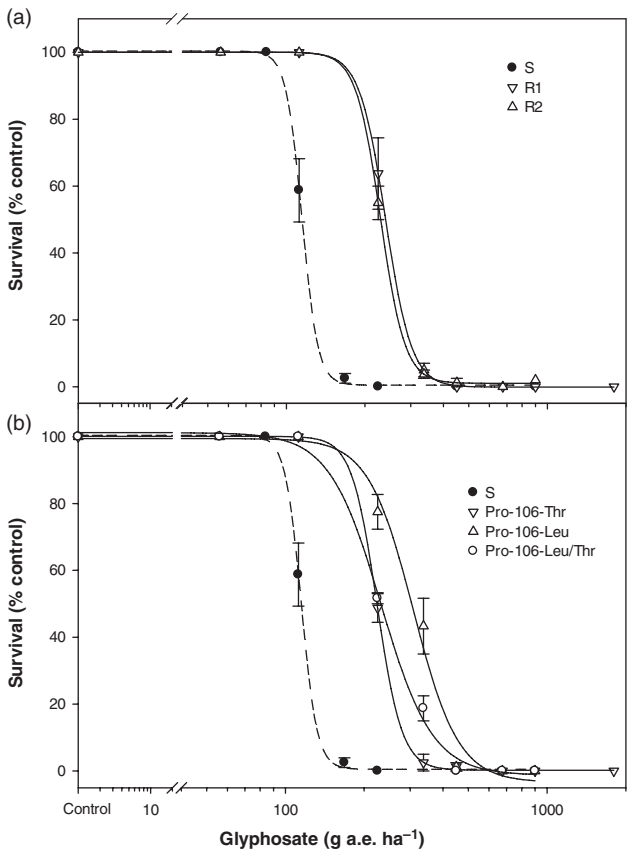

Figure 3. Glyphosate dose response of plants from (a) the original resistant populations R1 and R2 and (b) the subpopulations R*1, R*2 and R*2-2 containing the Pro-106-Thr, Pro-106-Leu and Pro-106-Leu/Thr EPEPS target-site mutation respectively. The experiment was conducted in a glasshouse with day/night temperature fluctuations at 31–17 °C/26–17 °C in summer (Janurary–February).

(Thr/Leu) is likely to be an allelic compound heterozygote. Thus, it is clear that even two allelic resistance EPSPS alleles in a polyploid *E. colona* plant are not enough to provide sufficient resistance at the field recommended glyphosate rate, under the experimental conditions used, if no other additional resistance mechanisms are involved.

Table 1. Foliar uptake and translocation of ^{14}C-glyphosate in plants of glyphosate-susceptible (S) and glyphosate-resistant (R1, R2) *Echinochloa colona* populations. Means (standard error) labelled with the same letter in a column within a time point are not significantly different (Tukey's test, $\alpha = 0.05$)

			Translocation (% absorbed)		
Time after treatment	Population	Folia uptake (% applied)	Treated leaf	Stem and new growth	Root
24 h	S	61 (4.5) a	31 (1.6) b	29 (3.1) a	41 (3.0) a
	R1	64 (3.2) a	33 (2.9) ab	36 (4.5) a	32 (2.2) a
	R2	60 (3.7) a	40 (1.1) a	30 (2.5) a	31 (2.6) a
48 h	S	66 (3.3) a	29 (1.0) a	33 (1.6) a	38 (0.9) a
	R1	59 (4.1) a	29 (2.0) a	36 (1.6) a	36 (2.8) a
	R2	61 (1.9) b	29 (2.0) a	37 (3.1) a	33 (2.9) a
72 h	S	63 (3.5) a	29 (2.1) a	35 (1.9) a	36 (3.5) a
	R1	54 (2.4) a	23 (2.5) a	34 (3.0) a	42 (4.0) a
	R2	58 (2.6) a	28 (2.6) a	31 (0.8) a	40 (2.7) a

Table 2. Comparison of level of resistance to glyphosate among resistant *Echinochloa colona* populations (R1, R2) and their progeny subpopulations (R*1, R*2, R*2-2) containing different EPSPS target-site mutation(s). SE from the non-linear regression analysis is in parentheses

