



Original Research Article

Inheritance of glyphosate resistance in *Echinochloa colona* from Australia

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ABSTRACT

Glyphosate resistant *Echinochloa colona* is widespread in fallow systems of northern Australia from intensive glyphosate use. The inheritance of glyphosate resistance and the potential for gene transfer through pollen from resistant to susceptible individuals were investigated. A glyphosate resistant population A533.1, containing a mutation in *EPSPS*, was used as the resistant parent and Echi S as the susceptible parent. Gene flow via pollen between adjacent susceptible and resistant individuals was examined by treating progeny from the susceptible individuals at 240 g ha⁻¹ glyphosate with 1.38% gene flow detected. Survivors were selfed and the progeny segregated 3:1 for survival to 240 g ha⁻¹ glyphosate, consistent with single dominant gene inheritance. Hand crosses between resistant and susceptible individuals produced a single F₁ seed which carried the mutation in *EPSPS*. The F₂ generation from the hand cross had a response to glyphosate, intermediate between the two parents and similar to that expected for a single largely dominant gene. Sequencing the *EPSPS* cDNA detected at least two *EPSPS* genes expressed in *E. colona*, only one of which carried the mutation. Glyphosate resistance in this population of *E. colona* is inherited as a single largely dominant allele.

Introduction

Echinochloa colona (L) Link. (junglerice) is a tropical to sub-tropical C₄ grass species that is a major weed in rice and horticulture throughout the tropics (Holm et al. 1977). *E. colona* seed germinates readily from the soil surface at temperatures above 25°C, provided there is sufficient moisture present (Chauhan and Johnson, 2009). In Australia, *E. colona* is a major weed of cotton and summer fallow in New South Wales (NSW) and Queensland (QLD) (Osten et al. 2007).

Glyphosate resistance has evolved in *E. colona* populations in Australia, principally as a result of intensive glyphosate application in summer fallow systems (Storrie et al. 2008; Gaines et al. 2012; Nguyen et al. 2016). Glyphosate resistant *E. colona* is now widespread across this region (Preston, 2015). In addition, glyphosate resistance in this weed species has been also found in three other countries including Argentina, USA and Venezuela (Heap, 2015). The mode of inheritance of herbicide resistance alleles can influence how they spread through populations. Single gene dominant traits are likely to spread faster than multi-gene or recessive traits (Jasieniuk et al. 1996; Diggle and Neve, 2001; Preston et al. 2009). The inheritance patterns of glyphosate resistance in weed species are variable. Reduced glyphosate translocation in annual ryegrass (*Lolium rigidum*) (Lorraine-Colwill et al. 2003; Wakelin et al. 2004), as a result of vacuolar sequestration (Ge et al. 2012), is inherited as a single, mostly dominant gene (Lorraine-Colwill et al. 2001; Wakelin and Preston, 2006). A similar mode of inheritance has been observed in horseweed (*Conyza canadensis*) (Zelaya et al. 2004) with the same resistance mechanism (Feng et al. 2004; Ge et al. 2010; Sammons and Gaines, 2014). Similarly, inheritance of target site resistance to glyphosate in goosegrass (*Eleusine indica*) was also due to a single incompletely dominant gene (Ng et al. 2004a); and resistance in this grass species was reported as mutations in the *EPSPS* gene (Pro₁₀₆ to Ser₁₀₆ and Pro₁₀₆ to Thr₁₀₆ substitutions) (Ng et al., 2004b). However, glyphosate resistance in some populations of *L. rigidum* (Simarmata et al. 2005) and hairy fleabane (*Conyza bonariensis*) (Okada and Jasieniuk, 2014) fitted to a two-gene model. Inheritance patterns of the gene amplification mechanism of glyphosate resistance are considerably more complicated in both palmer amaranth (*Amaranthus palmeri*) (Gaines et al. 2010; Chandi et al. 2012; Mohseni-Moghadam et al. 2013) and brome grass (*Bromus diandrus*) (Malone et al. 2016).

Gene movement, either via pollen or seed, plays an important role in the spread of genotypes of a plant species (Slatkin, 1987). A serious problem in herbicide resistance is the movement of resistant genes between crop fields (Christoffers, 1999). Previous studies have shown the extent of gene flow can be highly variable depending on population size, distance between populations, biology of species and the modes of seed and pollen dispersal (Ellstrand, 1992). For example, the majority of pollen movement in rice occurs over short distances (Messeguer et al. 2001), but gene flow can occur over much longer distances in forest trees (Kremer et al. 2012). Gene flow rates are high in most gymnosperms, but vary widely in angiosperms. In addition, allogamous species had significantly higher gene flow levels than autogamous species (Govindaraju, 1989). For weed populations, gene flow via pollen between herbicide resistant and susceptible plants has been demonstrated for many species (Stallings et al. 1995; Marshall et al. 2001; Murray et al. 2002; Volenberg and Stoltenberg, 2002). Gene flow can play an important role in increasing the frequency

