Research Article

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Characterisation of glufosinate resistance mechanisms in *Eleusine indica*

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Abstract

BACKGROUND: An *Eleusine indica* population has evolved resistance to glufosinate, a major post-emergence herbicide of global agriculture. This population was analysed for target-site (glutamine synthetase) and non-target-site (glufosinate uptake, translocation and metabolism) resistance mechanisms.

RESULTS: Glutamine synthetase (GS) activity extracted from susceptible (S) and resistant (R*) plants was equally sensitive to glufosinate inhibition, with IC_{50} values of 0.85 mM and 0.99 mM, respectively. The extractable GS activity was also similar in S and R* samples. Foliar uptake of [^{14}C]-glufosinate did not differ in S and R* plants, nor did glufosinate net uptake in leaf discs. Translocation of [^{14}C]-glufosinate into untreated shoots and roots was also similar in both populations, with 44% to 47% of the herbicide translocated out from the treated leaf 24 h after treatment. The HPLC and LC-MS analysis of glufosinate metabolism revealed no major metabolites in S or R* leaf tissue.

CONCLUSIONS: Glufosinate resistance in this resistant population is not due to an insensitive GS, or increased activity, or altered glufosinate uptake and translocation, or enhanced glufosinate metabolism. Thus, target-site resistance is likely excluded and the exact resistance mechanism(s) remain to be determined.

Keywords: glufosinate; glufosinate resistance; resistance mechanisms; *Eleusine indica*

1 INTRODUCTION

Glufosinate is a post-emergence, non-selective herbicide that is globally used for broad spectrum control of grass and broadleaf weed species. Glufosinate inhibits the activity of glutamine synthetase (GS), an enzyme that converts glutamate plus ammonia to glutamine and is thus essential for plant nitrogen metabolism.[1] Irreversible inhibition of GS by glufosinate[2] results in rapid accumulation of toxic ammonia, which inhibits photosynthesis and ultimately causes necrosis of the leaf tissue and plant death.[3,4] Glufosinate has been extensively used in minimum tillage systems, chemical fallows, as a pre-harvest desiccant in cropping systems and for burndown prior to crop emergence.[5,6] The commercialisation of transgenic glufosinate-resistant crops has allowed glufosinate to become widely used as a selective in-crop herbicide. Glufosinate-resistant crops have either the *bar* or *pat* gene, which encode the enzyme phosphinothricin acetyl transferase. This enzyme acetylates glufosinate to the non-toxic compound, *N*-acetyl-glufosinate.[7,8] Currently, glufosinate-resistant crops include canola (*Brassica napus* L.), maize (*Zea mays* L.), soybean (*Glycine max* L. Merr.) and cotton (*Gossypium hirsutum* L.).[6] Widespread adoption of glufosinate resistant crops further increases glufosinate usage and thus a higher risk of the evolution of glufosinate resistance in weeds.

Thus far, evolved glufosinate resistance has only been reported in *Eleusine indica* and *Lolium perenne* L. ssp. *multiflorum*.[9–13] A target-site GS gene mutation has been reported to impart for glufosinate resistance in *L. perenne* ssp. *multiflorum* populations.[12] This work investigates glufosinate resistance mechanisms in an *E. indica* population we have characterised.[9,13]

2 MATERIALS AND METHODS

2.1 Plant material

A susceptible (S) *E. indica* population and a glufosinate-resistant (R*) population from our previous research[9,13] were used in this study. Seeds were germinated on 0.6% (w/v) agar in a growth chamber under 30/25 °C (day/night), 12 h photoperiod with light intensity of 400 μmol m^{-2} s^{-1} and 75% humidity. After 4–7 d, seedlings were transplanted into pots containing potting mix (25% peat moss, 25% washed river sand, 50% mulched pine bark) and grown in the same conditions.