Population	LD_{50} (g a.e. ha^{-1})	LD_{50} R/S ratio	GR_{50} (g a.e. ha^{-1})	GR_{50} R/S ratio
S	115 (0.68)		88 (2.9)	
R1	240 (0.31)	2.08	102 (5.4)	1.16
R2	229 (0.87)	1.99	187 (15.5)	2.13
Pro-106-Thr (R*1)	224 (0.52)	1.95	197 (9.6)	2.24
Pro-106-Leu (R*2)	308 (15.8)	2.68	113 (1.87)	1.28
Pro-106-Leu/Thr (R*2-2)	232 (7.2)	2.02	106 (9.7)	1.20

3.4 Glyphosate dose response under warm and hot conditions

In trial experiments with the resistant populations, we observed variations in mortality response to the field rate of glyphosate (450 g a.e. ha^{-1}) when conducted in different temperature-controlled glasshouses. *E. colona* is a warm-season C_4 grass that performs well at higher temperatures. In order to investigate and confirm whether temperature affects glyphosate efficacy or resistance status, an additional experiment was conducted in a CER under warm (25/20 °C) versus hot (35/30 °C) conditions with similar humidity, using the S and the R*2-2 populations. For the S population, plants were clearly less susceptible to glyphosate under hot than under warm conditions (Fig. 4). For example, at 25/20 °C, S plants can be controlled at glyphosate rates of ≥112 g a.e. ha^{-1}, whereas there was 100% survival at this rate at 35/30 °C. Based on the LD_{50} ratio between the two temperatures (Table 3), S plants were 2.6-fold less susceptible to glyphosate at the higher temperature, requiring higher than the field rate (450 g a.e. ha^{-1}) to achieve total control. Similar results were observed for the R plants (Fig. 4, Table 3). For instance, at 25/20 °C, no R plants survived glyphosate rates of ≥337 g a.e. ha^{-1}, whereas at 35/30 °C, 90% of R plants survived this rate and 68% survived the field rate. The shift in LD_{50} value from the lower to higher temperature is 2.5-fold, requiring ≥900 g a.e. ha^{-1} to control the R plants. While the LD_{50} value for the S or R plants was affected by temperature, the LD_{50} R/S ratio remained similar regardless of the temperature (Table 3). These results clearly show that temperature affects glyphosate efficacy, and that glyphosate control of R and S *E. colona* populations is improved at lower temperatures.

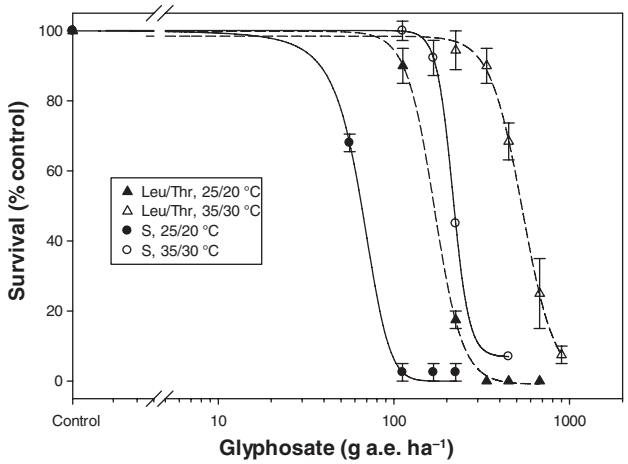

Figure 4. Glyphosate dose response, under warm (25/20 °C) versus hot (35/30 °C) conditions, of a herbicide-susceptible population (S) and a resistant subpopulation (R*2-2) containing Pro-106-Thr and Pro-106-Leu mutations.