of herbicide resistant genes within populations not being selected by herbicides (Busi et al. 2011). An increased frequency of resistant genes within a population can accelerate the evolution of resistance with herbicide use. Gene flow can also result in the spread of herbicide resistance within a population (Jasieniuk et al. 1996; Diggle and Neve, 2001). The aims of this study were to determine the mode of inheritance of glyphosate resistance in *E. colona* and to evaluate gene flow resulting from pollen exchange between resistant and susceptible individuals.

Materials and Methods

Plant material

Seeds of two *E. colona* populations A533.1 (R) and Echi S (S) were collected near Moree and Tamworth in New South Wales respectively (Nguyen et al. 2016). The populations were confirmed as *E. colona* through floristic characteristics (Jessop et al. 2006), and determined as resistant (A533.1) and susceptible (Echi S) to glyphosate at a single discriminating dose of 270 g a.e. ha⁻¹ via a dose response experiment in 2011 (data not shown). Of several populations of *E. colona* tested, A533.1 had the highest level of resistance to glyphosate with LD₅₀ ranging from 233 to 466 g a.e. ha⁻¹ and resistance level from 4 to 6.8-fold (Nguyen et al. 2016), and this population contained a mutation in *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) gene at codon 106 with a substitution of serine for proline (Nguyen et al. 2016). Seeds from two populations were treated with 95% H₂SO₄ for 30 minutes, rinsed under running water for 60 minutes and germinated on 0.6% (w/v) agar in a climate controlled cabinet with 12h light/dark periods at 22°C with 30 μmol m⁻²s⁻¹ during the light period.

Gene flow between resistant and susceptible individuals

At the one-leaf stage (8 days after germination), ten seedlings each of the R and S populations were transplanted into individual 25 cm diameter x 24 cm height pots (Masrac Plastics Pty Ltd., South Australia) containing standard potting soil. The pots were arranged into pairs containing one R and one S plant, with plants within the pair placed 30 cm apart and each pair placed at least 10 m apart. The plants were maintained outdoors, with all other *Echinochloa* spp. plants removed from the vicinity of the experiment to avoid pollen contamination. Prior to anthesis, two single spikes from each R and S individual were bagged with glassine bags to self-pollinate. Growth (flowering and seed maturity) and flower-head number of individuals were observed throughout the experiment. After flowering, all seeds from each individual were harvested separately, air dried and stored at room temperature for three to four months to break dormancy. Seeds from the bagged

spikes were also harvested and stored in isolated envelopes. The 100-seed weight from each individual plant was determined. Seeds were tested for germinability, as described above, once dormancy was relieved and the germination percentage recorded after 10 days. The data were analysed by ANOVA using SAS software ver. 6.03 for MS-DOS (SAS Institute, USA). LSD (least significant different) at the 5% level was used to separate means.

The frequency of gene flow was determined by testing the response of the F_1 seed from the susceptible parent of each crossing pair to glyphosate application. This experiment was conducted outdoors during the summer of 2013. The seed set on four S plants that had been planted adjacent to four R plants were germinated. At the same time, seeds from bagged spikes of the R and S plants that had been selfed were germinated. Seedlings at the one-leaf stage were transplanted into 35 x 29 x 6 cm trays (Masrac Plastics Pty Ltd., South Australia) at a density of 50 plants per tray. Seedlings of R and S selfs were planted at a density of 25 plants per tray. At the 3 to 4-leaf stage, these seedlings were treated with 240 g ha⁻¹ glyphosate (Roundup PowerMax®, Nufarm Australia Limited) plus 0.2% (v/v) non-ionic surfactant (alcohol alkoxyate, BS 1000, Crop Care), to identify resistant individuals in the F_1 generation. The glyphosate rate of 240 g a.e. ha⁻¹ was used in this experiment as it controlled completely the susceptible population in a previous dose response experiment (data not shown). Herbicide was applied with using a moving-boom laboratory twin nozzle boom sprayer (Hardi ISO F-110-01 standard flat fan, Hardi, Adelaide) positioned 40 cm above the seedlings at a water volume of 109.6 L ha⁻¹, a pressure of 250 kPa and a boom speed of 1 m s⁻¹. At 30 days after treatment, the number of survivors was recorded. Leaf material was taken from each surviving plant for sequencing the *EPSPS* gene and the survivors were transplanted into 20 cm diameter pots, transferred to a glass house and allowed to self to produce F_2 seed.

