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Non-target-site glyphosate resistance in *Echinochloa colona* from Western Australia

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ABSTRACT

An *Echinochloa colona* population from Western Australia has evolved resistance to glyphosate. This current study investigates the physiological, molecular and biochemical basis of glyphosate resistance in this population. To minimise genetic differences the susceptible (S) and resistance (R) phenotypes were isolated from within this resistant population. The S phenotype was found to accumulate significantly more shikimate in the leaf tissue than the R phenotype following glyphosate treatment. Target-site EPSPS gene sequencing revealed no resistance mutations and EPSPS gene expression was similar between the S and R phenotypes. Thus, glyphosate resistance in this population is unlikely target-site based. Similarly, there were no significant differences between the S and R phenotypes in glyphosate leaf uptake and translocation at the whole plant level. It is also unlikely that the resistance is associated with glyphosate metabolism as no major glyphosate metabolites were detected in leaf tissue of the S and R phenotypes. Despite much effort the exact glyphosate resistance mechanisms in this R population remain unclear, and novel resistance mechanisms are to be determined.

1. Introduction

Glyphosate, a non-selective herbicide active on many plant species, has been globally used for over four decades. Glyphosate competes with phosphoenolpyruvate (PEP) for the binding site in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate pathway (Steinrücken and Amrhein, 1980), disrupting production of the aromatic amino acids phenylalanine, tyrosine and tryptophan and secondary metabolic products (e.g. auxins, anthocyanins, lignin and phytoalexins) (Kishore and Shah, 1988; Herrmann and Weaver, 1999). This glyphosate inhibitory effect also causes accumulation of the shikimate (Holländer and Amrhein, 1980; Cole, 1985) and other acids derived from shikimate (Duke SO and Powles, 2008; Orcaray et al., 2012).

Since the first documented case of a weed evolving glyphosate resistance (Powles et al., 1998; Pratley et al., 1999), the number of glyphosate-resistant weed species has increased significantly, especially in cropping systems where glyphosate tolerant crops such as canola, corn, cotton, soybean and sugar beets have been adopted (Dill et al., 2008; Duke and Powles, 2009). To date, evolved resistance to glyphosate has been documented in at least 41 weed species worldwide (Heap, 2018).

Both target- and non-target-site based mechanisms endowing glyphosate resistance have been identified in resistant weed species (reviewed by Powles and Preston, 2006; Shaner, 2009; Powles and Yu, 2010, Sammons and Gaines, 2014). Single *EPSPS* gene mutations resulting in an amino acid substitution of Pro-106 by Ser, Thr, Ala or Leu that endow low to moderate level glyphosate resistance have been reported in various weed species (Baerson et al., 2002a,b; Ng et al., 2003; Yu et al., 2007; Kaundun et al., 2011). A double *EPSPS* gene mutation (Thr-102-Ile + Pro-106-Ser, also known as the TIPS mutation) has been identified in *Eleusine indica* that confers high level glyphosate resistance (Yu et al., 2015; Chen et al., 2015). In addition, *EPSPS* gene amplification has been increasingly documented as a target-site based resistance mechanism since first reported in *Amaranthus palmeri* (Gaines et al., 2010). Overexpression of *EPSPS* was also indicated as a possible resistance mechanism (Baerson et al., 2002; Dinelli et al., 2006). Non-target-site based glyphosate resistance mechanisms such as reduced leaf glyphosate uptake and/or glyphosate translocation, enhanced vacuolar sequestration have also been reported in various weed species (e.g. Lorraine-Colwill et al., 2003; Ge et al., 2012; and reviewed by Sammons and Gaines, 2014). Degradation of glyphosate to non-toxic compounds may also contribute to glyphosate resistance in a limited number of

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weed species such as *Cirsium arvense* (L.) Scop, *Convolvulus arvensis* L., *Equisetum arvense* L. and *Ipomoea purpurea* (L.) Roth (Duke, 2011).

Echinochloa colona (L.) Link, commonly known as awnless barnyardgrass or junglerice, is a widely distributed C4 annual summer weed species (Holm et al. 1991). Resistance to glyphosate and other herbicides of different modes of action (i.e. synthetic auxin, photosystem II, acetyl-coenzyme A carboxylase (ACCase), acetolactate synthase (ALS) and cellulose inhibitors) has been documented in this weed species (Heap, 2018). Thus far glyphosate-resistant *E. colona* populations have been identified in three continents, viz. Australia, North and South America (Heap, 2018). Mechanistic studies showed that glyphosate resistance in some of these *E. colona* populations was due to known *EPSPS* gene mutations resulting in amino acid substitutions of Pro-106-Thr, Pro-106-Ser and/or Pro-106-Leu (Alarcón-Reverte et la. 2013; 2014; Han et al., 2016; Nguyen et al., 2016). In Australia, glyphosate resistance in *E. colona* was first documented in 2007 in northern New South Wales. Since then, the number of resistant populations of this weed species has increased drastically, mainly in the Northern grain region of Australia (Preston, 2018). In Western Australia, the first confirmed glyphosate-resistant *E. colona* was found in the Tropical Ord River Region in north-west Australia (Gaines et al., 2012). The selected resistant phenotype of this population has a resistance index, based on survival and growth, of 8-fold relative to the susceptible phenotype (Goh et al., 2016). This study focuses on the identification of the glyphosate resistance mechanism in this *E. colona* population for better understanding of resistance and management. Target-site *EPSPS* gene mutation and *EPSPS* gene overexpression, and non-target-site glyphosate leaf uptake, translocation and metabolism were investigated.