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2.2 Glutamine synthetase assay

The glutamine synthetase inhibition assay was conducted following published methods[14,15] with modifications. When plants reached the three to five leaf stage (7 cm height), leaf material was harvested, snap-frozen in liquid nitrogen, and kept at −80 °C until further use. Leaf samples (4 g) were homogenised at 4 °C with a mortar and pestle in 12 mL 50 mM Tris–HCl (pH 7.5) extraction buffer containing 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.5% polyvinylpyrrolidone (PVP-40) and 10% (v/v) glycerol. The homogenate was then filtered through two layers of Miracloth (Calbiochem, San Diego, CA, USA) and centrifuged at 21 000 × *g* for 30 min at 4 °C. The supernatant was precipitated with ammonium sulfate at 60% saturation at 4 °C and centrifuged again at 21 000 × *g* for 30 min at 4 °C. The pellet was collected and dissolved in 1 mL extraction buffer. The enzyme extracts were desalted using Sephadex G25 PD-10 columns (GE Healthcare Life Sciences©) and used for the GS inhibition assay. A 115 µL aliquot of glufosinate stock was added to 935 µL reaction mixture containing 50 mM Tris–HCl, pH 7.5, 6 mM ATP, 10 mM $MgSO_4$, 20 mM hydroxylamine, 3.3 mM cysteine and 65.2 mM glutamate, to give final glufosinate concentrations of 0, 0.001, 0.01, 0.1, 1, 10 or 100 mM. Then, 100 µL of the enzyme extract was added to start the reaction. The mixture was incubated at 37 °C for 20 min, and the reaction was terminated by adding 350 µL 10% (w/v) ferric chloride solution in 0.2 N HCl. For background control samples, the reaction was terminated immediately after the addition of the enzyme extract without incubation. After centrifugation at 3000 × *g* for 5 min, absorbance of the mixture was measured at 595 nm after factoring in absorbance of the background control. A standard curve of 0.27–2.7 mM L-glutamic acid–γ-mono-hydroxamate was used as a reference. Protein concentration was determined following Bradford's method[15] using 0.2–1.4 mg bovine serum albumin as a standard. This experiment was conducted three times with two replicate samples each time. Specific GS activity was expressed as µmol product formed min^{-1} mg^{-1} protein.

2.3 [^{14}C]-Glufosinate uptake and translocation

Seeds of the S and R* populations were germinated and grown as described earlier, with the exception that the temperature was changed to 25/20 °C (day/night) to reduce chlorosis development following glufosinate treatment. At the three to five leaf stage, one droplet (1 µL) of [3,4-^{14}C]-glufosinate-HCl with a specific activity of 400 MBq $mmol^{-1}$ (Bayer AG, Frankfurt, Germany) diluted in commercially formulated glufosinate (Basta®; Bayer CropScience, Perth, Australia) with a resultant glufosinate concentration of 5 mM was applied to the midpoint of the adaxial surface of the youngest fully expanded leaf. This single droplet of glufosinate solution was at a concentration equivalent to 125 g ha^{-1} of the commercial glufosinate and contained 0.98 kBq ^{14}C. Previous dose–response experiments[13] have established that this is the lowest rate that discriminates S and R* individuals.

Treated plants were harvested (including roots) at 16, 24, 48 and 72 h after treatment (HAT), and differential visual symptoms (chlorosis) were observed for S and R* plants at 48 and 72 HAT. Unabsorbed radioactivity was determined by rinsing the treated leaf portion with 10 mL of 0.1% (v/v) Triton X-100. A 2 mL aliquot was taken from the leaf rinse and mixed with 3 mL of liquid scintillation cocktail (IRGA Safe Plus; Perkin-Elmer, Waltham, MA, USA) and the radioactivity quantified by liquid scintillation spectrometry (LSS) (Packard 1500, Tri-Carb®; Downers Grove, IL, USA). The plants were pressed and oven dried at 60 °C for 72 h and then exposed overnight to a phosphor imager plate (BS 2500; FujiFilm, Kanagawa, Japan) at room temperature for visualisation of glufosinate translocation, using a phosphor imager (Personal Molecular Imager™; Bio-Rad Laboratories, Inc., Hercules, CA, USA). After imaging, each individual plant was divided into three parts: roots, untreated shoot and treated leaf and combusted in a biological sample oxidiser (RJ Harvey Instrument Corporation, Hillsdale, NJ, USA). Radioactive CO_2 produced was trapped in an absorbent mix of Carbosorb E and Permafluor E (1:1, v/v) (Perkin-Elmer) and radioactivity in oxidised samples was quantified using LSS. Average total recovery of applied ^{14}C (^{14}C in leaf wash plus $^{14}CO_2$) was 88%. Leaf glufosinate uptake was calculated from the percentage of radioactivity that was recovered from the leaf wash solution. Glufosinate translocation was expressed as the percentage of absorbed radioactivity (i.e. recovered from combustion only) present in each plant part. The experiment had four to six replicates per treatment.