Hand crossing of resistant and susceptible individuals

Seedlings of the R population and S population were grown outdoors in 25 cm in diameter pots (Masrac Plastics Pty Ltd., South Australia) in the 2012/13 summer. Prior to anthesis, the anthers of 300 S flowers were removed and the flowers covered with glassine bags to exclude unwanted pollen. When the stigmas of the emasculated flowers were exposed, pollen from R plants was applied onto the stigmas. Only two fertilized flowers set seed. The two fertilized mature seed were harvested, air dried and stored at room temperature. A single F_1 seed was germinated and planted in a 25 cm diameter pot and allowed to set seed to produce F_2 progeny. Meanwhile, the remaining seed did not germinate due to damage after putting on agar medium.

Shikimate accumulation in leaf discs

Shikimate accumulation was measured as described by Shaner et al. (2005) with minor modifications. Five leaf discs (5 mm diameter) were excised from the youngest fully mature leaf of the F₁ plant produced via hand crossing, and of the two parental plants (R and S) using a cork borer and placed in wells of a 400 µL flat-bottomed microtiter plate containing 200 µL of 10 mM phosphate buffer at pH 7 including 0, 50, 200, 500 and 1000 µM glyphosate. The plates were incubated under fluorescent lights (553 µmol m⁻²s⁻¹) at 25°C for 16 h. At the end of the incubation period, 50 µL of 50 mM HCl was added to each well and the plates were wrapped up in cling film to minimise evaporation. The samples were frozen and thawed through two cycles of -20°C for 90 minutes and 60°C for 20 minutes until the leaf tissue had turned grey-green. After this, 25 µL of extract from each sample was transferred to a clean microtiter plates, a mixture (100 µL) of 0.25% (w/v) periodic acid and 0.25% (w/v) sodium m-periodate was added to each well and the samples were incubated at room temperature for 60 minutes. To stop the reaction, 100 µL of newly made quench buffer (mixture of 0.6 M NaOH and 0.22 M Na₂SO₃) was added. A standard curve of shikimic acid: 1, 2.5, 5, 10, 25 and 50 µM, were included for each experiment. Absorbance of the final reaction sample was measured at 380 nm. The optical density of the 0 glyphosate samples was subtracted from all other samples to determine shikimate accumulation due to glyphosate. Shikimate accumulation was calculated from a standard curve and presented as nmol of shikimate cm⁻². The experiment was repeated five times with five leaves per plant each time as replicates. The data were analysed as a one-phase association using Prism 6 ver. 6.00 (©1992-2012 GraphPad Software, Inc., USA) to compare the differences between parental plants and the F₁ plant.

Segregation test for resistance

This experiment was conducted outdoors. F₂ seeds of the 28 putative F₁ crossed individuals from the gene flow experiment were germinated and transplanted into 8.5 x 9.5 x 9.5 cm pots at a density of nine seedlings per pot. The number of F₂ plants from each F₁ individual ranged from 13 to 27. The parental R and S populations were also included. At the 3-4 leaf stage, these plants were treated with glyphosate at 240 g ha⁻¹. At four weeks after glyphosate treatment, survival and mortality rates were scored and segregation was tested against a single-gene model with a dominant allele using a G-test with Williams correction (Sokal and Rohlf, 1981). The expected survival and mortality rates were corrected for the low rate of survival of the S parents in this experiment.

Dose response to glyphosate

Dose response experiments were conducted outdoors on the F₂ progeny from the hand cross and the two parental populations R and S. Seedlings were transplanted into 8.5 x 9.5 x 9.5 cm pots with a density of nine seedlings per pot and three replicate pots per treatment. Glyphosate was applied at 10 rates of 0, 40, 80, 120, 240, 360, 480, 600, 800 and 1000 g a.e. ha⁻¹ with non-ionic surfactant at 0.2% (v/v). Survival was assessed at 21 days after glyphosate treatment, with plants having new green leaf tissue considered as survivors.

EPSPS gDNA sequencing

Young green leaf tissue was sampled from the parental R and S plants and F₁ plants in the gene flow experiment, the single F₁ plant and 30 F₂ individuals from hand crossing; and DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Australia) in accordance with the manufacturer's instructions. PCR was conducted in 25 µl volumes containing 80 -100 ng DNA, 1 × High Fidelity PCR Buffer [600 mM Tris-SO₄ (pH 8.9), 180 mM (NH₄)₂SO₄], 0.4 mM dNTP mixture, 4 mM MgSO₄, 0.4 µM 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 GGAGGGCMTAGTGCCAAG-3') primer were used for amplification of an approximately 500 bp fragment of the *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase). 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 sequenced at the Australian Genome Research Facility (AGRF) Ltd., Australia using the same primers used for amplification. DNA sequence data was assembled compared and analysed using ContiExpress from the Vector-NTi Advance 11.5 programs (Invitrogen, USA).