2. Materials and methods

2.1. Plant material

Identification of glyphosate susceptible (S) and resistant (R) phenotypes from within an *E. colona* population collected from the Tropical Ord River region (15°30′S, 128°21′E) of Western Australia (WA) was conducted. The selection of S and R phenotypes with similar genetic background was conducted in a previous study by Goh et al. (2016).

2.2. Shikimic acid measurement

Differences in shikimate accumulation in S and R phenotypes after glyphosate treatment is an indicator of glyphosate-resistance (Singh and Shaner, 1998). At the 2-leaf stage, seedlings of the S and R phenotypes (n = 100) were transplanted into plastic trays (34 × 28 cm) containing potting mixture and grown in glasshouse conditions (at constant temperature 30 °C). At the 4- to 5-leaf stage, plants were treated with glyphosate at 540 g ha^{-1}. Treated plants were moved outdoors during the normal warm summer growing conditions for *E. colona*. Fresh leaf tissue (about 1 g) was harvested snap-frozen at 0, 1, 2, 3 and 5 days after treatment (DAT) and stored at −20 °C prior to analysis.

Shikimic acid extraction and measurement were performed according to Yu et al. (2009) with some modifications. A standard curve was constructed with shikimic acid standard (Sigma-Aldrich, USA) at a range of 0–420 μg mL^{-1} (0–2.4 mM). Shikimate concentration in leaf tissues was quantified against the standard curve. Shikimic acid concentration was expressed as mg per gram of leaf fresh weight.

This experiment was arranged in a completely randomised design using replicate plants (n = 3) of each S and R phenotype. The experiment was repeated at least twice with similar results (ANOVA, $P > 0.05$), so all data were pooled. Accumulation of tissue shikimate was fitted to a non-linear regression exponential model:

$$y = a(1 - b^t)$$

where y is accumulated shikimic acid at different days (t) after glyphosate application, a is the upper limit of shikimic acid concentration and b is rate of increase in shikimic acid.

Estimates of shikimic acid accumulation of the S and R phenotypes was subjected to one-way ANOVA with Tukey's test (SAS 9.3, SAS Institute Inc., Cary, NC, USA). Non-linear regression models were subjected to Fisher's test using GraphPad Prism version 5.00 (GraphPad Software, San Diego, California, USA, www.graphpad.com) to determine whether the two fitted curves were statistically different.

2.3. Evaluation of target-site resistance mechanisms

2.3.1. EPSPS gene sequencing of the highly conserved region

The 2- to 3-leaf stage seedlings of the S and R phenotypes were transplanted into 20-cell plastic trays containing potting mixture and grown in a heated glasshouse (at constant 30 °C). At the 3- to 4-leaf stage, they were treated with 540 g glyphosate ha^{-1}. Leaves of surviving plants (12 R individuals) were snap-frozen for DNA extraction. Bulked leaf material from the S phenotype (25 individuals) without glyphosate treatment was used as a control. Genomic DNA was extracted from leaf tissues of both phenotypes (Yu et al., 2008). The forward primer 5′-GCGGTAGTTGTTGGCTGTGGTG-3′ and the reverse primer 5′-TCAATCCGACAACC AAGTCGC-3′ from Ng et al. (2003) were used to amplify a 292 bp (including 90 bp intron) fragment, which covers the highly conserved region where potential point mutations conferring glyphosate resistance have been identified (Padgette et al., 1996; Sammons and Gaines, 2014).

A polymerase chain reaction (PCR) was conducted in a 25 μL volume that consisted of 2 μL of genomic DNA, 0.5 μM of each primer and 12.5 μL of 2 × GoTaq Green Master Mix (Promega Corp., USA). The PCR was run in a thermal cycler (Mastercycler, Eppendorf, Germany) with the following profile: 94 °C for 4 min, 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, followed by a final extension step of 5 min at 72 °C. The PCR product was purified from agarose gel and sequenced from both directions using a commercial sequencing service. All the sequence results were visually examined using the chromatogram files, aligned and compared between the S and R phenotypes.