4 DISCUSSION

Three target-site EPSPS mutations (Pro-106-Ser, Pro-106-Thr and Pro-106-Leu) have been previously reported in glyphosate-

Table 3. LD_{50} values of susceptible (S) and resistant (R*2-2) *Echinochloa colona* populations in response to glyphosate treatment under warm (25/20 °C) versus hot (35/30 °C) conditions. SE from the non-linear regression analysis is in parentheses

	LD_{50} (g a.e. ha^{-1})				
Population	25/20 °C	R/S ratio	35/30 °C	R/S ratio	LD_{50} ratio (high/low temperature)
S	65 (2.41)		170 (3.25)		2.6
R*2-2	216 (0.17)	3.3	539 (19.2)	3.2	2.5

resistant *E. colona*.[14,15,22,23] Here, we identified the same mutations Pro-106-Thr and Pro-106-Leu in *E. colona*, and demonstrated that, depending on the environmental conditions, target-site Pro-106 mutation(s) may or may not be sufficient to enable plant survival at recommended glyphosate field rates in the polyploidy weed species *E. colona*.

Target-site mutation often endows herbicide resistance. There can be strong target-site mutations that confer high-level (>20–100-fold) herbicide resistance, such as various mutations endowing resistance to acetolactate synthase (ALS)-inhibiting or acetyl coenzyme A carboxylase (ACCase)-inhibiting herbicides, and weak mutations that provide a marginal level (2–5-fold, as compared with susceptible counterparts) of resistance, such as Pro-106 mutations in the EPSPS gene endowing resistance to glyphosate.[19] The likely reason why often the weak EPSPS Pro-106 mutations were selected in the field is that glyphosate competes with the substrate PEP for the binding site in EPSPS synthase,[31] and therefore it is unlikely for a mutation that endows high-level glyphosate resistance without comprising the catalytic capacity to be selected in nature.[21] Indeed, since first identified (Pro-106-Ser) in *Eleusine indica*,[27] four Pro-106 mutations (Pro-106-Ala, Pro-106-Thr, Pro-106-Ser and Pro-106-Leu) have been reported in glyphosate-resistant populations of six weed species.[20] These weed species include the diploid species *Amaranthus tuberculatus*, *Digitaria insularis*, *E. indica*, *Lolium rigidum* and *L. multiflorum*,[24,27,29,30,32,33] and the polyploid species *E. colona*.[14,15] Each of these 106 mutations generally provides only a low level (2–4-fold) of glyphosate resistance, enabling plant survival at certain glyphosate rates.[24,30] For example, in diploid *E. indica*, homozygous Pro-106-Ser mutation conferred only a moderate level (2.8-fold based on the GR_{50} resistant/susceptible ratio) of glyphosate resistance, which was nevertheless sufficient for plant survival at the recommended glyphosate field rate of about 1000 g a.e. ha^{-1}.[24] Here, we showed that the Pro106 mutations in polyploidy species (at least in the *E. colon* populations tested) may not be able to endow plant survival at the glyphosate field rate.

It is expected that the level of resistance conferred by the Pro-106 mutations in diploid species may also be affected by EPSPS copy numbers. For example, in the diploid *L. rigidum*, EPSPS exists as a small gene family,[30] and in the diploid *Conyza canadensis* there are two EPSPS gene copies.[34] Therefore, even in diploid species, the effect of a single Pro-106 mutation in one gene copy may be diluted by additional wild-type EPSPS gene copies.

Similarly yet more evident in polyploid species, the level of resistance endowed by a herbicide target-site mutation (especially for semi-dominant mutant alleles) can be much lower than in diploid species owing to the dilution and/or expression effect by multiple EPSPS copies, as has been demonstrated for resistance to ACCase-inhibiting herbicides in hexaploid *Avena fatua*.[35] For instance, hexaploid *E. colona* is expected to have at least three homoeologous EPSPS gene copies (although not determined in this study). *E. colona* plants homozygous for a given EPSPS mutation at one EPSPS locus would still have two-thirds of the total EPSPS expressed as wild-type susceptible EPSPS. This would further reduce the already low level of resistance endowed by various EPSPS Pro-106 mutations. This also explains why *E. colona* plants even possessing one or two EPSPS Pro-106 mutations can still be controlled by the glyphosate field rate of 450 g a.e. ha^{-1} under mild temperature glasshouse conditions (Figs 3 and 4).