EPSPS cDNA cloning and sequencing

EPSPS was sequenced from cDNA of one individual from the parent R and S populations as well as two F₂ individuals from the gene flow experiment; one F₂ from Family 10, where a Pro 106 to Ser mutation was identified in sequencing gDNA and one F₂ individual from Family 28, where no Pro 106 to Ser mutation was detected in sequencing gDNA. Total RNA was extracted using the Isolate II RNA Plant Kit (Bioline, Australia) and cDNA was synthesized using the Tetro cDNA Synthesis Kit (Bioline, Australia) in accordance with manufacturer's instructions. *EPSPS* was amplified from the

cDNA as described above and cloned using the pGEM-T Easy Vector system (Promega, USA). The presence of the *EPSPS* fragment in DNA isolated from resultant colonies was confirmed by PCR. The PCR conditions were as described above, except template cDNA was replaced with a single clone colony and the initial denaturing step increased to 10 min to aid cell lysis. Plasmids containing the *EPSPS* fragment were sequenced at the AGRF (Australia) using the T7 primer (5'-TAATACGACTCACTATAGGG-3'). Sequences were aligned using Clustal X software ver. 1.83.40 (Thompson et al. 1997). Geneious 8.1.3 (<http://www.geneious.com>) (Kearse et al. 2012) was used to generate an unrooted UPGMA dendrogram using Jukes-Cantor p-distance values.

Results and Discussion

Plant growth of the parental populations

There were no differences in the growth patterns of the R and S parental populations. Flowering occurred between 35 and 37 days after transplanting and seed maturation between 62 and 68 days after transplanting. The flowering time of resistant and susceptible plants was similar, increasing the probability of pollen exchange between the two populations. There were significant differences in flower-head number, 100-seed weight and seed germination between the resistant and susceptible populations. The R population produced a smaller number of panicles than the S population. The 100-seed weight of the S population was 0.12 g while that of R population was 0.10 g. The germination rate of seed from the S population was higher than that of the R population (80.9% compared with 62.7% respectively) (Table 1).

Table 1. Flower-head number, 100-seed weight and seed germination of the R (A533.1) and the S (Echi S) populations of *E. colona* in the gene flow experiment.

Population	Flower-head number	100-seed weight (g)	Seed germination (%)
A533.1 (R)	203.0	0.10	62.7
Echi S (S)	286.4	0.12	80.9
LSD (5%)	36	0.005	17.6

Gene flow frequency

The gene flow frequency was determined as the survival rate of seed from the S plants in each pair at 240 g ha⁻¹ of glyphosate. At 30 days after glyphosate application, 28 survivors were recorded out of 2024 plants grown, accounting for a gene flow frequency of 1.38%. Meanwhile, all R control

plants (80 plants) survived and all S control plants (96 plants) were killed (Table 2); this demonstrates that the genotype of parental plants of these progenies were homozygous. The gene flow frequency among four different parental pairs was similar, ranging from 1.35% to 1.47% (Table 2). This suggests that homogeneous cross-pollination occurred in the crossing pairs.

Table 2. Survival of F₁ progenies from four parental susceptible plants in the gene flow experiment to determine gene flow frequency between R and S *E. colona* plants

Parental pair	Number of plants tested	Survivor	Gene flow frequency (%)
1	68	1	1.47
2	554	8	1.44
3	511	7	1.37
4	891	12	1.35
Total	2024	28	1.38
A533.1 (R control)	80	80	
Echi S (S control)	96	0	

This study has shown using glyphosate resistance as a marker that there is a low frequency (1.38%) of pollen movement between plants of *E. colona*. Cross-pollination is an essential prerequisite in grass weed species for gene flow to occur. Full self-pollination happens when anthers dehisce inside the flower before the flowers opened (Motten and Antonovics, 1992; Nunez-Farfan et al. 1996). However, in *E. colona* no pollen dehiscence occurred in the anthers at the opening of the flowers, but pollen grains can be found adhering to the stigmas of the flowers after anthesis (data not shown). The frequency of gene flow via pollen between plant populations has been reported to vary. Levin (1984) considered gene flow to be limited and likely far lower than 1%, while Ellstrand (1992) argued that this rate was normally over 1%. In a study of cultivated rice (*Oryza sativa* L.), Messeguer et al. (2001) determined gene flow from genetically modified rice to conventional rice with a percentage from 0.01 to 0.53%. Volenberg and Stoltenberg (2002) used the acetyl-CoA carboxylase (ACCCase) herbicide resistant trait of giant foxtail (*Setaria faberi*) as a marker in identifying the gene flow from resistant to susceptible individuals, and showed an outcrossing rate ranging from 0.24 to 0.73%. The ACCCase resistance trait was likewise used by Murray et al. (2002) in wild oat (*Avena fatua*) with an outcrossing rate ranging from 0 to 12.3%. A study of glyphosate resistance in *C. canadensis* also determined that the resistant gene flow

frequency reached 1.1 to 3.8% (Davis et al. 2010). The level of gene flow found in *E. colona* in this study is similar to that found in other predominantly autogamous weed species.