2.3.2. EPSPS gene expression

Seedlings of the S and R phenotypes at the 2- to 3-leaf stage were transplanted into plastic trays (33.5 × 28 × 6 cm) and grown in a heated glasshouse (30 °C). When plants reached the 4- to 5-leaf stage, approximately 100 seedlings were glyphosate treated at 540 g ha^{-1}. Bulked shoot material from approximately 15 untreated or treated S and R plants per replicate (n = 4) was collected 24 h after glyphosate treatment. Samples were snap-frozen in liquid nitrogen and kept at −80 °C until use.

RNA was extracted using the protocol of the Isolate II RNA Plant Kit (Bioline Australia Pty Ltd.). The concentration of the extracted RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Australia). The RNA sample was diluted and normalised to about 10 μg μL^{-1} and possible contaminating DNA was removed using a Turbo DNA-free kit (Life Technologies Australia Pty Ltd., Australia). About 2 μg total RNA was used for cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen). A 132 bp fragment of eukaryotic translation initiation factor 4B gene (*EIF4B*), that was amplified from a pair of primers (the forward primer 5′-CCAGTCCCTTTTTGTTTTGGA-3′ and the reverse primer 5′-CTACAGCATACAGAGGTGATCAAT-3′) was

tested and used as an internal control gene (Iwakami et al., 2014). The forward primer '5-GCAAGTTCCCGGTTGAGAAGG-3' and the reverse primer 5′TCCACCAGCAGCAGTCACGGC-3′ were designed (based on a single 292 bp fragment sequence) to amplify a 106 bp cDNA fragment of the *EPSPS* gene. Primer efficiency and slope were 85% and −3.755 (R^2 = 0.99) for *EIF4B* and 104% and −3.233 (R^2 = 0.96) for *EPSPS*, respectively. No amplification products were observed in control samples. Quantitative real time PCR was performed in a 20 μL reaction volume consisting of 5 μL cDNA (50 ng) as template, 5 μL 0.5 μM of each primer and 10 μL 2 × SensiFAST SYBR Lo-ROX Mix (Bioline, Australia). Negative controls consisting of primers with no template were included. The PCR was run on a 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with the following profile: the initial polymerase activation at 50 °C for 20 s and 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The experiment involved two glyphosate treatments (untreated and treated) and two phenotypes (S and R) with four biological replicates of 15 plants each, and the experiment was repeated with similar results. The expression of the target *EPSPS* gene relative to the internal control gene (*EIF4B*) in the glyphosate untreated and treated S and R phenotypes was determined. Relative gene expression was assessed using the following equation:

$$\text{Fold change} = 2^{-\Delta C_T} \text{or} 2^{-\Delta\Delta C_T}$$

where ΔC_T is the difference in threshold cycles for sample and reference where ΔC_T = [Avg. $C_{T(Sample)}$ -Avg. $C_{T(EIFB4)}$] and $\Delta\Delta C_T$ = [$\Delta C_{T(Treated)}$-$\Delta C_{T(Untreated)}$].

2.3.3. Full EPSPS cDNA cloning and sequencing

To examine other possible resistance-endowing mutations outside the highly conserved region of the *EPSPS* gene, two primer pairs were designed to obtain the *E. colona* EPSPS full coding sequence based on our unpublished *E. colona* RNA-sequencing data, which include two forward primers (EC-EPSPS1-F: 5′-ATGCGCGGGCGCCGGACGTCG-3′ and EC-EPSPS3-F: 5′-ATGCGGCGCGGGGCGTCGGTG-3′) and a shared reverse primer (EC-EPSPSR: 5′- TTAGTTCTTGACGAATGTGCTCAGCA CATCGAAGTAGT -3′). The amplified cDNA fragment was cloned into the pGEM-T vector (Promegam, Madison, WI) and transformed into *E. coli* competent cell (strain JM109). White colonies were used as templates for PCR re-amplification to confirm the putative inserts. Plasmids were extracted from white colonies that contained the right inserts and sequenced. Six clones from each three S and three R samples were analysed and sequences compared.