Does this necessarily mean that one or two weak EPSPS resistant alleles in polyploid weed species are not sufficient for endowing resistance at the recommended glyphosate rates? Our results reveal that the weak EPSPS mutations in polyploidy *E. colona* can enable plant survival at field or higher glyphosate rates under higher temperature conditions which reduce glyphosate efficacy (Fig. 4). It has been demonstrated that a number of extreme environmental conditions, including high air temperature,[36] low soil moisture content[37] and low relative humidity,[38,39] reduce glyphosate efficacy. Especially in glasshouse-grown *E. colona*, highest glyphosate efficacy was achieved under warm conditions (25/20 °C), whereas the lowest efficacy occurred under hot conditions (35/30 °C),[40] which is similar to our results. Although not examined in our study or in other studies, reduced glyphosate efficacy at high temperatures may be due to increased glyphosate upward movement caused by higher rates of plant transpiration, thus decreasing glyphosate translocation. *E. colona* is the most common weed of summer fallows in the north-eastern grain region of Australia;[10] extreme growing conditions have been recorded, and higher temperatures (over 35 °C) in summer are quite common.[40] In addition, other factors reducing glyphosate rates (e.g. adverse weather conditions, poor herbicide application and large plant sizes) also contribute to reduced glyphosate efficacy. Therefore, even a weak EPSPS resistance mutant allele in a polyploid background can be very successful in conferring resistance at the field or higher glyphosate rates, subject to environmental conditions.

It is evident that under mild and unstressed glasshouse conditions (e.g. 25/20 °C), where glyphosate efficacy is high, one or two EPSPS resistance alleles in *E. colona* plants is not enough to endow glyphosate resistance at the field glyphosate rates. However, such EPSPS mutants can survive field or higher glyphosate rates when glyphosate is applied in the field under extreme or unfavourable environmental conditions. In this case, higher glyphosate rates (e.g. 900–1000 g a.e. ha^{-1}) were required to control the mutant plants.

As expected, target-site resistance is unlikely to be affected by temperature, and therefore poor glyphosate control in both the S and R *E. colona* populations at higher temperatures is due to reduced glyphosate efficacy (Table 3, Fig. 4a). This is different to those studies in which temperature only had an effect on glyphosate response of resistant plants.[41] This is because the non-target-site-based glyphosate resistance mechanism

(enhanced vacuole sequestration) operates more efficiently at cool temperatures.[20,42,43] Nevertheless, all these studies suggest that glyphosate application at relatively low temperatures would be beneficial to better weed control. This observation has practical importance for determining environmental (temperature) conditions where glyphosate efficacy is optimum.

In other cases where a higher level (5–10-fold or more) of glyphosate resistance is observed for plants possessing the Pro-106 mutations, additional non-target-site resistance mechanisms were also identified.[29,44,45] For example, in *E. colona*, higher levels (6–12-fold) of glyphosate resistance were reported as a result of Pro-106-Ser or Pro-106-Thr mutation.[14,15,23] This was probably due to as yet unidentified additional non-target-site resistance mechanisms, polyploidy (tetraploid versus hexaploid) or levels of expression of the mutant EPSPS allele(s) in the polyploid genome.

In summary, target-site EPSPS Pro-106 mutations were identified in two glyphosate-resistant polyploid *E. colona* populations. These are weak target-site mutations that were known to provide a level of resistance in diploid weed species. In polyploid plants such as *E. colona*, the level of resistance endowed by the same mutations can be lower, with one or two mutant alleles being insufficient for plant survival at glyphosate label rates under mild temperature conditions. Nevertheless, such mutants can result in glyphosate control failure if treated during periods of high temperatures. Therefore, better summer weed control by glyphosate would be achieved under relatively mild temperature conditions with higher glyphosate efficacy, and in extreme hot conditions higher glyphosate rates (≥900 g a.e. ha^{-1}) are required to control EPSPS Pro-106 mutants. However, if other glyphosate-resistant mechanisms (e.g. reduced translocation, gene amplification) co-occur, the maximum recommended glyphosate rate (1035 g a.e. ha^{-1}, Roundup Ready) in crop may not be effective. Other management strategies such as double knock with glyphosate and paraquat and other tactics[11] have to be employed.

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