The effect of distance on gene flow between pollen donor and acceptor plants has been studied by many authors and gene flow reduces greatly with distance (Ellstrand, 1992). The impact of distance and wind on gene flow was studied by Messeguer et al. (2001) on *O. sativa*, who found the gene flow rate was higher with plants in close proximity and down wind. A distance of 75 m between pollen donor and acceptor fields produced a dramatic drop in gene flow of *meadow fescue* (*Festuca pratensis*) (Rognli et al. 2000). For common sunflower (*Helianthus annuus*), gene movement from imazethapyr resistant plants to susceptible plants occurred at a distance of 15.5 m (Marshall et al. 2001). In addition, Ellstrand (1992) suggested that within a small population, gene flow levels were likely to be higher compared to populations of larger size. In the present study, resistant and susceptible plants were grown as adjacent pairs with only one plant for each biotype in the pair and the distance between two individuals was <1 m in order to maximise cross-pollination. Therefore, the probability of identifying gene flow would be high and the frequency of gene flow by pollen identified in this experiment is likely to be much higher than that between isolated populations.

Shikimate accumulation in F₁ plants

From a single seed obtained from the hand cross between resistant and susceptible plants, the non-destructive shikimate assay was used to confirm whether this plant was likely to be an F₁ hybrid. It has been previously shown that F₁ hybrids of *C. canadensis* (Zelaya et al. 2004) and *L. rigidum* (Preston et al. 2006) have intermediate shikimate accumulation compared with the parents. In this study, the single seed was confirmed to be a hybrid by both sequencing the *EPSPS* gene (data not shown) and shikimate accumulation in the presence of glyphosate (Figure 1). Shikimate accumulated to higher concentrations in leaf discs from the susceptible plants than the resistant plants. Shikimate accumulation in the F₁ cross was intermediate between that of the two parents. This is consistent with the F₁ cross being a heterozygous individual. In other weed species, such as *C. Canadensis* (Zelaya et al. 2004), *A. palmeri* (Gaines et al. 2011) and *L. rigidum* (Lorraine-Colwill et al. 2001; Preston et al. 2006), the response to glyphosate of F₁, F₂ or backcross populations was intermediate between that of their parental resistant and susceptible populations. A combination of survivors from the gene flow experiment and the hand-cross were used to examine the inheritance of glyphosate resistance in *E. colona*.

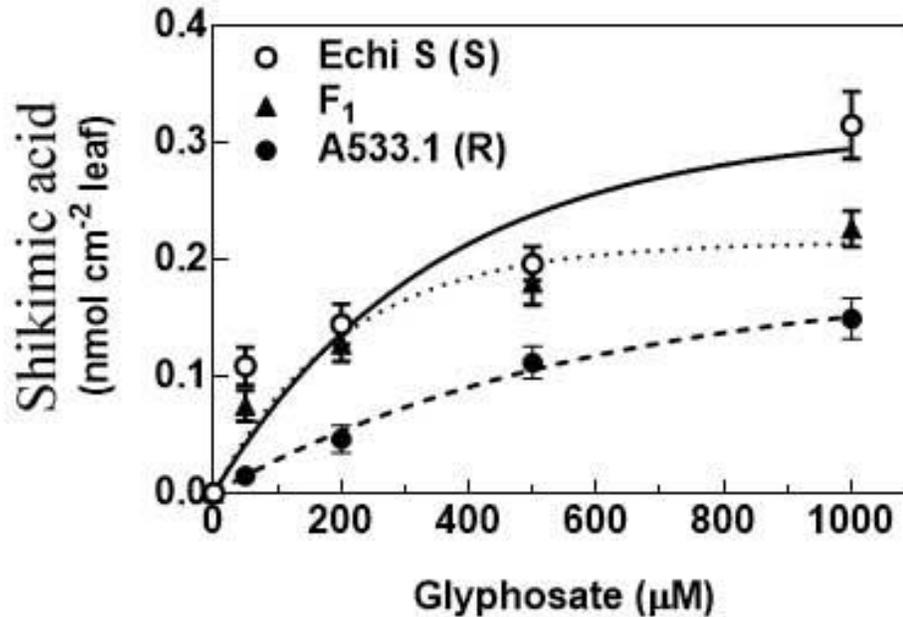


Figure 1. Shikimate accumulation in leaf discs of S (Echi S ○) and R (A533.1 ●) plants of *E. colona* and the F₁ cross (▲) in response to glyphosate. Each data point represents the mean amount of shikimate from five experiment times with 5 replicates per each glyphosate rate for each experiment ± SE.