2.4. Evaluation of non-target-site resistance mechanisms

2.4.1. Leaf uptake and translocation of glyphosate

At the 3-leaf stage, the midpoint of the adaxial surface of the second fully expanded leaf was spotted with a single droplet (1 μL) of ^{14}C-labelled glyphosate solution (with a specific ^{14}C radioactivity of 55.18 mCi mmol^{-1}, PerkinElmer, Inc., Boston, USA) using a micropipette. The ^{14}C-glyphosate treatment solution (5.04 mM) was made up of 0.44 mM ^{14}C-glyphosate in a diluted commercial glyphosate formulation plus 0.25% (v/v) non-ionic surfactant BS1000 (1000 g L^{-1} of alcohol alkoxylate). This treatment solution with 0.89 kBq μL^{-1} of ^{14}C-glyphosate is equivalent to a discriminating glyphosate dose of 123 g ha^{-1}, for the S and R phenotypes. At 24, 48 and 72 h after ^{14}C-glyphosate treatment (hours after treatment, HAT), S and R seedlings were harvested. Plants were removed from soil and aboveground (leaf and stem) and root material collected. To remove unabsorbed ^{14}C-glyphosate, the ^{14}C-glyphosate treated leaf surface of each plant was rinsed in 20 mL washing buffer containing 20% (v/v) methanol and 0.2% (v/v) Triton X-100. The aliquots of the leaf wash (5 mL) were mixed with 5 mL of scintillation solution (IRGA-Safe Plus, PerkinElmer, Inc., MA, USA) and the radioactivity was quantified using a liquid scintillation counter (LSC) (Packard, Tri-Carb 1500, USA). Roots of individual plants were rinsed in 50 mL washing buffer, and radioactivity in the recovered buffer quantified.

The harvested plant samples were blotted dry between two sheets of paper towels, and oven dried for 2 days at 60 °C. Translocation of ^{14}C-glyphosate was visualized by phosphor-imaging (Personal Molecular Imager System, Bio-Rad Laboratories, Inc., California, USA). After imaging, the plant samples were separated into three sections, that is treated leaf, roots, and untreated shoot and leaves, and combusted in a biological oxidiser (RJ Harvey Instrument Corporation, Hillsdale, NJ). The average combustion efficiency was 111.5 ± 0.5% (within the acceptable combustion efficiency that ranged from 95 to 130%). ^{14}C-glyphosate in plant sections was oxidized to $^{14}CO_2$, which was trapped in a 15 mL scintillation cocktail containing a 1:1 mixture of carbon dioxide absorber (Carbo-Sorb E, PerkinElmer, Inc., MA, USA) and Permafluor E$^+$, PerkinElmer Life and Analytical Sciences, CT, USA) and quantified by LSC. The average recovery of applied ^{14}C-glyphosate from leaf plus root wash and combustion was 95 ± 1%. The total radioactivity recovered from all plant parts (excluding leaf and root wash) was considered to be the foliar uptake of glyphosate. Radioactivity present in all plant sections except the treated leaf was considered as translocation and expressed as percentage of total ^{14}C absorbed.

Treatments were arranged in a completely randomized design using replicated plants (n = 5) per harvest and per phenotype at each time point, and the experiment was repeated using slightly different ^{14}C-glyphosate radioactivity with similar results. Percentage data from absorption and translocation experiments were arcsine transformed to meet analysis of variance (ANOVA) assumptions (i.e. data are homoscedastic and normal distributed). Means were separated using Tukey's test (two-way ANOVA with phenotype, HAT and its interaction as sources of variation for absorption; meanwhile three-way ANOVA with phenotype, HAT, plant sections and its interactions as sources of variation for translocation) at the 5% level of probability using the ANOVA procedure of the SAS package. As analyses of transformed data gave results identical to those obtained with untransformed data, the analyses of the original, untransformed data are reported.

2.4.2. Glyphosate metabolism

At the 3-leaf stage, S and R plants were treated with ^{14}C-glyphosate following the procedures described above. Shoot material of six treated plants per phenotype (approximately 0.5 g fresh weight) were harvested at 48 and 72 HAT. In addition, three untreated plants per phenotype were also collected and bulked. The treated leaf surface of each seedling was rinsed in 20 mL washing buffer containing 20% (v/v) methanol and 0.2% (v/v) Triton X-100 to remove unabsorbed ^{14}C-glyphosate, and the radioactivity in the leaf wash was measured as described previously. Plant samples were then blotted dry, snap-frozen in liquid nitrogen and kept at −80 °C until use. Frozen material was ground and homogenized in 1 mL of 10% (v/v) cold methanol with a pre-chilled mortar and pestle for 5 min. The homogenate was transferred to a 10 mL centrifuge tube and centrifuged at 8000 g for 15 min at 4 °C. The supernatant was decanted and the pellet was washed and re-extracted twice with 0.5 mL 10% (v/v) cold methanol. The three portions of supernatant were pooled (about 3 mL), re-centrifuged at 9000 g for 15 min and then transferred to a new 5 mL tube. The untreated plants were spiked with ^{14}C-glyphosate and extracted following the same extraction procedure. The supernatant was evaporated to dryness under vacuum with a SpeedVac Concentrator (SVC-100H, Savant, Farmingdale, NY). The dried residue was resuspended in 250 μl 10% (v/v) cold methanol and was concentrated to 50 μl. The concentrated liquid was centrifuged at 14,000 g for 5 min and quantified by LSC to determine the total radioactivity for each sample. The mean recovered radioactivity was 78.8 ± 0.3% of the total applied dose.