2.4 Glufosinate metabolism

Plants were grown as described previously. Three- to five-leaf stage S and R* seedlings were first foliar-sprayed at sub-lethal doses of 20 and 80 g ha^{-1} glufosinate, respectively, to boost glufosinate metabolism, and kept overnight in the growth chamber. This pre-treatment with herbicide at a sub-lethal dose was based on our previous experience in herbicide metabolism studies with *Lolium rigidum*.[16,17] The plants were then treated with a solution containing [^{14}C]-glufosinate-HCl (3.9 kBq in 10 µL) with 0.3% (v/v) BioPower adjuvant (Bayer AG, Frankfurt, Germany), prepared as described earlier but with a final glufosinate concentration of 0.86 mM (concentration equivalent to 20 g ha^{-1}). The treatment solution was applied in 10 small droplets along the adaxial surface of the youngest fully expanded leaf. The treatment solution was allowed to dry before the plants were returned to the growth chamber. Transgenic glufosinate-tolerant tobacco plants (supplied by Bayer AG, Frankfurt) were also included in this treatment as a positive control. Treated leaves were harvested at 24, 48 and 72 HAT, rinsed as described previously, blotted dry and stored at −80 °C until extraction and analysis. One leaf of six plants of each population were pooled together as one replicate sample, with two replicates extracted per time point per population. The samples were inserted in a 96-well round bottom plate. A metal bead and 600 µL of extraction buffer (water:methanol 90:10, v/v) were added into each well containing the sample. The plate was then sealed with a rubber cap and homogenised for 10 min using a TissueLyser (Qiagen, Hilden, Germany). Following homogenisation, the homogenate was centrifuged at 5700 × *g* for 10 min. The supernatant was transferred into a new 96-well square bottom plate and evaporated to dryness. The 96-well round bottom plate was refilled with 600 µL extraction buffer, and extraction was repeated twice as described. In the final extraction the extraction buffer was replaced with 80% acetone. The samples in the 96-well square bottom plate were then resuspended in 200 µL extraction buffer, shaken and sonicated for 5 min each. They were then transferred into a 96-well filtration plate and centrifuged at 780 × *g* for 10 min. The recovered radioactivity in the filtrate was determined using LSS (recovery was >80% with an average of 85%). A non-treated control sample, spiked with ^{14}C-labelled glufosinate prior to extraction, was also included.

Glufosinate metabolism was examined using HPLC with a strong anion exchange (SAX) column (Phenomenex PhenoSphere 5u SAX 80A column, 250 × 4.6 mm) based on the method of Jansen *et al.*[18]

The chromatography was performed at a flow rate of 1.0 mL min^{-1} with a mobile phase consisting of 90% 10 mM KH_2PO_4:10% acetonitrile (pH 2.8) for 45 min (isocratic), followed by a 15 min linear gradient to 90% 50 mM KH_2PO_4:10% acetonitrile (pH 2.8), and isocratic 90% 50 mM KH_2PO_4:10% acetonitrile (pH 2.8) for 10 min. The system was then brought back to its initial conditions in a 15 min linear gradient and held for another 65 min before the next run. An in-line radioactivity detector was used for radioactivity peak determination. Each sample was normalised to give about 1050 Bq per injection into the HPLC.

The parent herbicide [^{14}C]-glufosinate-HCl, its non-radiolabelled reference standard glufosinate-ammonium (GA) and its metabolite reference standards (non-radiolabelled) were injected both individually and as a mixture. The reference standards were 2-acetamido-4-methylbutanoic acid (NAG), 4-methylphosphinyl-2-oxobutanoic acid (PPO), 3-methylphosphinylpropionic acid (MPP), 2-methylphosphinylacetic acid (MPA) and 4-methylphosphinylbutanoic acid (MPB). All standards were provided by Bayer AG. Detection of non-radiolabelled reference standards was carried out using an inline UV–visible spectrophotometer at 210 nm to establish retention times. Preliminary experiments revealed that shifts in retention times occurred following injections. Thus a mixture of standards was analysed before and after each set of sample runs. This experiment was repeated.

In order to identify other possible metabolites, plants treated with non-radiolabelled glufosinate were also analysed using liquid chromatography–mass spectrometry (LC-MS). For this purpose, the S and R* seedlings at the three to five leaf stage were treated with 20 and 80 g ha^{-1} glufosinate, respectively, and kept overnight in the growth chamber. At 24, 48 and 72 HAT, the youngest fully expanded leaf on each plant was harvested and processed as described previously. For comparison extracts of non-glufosinate treated plants were also prepared in the same way.