Segregation for glyphosate resistance in progeny of gene flow survivors

The F₂ progeny from the 28 selfed survivors of the gene flow experiment were tested for segregation for glyphosate resistance using 240 g a.e. ha⁻¹ glyphosate. All R plants survived at this rate (Table 3); whereas, only 15% of the S plants survived. No difference in response to glyphosate between the F₂ families was observed ($p = 0.992$), so data across families were pooled. When corrected for the expected survival of plants segregating for susceptibility, the F₂ progeny segregated at a 3:1 survival : mortality ratio (Table 3) indicating resistance was due to a single major gene that was dominant at this rate of glyphosate.

Table 3. Segregation of the F₂ progenies from selfed F₁ survivors of *E. colona* from the gene flow experiment after glyphosate treatment at of 240 g a.e. ha⁻¹

Plant no.	Number of F ₂			G statistic (3:1)	P
	progeny tested	Alive	Dead		
Echi S (S)	26	4	22		
A533.1 (R)	21	21	0		
1	25	22	3	1.402	0.236
2	18	15	3	0.223	0.636
3	26	20	6	0.056	0.814
4	21	15	6	0.629	0.428
5	24	19	5	0.001	0.970
6	23	18	5	0.005	0.946
7	24	19	5	0.001	0.970
8	27	22	5	0.114	0.736
9	24	19	5	0.001	0.970
10	27	23	4	0.694	0.405
11	26	21	5	0.058	0.810
12	25	19	6	0.116	0.734
13	24	18	6	0.201	0.654
14	13	9	4	0.633	0.426
15	27	21	6	0.018	0.893
16	25	20	5	0.020	0.888
17	27	21	6	0.018	0.893
18	27	21	6	0.018	0.893
19	26	19	7	0.480	0.488
20	27	19	8	1.051	0.305
21	25	22	3	1.402	0.236
22	26	19	7	0.480	0.488
23	26	23	3	1.624	0.203
24	24	18	6	0.201	0.654
25	26	19	7	0.480	0.488
26	27	19	8	1.051	0.305
27	27	20	7	0.345	0.557
28	27	24	3	1.856	0.173
Total	694	544	150	0.089	0.765
Homogeneity				13.18	0.992

The inheritance of glyphosate resistance was demonstrated as due to a single, nuclear, partially dominant and monogenic trait in *L. rigidum* from Australia (Lorraine-Colwill et al. 2001; Wakelin and Preston, 2006), *E. indica* from Malaysia (Ng et al. 2004a), and in *C. canadensis* and *C. bonariensis* from the USA (Zelaya et al. 2004; Okada and Jasieniuk, 2014). However, in glyphosate-resistant *L. rigidum* from the USA (Simarmata et al. 2005), other populations of *C. bonariensis* from the USA (Okada and Jasieniuk, 2014), *A. palmeri* from the USA (Chandi et al. 2012; Mohseni-Moghadam et al. 2013) and *B. diandrus* from Australia (Malone et al. 2016), glyphosate resistance was not monogenic. This variation in inheritance patterns is partly related to resistance mechanism present; with gene amplification having complex inheritance (Chandi et al. 2012; Mohseni-Moghadam et al. 2013; Malone et al. 2016). The glyphosate-resistant *E. colona* studied here contained a target site mutation within *EPSPS* and was inherited as a single, mainly dominant allele (Table 3). Dominance of resistance alleles can be dose dependent (Preston and Malone, 2015) having less dominance at higher rates. Therefore, using a single rate of herbicide may be insufficient for inheritance studies. In the case of *E. colona*, a dose response on the F₂ population from the hand cross showed that while glyphosate resistance was dominated by a single, mainly dominant gene, additional gene(s) were likely to contribute to survival at low rates of glyphosate (Table 3). The importance of these gene(s) to survival of *E. colona* populations treated with glyphosate in the field is unclear, as they only seemed to provide additional resistance at low rates of glyphosate application. As reported by Busi and Powles (2009) on glyphosate resistant *L. rigidum*, cross-pollination speeded up the accumulation of additional glyphosate resistance genes and these genes contributed to the survival of filial plants at low glyphosate rates.