TLC was performed using 20 × 20 cm aluminium-backed silica gel plates with a particle size of 25 μm (Sigma-Aldrich, Australia). A 1 μl droplet of concentrated plant extract (about 0.3 nCi of C14-glyphosate) and diluted ^{14}C-glyphosate standard was spotted onto the TLC silica gel plate. The TLC plate was developed in a solvent system containing

isopropanol: 5% (v/v) ammonia in 1:1 ratio (Selvi et al., 2013). After development for a run length of 15 cm (which took about 6.5 h), the plate was removed and air-dried overnight. Then, the TLC plate was exposed to an imaging plate for 48 h and the results from TLC analysis were visualised with the phosphor imager and the retention factor value estimated (*Rf*, the ratio of the distance moved by the ^{14}C-labelled compounds compared with that of the solvent). Parent glyphosate in the samples was identified by comparing their *Rf* value with the ^{14}C-glyphosate standard. The ^{14}C-glyphosate standard was detected with a *Rf* value of 0.36. The experiment was repeated at least three times with similar results.

3. Results

3.1. Differential shikimate accumulation in the S and R phenotypes

For the treated S phenotype, leaf shikimic acid level increased dramatically (33-fold) over time with a steeper slope (b = 0.9) (Fig. 1). In contrast, increase in shikimic acid in the R phenotype was observed only at 2 DAT and then remained unchanged over the next three days (Fig. 1). Based on the fitted curves, the R phenotype accumulated three times less shikimic acid than the S phenotype at 5 DAT, thus confirming glyphosate resistance in the R phenotype.

3.2. Absence of target-site EPSPS gene mutations and overexpression

Initially, a single 292 bp DNA fragment of the highly conserved region of EPSPS was amplified from 12 individual R plants, aligned and compared with the bulked S samples. No known glyphosate resistance endowing mutations were found in the highly conserved region flanking amino acid position from 95 to 109, according to *Arabidopsis thaliana EPSPS* cDNA numbering. In addition, three full EPSPS coding sequences (1398bp, 1398bp, 1401bp, respectively) were obtained from the S and R plants, and there were no single nucleotide polymorphisms between the S and R sequences that resulted in amino acid substitutions.

Relative *EPSPS* gene transcript levels in pooled S and R samples were determined using quantitative RT-PCR. There was no difference in basal or glyphosate induced *EPSPS* transcript levels detected in S or R samples (Table 1). These results show that target-site glyphosate resistance mechanisms such as *EPSPS* gene point mutation and overexpression are not responsible for glyphosate resistance in these R samples analysed.

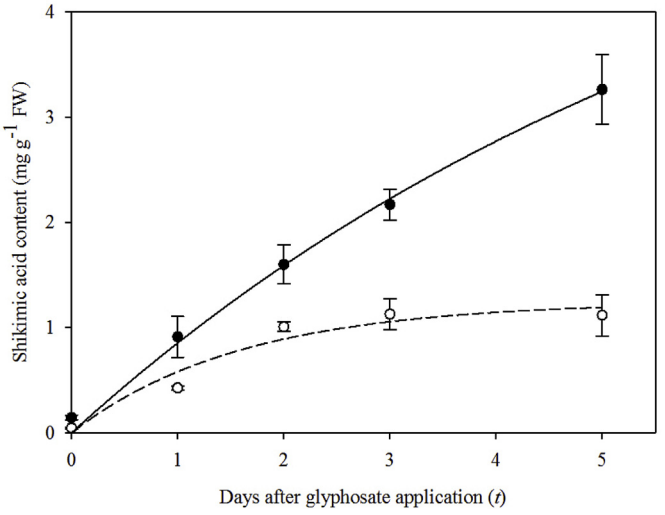

Fig. 1. Shikimate accumulation in the leaves of untreated (day 0) and treated glyphosate-susceptible (S) and -resistant (R) *Echinochloa colona* plants over time. Each data point represents mean values of three replications. Bars designate the standard error of each mean. Data were fitted to the exponential regression model (S: y = 6 [1-0.9^t], R^2 = 0.928; R: y = 1-0.5^t, R^2 = 0.837).

Table 1
Basal level and induction (treatment) of *EPSPS* gene expression in glyphosate-susceptible (S) and -resistant (R) *Echinochloa colona* phenotypes 24 h after glyphosate treatment.[a]

EPSPS gene expression	Phenotype	ΔC_T*	$2^{-\Delta C_T}$ +
Basal level	S	1.7 (0.8) a	0.3
	R	1.5 (0.3) a	0.3
		$\Delta\Delta C_T$*	$2^{-\Delta\Delta C_T}$ +
Induction	S	0.1 (0.8) a	0.9
	R	0.1 (0.7) a	0.9

[a] Values in parentheses are standard errors of the mean (n = 4). Means within a column followed by the same letters are not significantly different according to *t*-test (α = 0.05).

Table 2
Foliar absorption of ^{14}C-glyphosate in glyphosate-susceptible (S) and -resistant (R) *Echinochloa colona* phenotypes as a function of time.

Phenotype	Absorption (SE) (% of total recovered)[a]		
	24 HAT	48 HAT	72 HAT
S	67 (3)	62 (2)	61 (3)
R	62 (4)	59 (1)	60 (3)
P value	0.4	0.2	0.8

[a] Values in parentheses are standard errors of the mean (n = 5). HAT = hours after treatment, SE = standard errors of the mean.

3.3. No difference in foliar uptake and translocation of ^{14}C-glyphosate

About 60–70% of ^{14}C-label was absorbed by adaxial leaf surfaces of both S and R phenotypes and remained constant over the assessed time intervals (Table 2). The highest amounts of the absorbed radiolabelled glyphosate were retained in the treated leaf (Figs. 2 and 3). The amount of ^{14}C-glyphosate translocated out of the treated leaf ranged from 45 to 60% of the total absorbed (Fig. 3). It was observed that considerably more ^{14}C-glyphosate moved downward to the roots than upward to the shoots (Figs. 2 and 3). However, no significant differences in the quantified amounts of ^{14}C-labelled glyphosate in the treated leaf, untreated shoot and leaves and roots were found between the S and R phenotypes, except at 48 HAT when less glyphosate (23%) was retained in treated leaf of R than S plants due to increased movement into the root (Fig. 3). In addition, ^{14}C activity in the root wash only accounted for less than 5% of total applied in both S and R samples (not shown). Thus, no altered glyphosate translocation or possible root extrusion was observed in S vs R phenotypes.

3.4. No major glyphosate metabolism

Glyphosate was ^{14}C labelled at the phosphonomethylene group. A potential degradation pathway of ^{14}C-glyphosate will produce radioactive metabolites such as aminomethylphosphonic acid (AMPA), sarcosine and glycine. However, no major glyphosate metabolites were detected by TLC in the S or R shoot extracts at 48 and 72 HAT (Fig. 4). This result indicates that glyphosate metabolism is unlikely associated with glyphosate resistance in the R phenotype studied.

4. Discussion

This glyphosate-resistant *E. colona* population from the northern region of WA has a moderate level of glyphosate resistance (Goh et al., 2016). We studied possible physiological (glyphosate uptake and translocation) and biochemical (shikimic acid content and glyphosate

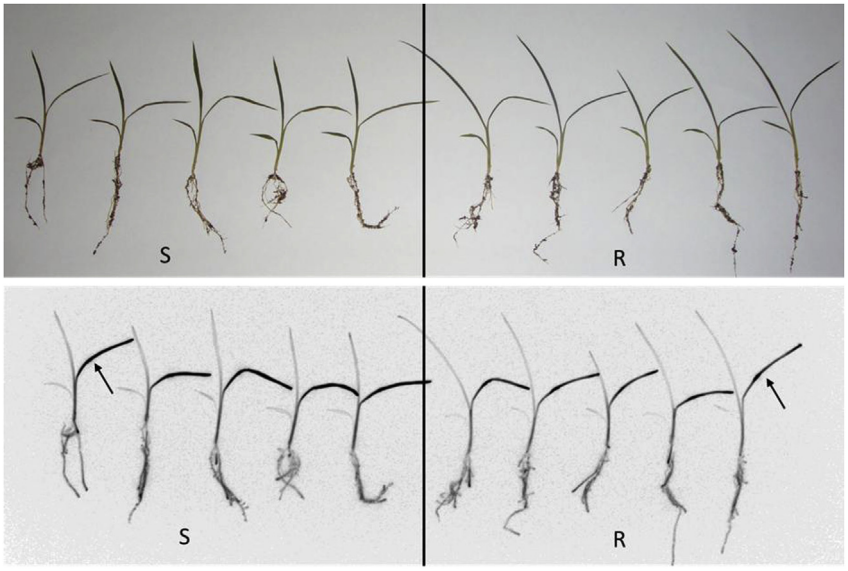

Fig. 2. Camera image (upper panel) and the ^{14}C-glyphosate radioactive image (lower panel) of the glyphosate susceptible (S) and resistant (R) *Echinochloa conlona* seedlings, 48 h after treatment. ^{14}C-glyphosate was applied as a droplet to the midpoint of the second expanded leaf of each 3-leaf stage plant (arrowed).

metabolism or EPSPS target-site mutation) basis of glyphosate resistance in this population. As expected, in comparison to the S phenotype, the R phenotype exhibited a much slower shikimate accumulation over time after glyphosate treatment (Fig. 1). This result further confirms glyphosate resistance at a physiological level in this *E. colona* population. Estimation of progressive shikimate accumulation over time has been commonly used to assess EPSPS inhibition and its effects on plants and as a rapid and reliable diagnostic tool to identify glyphosate S and R phenotypes (Shaner et al., 2005).