Dose response to glyphosate of F₂ progeny

To confirm the segregation pattern in the F₂ generation, a dose response experiment was conducted on the resistant and susceptible parents and the F₂ progeny. The S population was easily controlled by glyphosate with more than 85% of the population controlled at 240 g a.e. ha⁻¹ glyphosate. On the contrary, no mortality of the R population occurred at this rate of herbicide. The response of the F₂ population was intermediate to that of the two parents (Figure 2). The response of the F₂ population to glyphosate was compared to the expected response for a single dominant gene. The expected response was calculated by summing 0.25 x the response of the susceptible population and 0.75 x the response of the resistant population. At intermediate rates of glyphosate, the response of the F₂ population fitted the predicted response. However, at lower and higher rates there was a deviation from the predicted response. At the highest glyphosate rate there was more mortality than expected. As the model assumed full dominance of the resistance allele, this result

indicates the resistance allele is not completely dominant. However, at the lower rates, there was less mortality than expected. This suggests the involvement of a major allele contributing to glyphosate resistance. However, it is likely that additional genes contribute to resistance at low glyphosate rates.

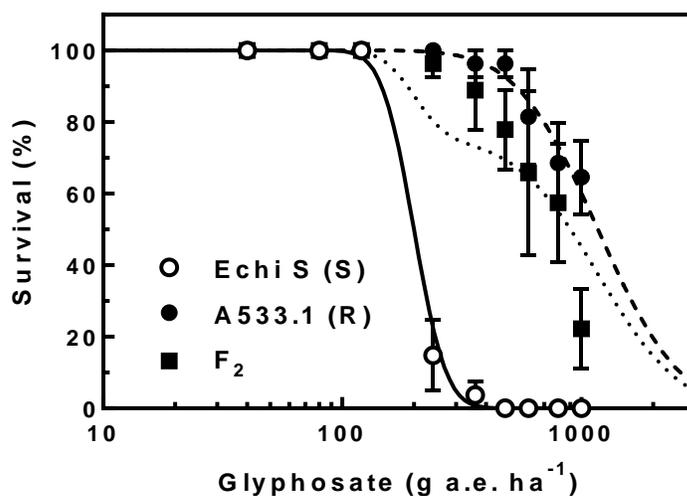


Figure 2. Glyphosate dose response of S (Echi S ○) and R (A533.1 ●) populations of *E. colona* and the F₂ population (■). Each data point represents the mean percent survival from 3 replicates for each glyphosate rate ± SE. The solid and dashed lines are the probit curves for the susceptible and resistant populations respectively. The dotted line is the predicted response for the F₂ population segregating for a single dominant allele.

Detecting an EPSPS gene mutations in F₁ and F₂ progeny

As conflicting results were found in the segregation test and dose response experiment, *EPSPS* gene sequencing was carried out to further investigate inheritance. When sequencing was conducted from genomic DNA, all individuals from the resistant parent line sequenced were found to contain a mutation in *EPSPS* at codon 106, with a replacement of T for C in the first nucleotide of the codon leading to a predicted substitution of serine for proline. All susceptible parent individuals tested did not contain a mutation. However, when the 28 F₁ survivors from the gene flow experiment were tested it was found that not all of the survivors contained a mutation, with no mutation detected in *EPSPS* in five resistant F₁ individuals (plants 24-28 in Table 3). The same mutation was detected in the F₁ seedling generated from the hand cross. The mutation was the same as in the paternal R biotype: a single nucleotide substitution of T for C resulted in substitution of serine for proline at position 106 of the *EPSPS* gene. Similarly, this mutation was also detected in all 30 F₂ individuals from the hand-cross sequenced.

EPSPS cDNA sequencing and cloning

While the segregation results show that all progeny from the 28 F₁ families from the gene flow experiment segregated in a 3:1 survival : mortality ratio, in five families (from no. 24 to no. 28) a mutation in the *EPSPS* gene was not detected by PCR on genomic DNA. Therefore, sequencing of *EPSPS* cDNA from the resistant, susceptible and F₂ individuals was undertaken to clarify the inheritance of the target site mutation.

EPSPS cDNA sequence was obtained from 19 clones of the R parent, 17 from S parent, 7 from an F₂ individual of Family 10 (where a mutation was detected by genomic sequencing) and 4 from an F₂ individual from Family 28 (where a mutation was not detected by genomic sequencing). As expected, none of 17 sequences obtained from the S plant contained a mutation. However, for all other samples, some but not all cDNA clones contained a mutation. The Pro 106 to Ser mutation was identified in 13 of the 19 clones from the R plant, 2 out of 7 clones from the F₂ individual from Family 10 and 1 of 4 clones from the F₂ individual from Family 28 (Table 4). This showed that the target site mutation identified was segregating with glyphosate resistance, but also that more than one *EPSPS* gene was being expressed in *E. colona*.