Previous studies have shown that amino acid substitutions at Pro-106 in the *EPSPS* gene generally confer low to moderate level (2- to 4-fold) glyphosate resistance in many weed species (Kaundun et al., 2008, and reviewed by Sammons and Gaines, 2014). The EPSPS Pro-106-Ser or Pro-106-Leu substitutions have also been identified in glyphosate-resistant *E. colona* from California and Australia (Alarcón-Reverte et al., 2013; Han et al., 2016; Nguyen et al., 2016). In this glyphosate-resistant *E colona*, three full length EPSPS cDNA transcripts were cloned and sequenced, and no nucleotide changes were detected that resulted in amino acid substitutions in S vs R sequences. Thus, glyphosate resistance in this population is not due to target-site *EPSPS* gene mutation. Likewise, *EPSPS* gene expression between the pooled S and R samples of *E. colona* was similar (Table 1), suggesting that *EPSPS* overexpression does not contribute to glyphosate resistance in this R phenotype. Other target-site resistance mechanisms such as *EPSPS* gene overexpression or gene amplification (duplication) are known to endow glyphosate resistance. For example, higher *EPSPS* mRNA expression was observed in glyphosate-resistant plants of *Conyza canadensis*, *Dicliptera chinensis* and *L. rigidum* (Baerson et al., 2002; Yuan et al., 2002; Dinelli et al., 2006). The *EPSPS* gene amplification with EPSPS relative genomic copy numbers ranging from three to 160 has been shown to endow resistance in *Amaranthus* spp. (Gaines et al., 2010, 2011; Nandula et al., 2014) *Kochia scoparia* (Wiersma et al., 2015) and *Lolium perenne* (Salas et al., 2012), *Eleusine indica* (Chen et al., 2015) and *Bromus diandrus* (Malone et al., 2016). In our study, as no *EPSPS* gene overexpression was found in the pooled R vs S samples, gene amplification as a major glyphosate resistance mechanism in the R phenotype can be ruled out. However, direct measurement of EPSPS enzyme activity can further verify whether EPSPS overexpression and/or amplification is a responsible mechanism involved in the studied R population. For example, enhanced basal EPSPS activity (1.4-fold) without increased gene transcription was detected in one glyphosate-resistance *E. colona* population from California (Alarcon-Reverte et al., 2014).

Glyphosate metabolism is rarely found to be associated with glyphosate resistance in field evolved glyphosate-resistant weedy species except for *C. canadensis* and *Digitaria insularis* (reviewed by Sammons and Gaines, 2014). The absence of detectable metabolites of glyphosate in the studied R phenotype (Fig. 4) indicates that glyphosate metabolism is not a resistance mechanism in this R *E. colona*. Previous studies showed that glyphosate metabolism did not contribute to glyphosate resistance in *C. Canadensis* (Feng et al., 2004) and *L.* rigidum (Feng et al., 1999; Lorraine-Colwill et al., 2003).

Restricted whole-plant glyphosate translocation has been one of the earliest and often reported mechanisms endowing glyphosate resistance in plants, generally conferring a moderate level of resistance (Preston and Lorraine-Colwill et al., 2003; Kaundun et al., 2008; Preston and Wakelin, 2008; Vila-Aiub et al., 2012). In addition to the impaired glyphosate translocation, reduced glyphosate foliar uptake was also observed in *Lolium multiflorum* and *Sorghum halepense* (Michitte et al., 2007; Nandula et al., 2008; Vila-Aiubet al. 2012). However, in the present study, ^{14}C-glyphosate foliar uptake and long distance translocation is similar between the S and R phenotypes (Figs. 2 and 3, Table 2), indicating that impaired glyphosate uptake and translocation are not responsible for glyphosate resistance.

In summary with this particular glyphosate-resistant *E. colona* population the mechanistic basis of resistance is not due to altered glyphosate uptake, translocation, metabolism or mutations or amplifications of the EPSPS gene. Thus the exact glyphosate resistance mechanism remains elusive in this resistant population. Potential mechanisms such as reduced cellular uptake and/or reduced chloroplast membrane permeability for glyphosate, as well as other novel detoxification mechanisms such as aldo-keto reductases (Vemanna et al., 2017) and anti-oxidative machinery (Maroli et al., 2015) remain to be explored. Single S and R lines have been generated and genetic

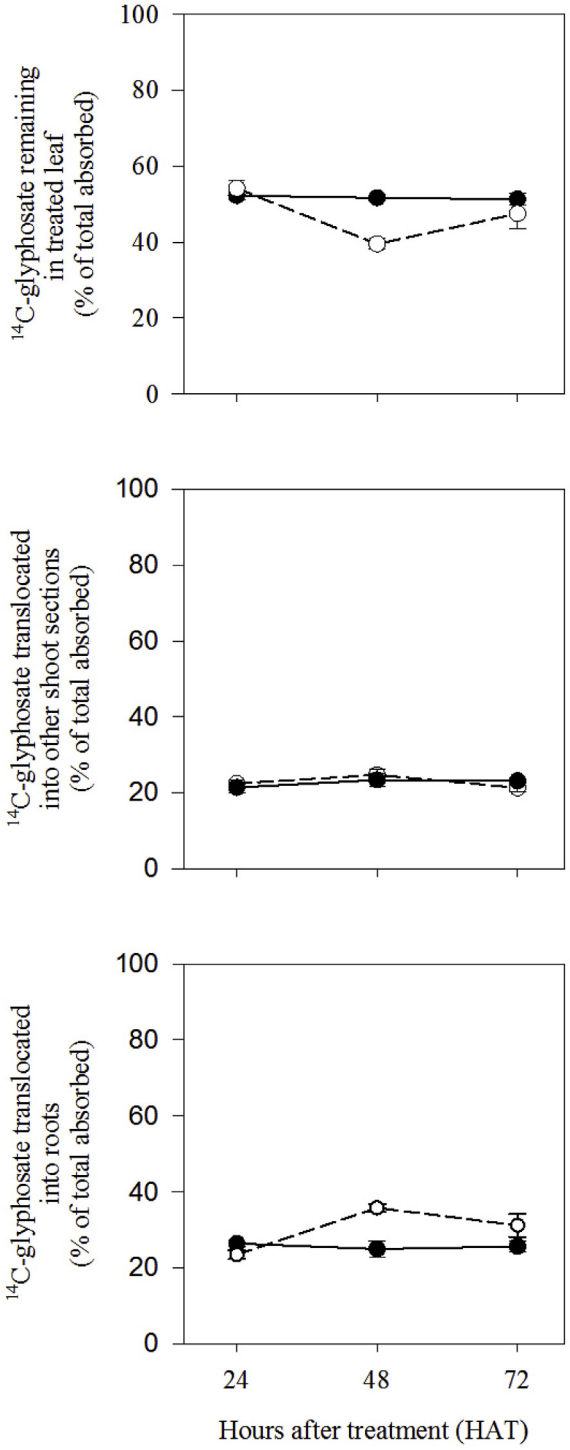

Fig. 3. Translocation and distribution of ^{14}C-glyphosate in glyphosate-susceptible (S) and –resistant (R) *Echinochloa colona* phenotypes at 24, 48 and 72 h HAT. Values are means (n = 5) and bars denote the standard error of the mean.

approaches (RNA sequencing and genome-resequencing) will be pursued in an attempt to identify new mechanisms/candidate genes responsible for glyphosate resistance in this *E. colona* population. Glyphosate resistance in *E. colona* has rendered glyphosate less effective, and current and future management practices should involve both mechanical cultivation and strategic herbicide rotation (e.g. per-emergent herbicides and ACCase, PSII herbicides) to reduce weed seedbank and seedling emergence.

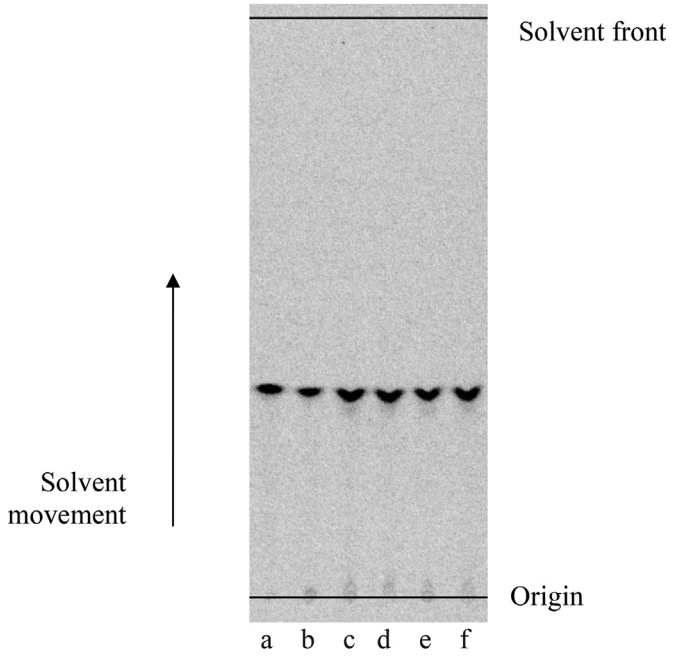

Fig. 4. Phosphor image of thin layer chromatography analysis of shoot extracts from the glyphosate-susceptible (S) and -resistant (R) *Echinochloa colona* phenotypes. a, ^{14}C-glyphosate standard; b, ^{14}C-glyphosate standard spiked in the S and R extracts; c-d, the S and R extracts at 48 h HAT; e-f, the S and R extracts at 72 HAT.

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