Table 4. Mutation at position 106 detected in cDNA from *EPSPS* gene after cloning of resistant and susceptible plants, and F₂ progenies of *E. colona*

Plant		Number of sequences	Sequences with mutation
A533.1	(R)	19	13
Echi S	(S)	17	0
F ₂ from Family 10	(R)	7	2
F ₂ from Family 28	(R)	4	1

E. colona is a tetraploid or hexaploid grass species (Alarcón-Reverte et al. 2015; Haroun and Alotaibi, 2015; Han et al. 2016) with 54 chromosomes ($2n = 6x = 54$) (Yabuno, 1983), suggesting that 9 is the base chromosome number of this grass species. Therefore, if a mutation occurs on only one *EPSPS* allele it is possible that this mutation may not be detected via genomic DNA sequencing, due to the mutation carrying allele being diluted or masked by the non-mutant *EPSPS* alleles. This may explain the discrepancy seen when comparing of the results from the genomic sequencing versus the cDNA cloning of *EPSPS* in *E. colona*. All cDNA sequences were checked for SNPs in

As mentioned above, *E. colona* is a polyploid species. This means that it is likely to have more than one *EPSPS* gene, making sequencing from genomic DNA potentially confusing. Tobacco (*Nicotiana tabacum* L.), an allotetraploid ($2n=4x=48$) contains at least two *EPSPS* genes (Goldsbrough et al. 1990). In *E. colona*, no mutation within *EPSPS* was detected in 5 of the survivors of glyphosate application from the gene flow experiment using genomic DNA, despite these plants and their progeny being resistant to glyphosate (Table 3). Sequencing from cDNA clones identified a mutation within *EPSPS* in the R population as well as two F₂ families, including one where the mutation could not be observed when sequencing genomic DNA (Table 4). In the allohexaploid species *A. fatua* ($2n=6x=42$) there are three homoeologous ACCase genes and each gene can contain a mutation offering herbicide resistance although the resistance was not strong when only one gene contains a mutation (Yu et al., 2013). However, in another allohexaploid species of *Echinochloa crus-galli* var. *formosensis* ($2n=4x=36$) (Yabuno, 1983), six ACCase genes and three ALS genes were identified, but target-site resistance was not found and the resistance mechanism was suspected as non-target-site based (Iwakami et al. 2015). Identification of target site mutations in polyploid species through sequencing gDNA could miss identifying target site mutations some of the time. According to Iwakami et al. (2012), the existence of target-site resistance to ACCase inhibitors in polyploid species *Echinochloa phyllopogon* could be affected by the presence of three active ACCase genes and variation of their relative expression levels. There may also be variation in the level of resistance provided by a target site mutation depending on the relative expression of the genes carrying the mutation and other genes. This may also complicate comparisons of resistance levels in whole plants with mutations present in the genome. For example, Han et al. (2016) reported *E. colona* populations with target site mutations having low levels of resistance to glyphosate, and concluded this mechanism was insufficient for field level resistance in this species.

Comparison of SNPs in the sequences from the cDNA clones showed that at least two *EPSPS* genes were expressed in *E. colona*. One of these genes did not contain a target site mutation in the resistant population (Figure 3). In the resistant population, cDNA clones with the target site mutation were 68% of the total, suggesting this allele is preferentially expressed in *E. colona*. As expected, the frequency of cDNA clones with target site mutations was less in the segregating F₂ populations and these clones belonged to the same *EPSPS* gene as present in the resistant parent (Table 4). Additional research is required to understand the contributions of the target site mutations to resistance in *E. colona*.

Conclusion

While *E. colona* is generally considered self-pollinated, this study demonstrated a natural outcrossing frequency of 1.38%, as measured by the transfer of glyphosate resistance from resistant plants to susceptible plants. This amount of outcrossing through pollen could spread resistance locally within populations, but is unlikely to contribute to spread of resistance between populations. The identification of a mutation in the *EPSPS* gene of the F₁ progenies proved the glyphosate resistance trait came from the resistant parent via pollen. Segregation of the F₂ progenies from both the gene flow survivors and from a hand cross occurred at a 3:1 resistance:susceptibility ratio demonstrating the F₁ individuals were heterozygotes and the glyphosate resistance inheritance in *E. colona* was likely due to a single and dominant gene. However, other genes may be contributing to resistance at low glyphosate rates. Analysing the cDNA sequence indicated the presence of at least two *EPSPS* genes were expressed in this grass species, only one of which carried a mutation in the resistant individuals.

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Conflict of Interest

Authors declare no conflict of interest.

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