LC-MS analysis of all these samples was performed on a Waters QTOF premier mass spectrometer (Waters, Manchester, UK) connected to Waters 2795 HPLC System (Waters, Milford, MA, USA) via an electrospray interface. Ten microlitres of each sample was injected. Chromatographic separation was achieved at a flow rate of 1 mL min^{-1} on a 150 × 4.6 mm I.D Nucleodur® HILIC column with a particle size of 3 µm (Macherey-Nagel, Düren, Germany). The column temperature was kept at 40 °C.

A binary gradient elution with eluent A acetonitrile and eluent B Milli-Q water, both containing 0.5% (v/v) formic acid was used. Initial conditions of 90% A were held for 0.5 min, before a linear gradient to 50% A over 9.5 min, followed by a hold time of 4 min before immediate change back to 90% A. The column was then equilibrated with 90% A for 6 min before next injection.

Ionisation was achieved with an electrospray interface operated in the negative ion mode at a capillary voltage of 2.2 kV. Further ion source parameters were set as follows: ion source temperature (80 °C), desolvation gas temperature 450 °C and the desolvation gas flow (450 L h^{-1}). Data evaluation was done with the Waters software MetaboLynx® XS (Waters, Manchester, UK), by comparing the obtained LC-MS data of glufosinate treated and non-treated samples.

2.5 Leaf disc glufosinate uptake experiment

2.5.1 Plant material

Net uptake and efflux of glufosinate were investigated using a leaf disc system. Seeds of S and R* were germinated on 0.6% (w/v) agar in a glasshouse with mean day/night temperature of 30/25 °C and 75% humidity during September to November 2015. After 4–7 d, the seedlings were transplanted into pots containing potting mix and grown in the same conditions.

2.5.2 Leaf disc sampling and infiltration

Two young fully expanded leaves were collected from each plant (with a total of 13 plants used per biotype. One leaf disc toward the base of each leaf (avoiding the midrib) was sampled using a 4 mm cork borer. Each set of 25 fresh leaf discs were weighed and vacuum infiltrated using a 10 mL syringe in a solution containing 0.1% Tween 80 and 10 mM sucrose (hereinafter defined as the medium). The infiltrated leaf discs were kept in the medium and stored at room temperature under low light conditions up to 2 h until used.

2.5.3 Measurement of ammonia accumulation in leaf discs

Glufosinate concentration discriminating S and R* plants in the leaf disc system was determined by measuring ammonia accumulation in leaf discs in the presence of a series of glufosinate concentrations (1.56–100 µM, technical grade) in a 96-well plate.[19] Leaf discs were placed in wells with 200 µL of different glufosinate concentrations diluted in the sucrose/Tween 80 medium. The plate was then sealed with two layers of micropore tape and placed in an incubation chamber under 150 µmol m^{-2} s^{-1} of light intensity for 16 to 24 h. The incubation was stopped by placing the plate in −80 ° C and then thawing it (two cycles) to lyse the cell membranes. A 50 µL aliquot of solution from each well was transferred to another plate and mixed with 150 µL of water. Premixed phenol nitroprusside solution (100 µL, P6994; Sigma–Aldrich, St. Louis, MO, USA) was then added, followed by 50 µL of premixed alkaline hypochlorite solution (A1727; Sigma–Aldrich). The solution was then mixed and left at room temperature for at least 30 min and the colour development was assayed in a microplate reader as the absorbance, *A*, at 630 nm. Two replicates were included in each experiment and the experiment was repeated once. Ammonia content was determined using 0.2–2.4 µg ammonium chloride as a standard.

2.5.4 [^{14}C]-glufosinate incubation and efflux

A significant difference in ammonia content between the S and R* leaf discs was found at a glufosinate concentration of 5 µM after 16 h of incubation (4.68 µg and 2.18 µg ammonia, respectively) and thus this concentration was chosen for the subsequent study. Glufosinate incubation and efflux protocols were based on Gougler and Geiger[20] with some modifications. Leaf discs of each replicate (25 leaf discs) were placed into wells (five leaf discs per well) of a 24-well microtiter plate containing 1 mL [^{14}C]-glufosinate (2.41–2.92 kBq; Bayer AG, Frankfurt). A well containing only the leaf discs and the medium with no radiolabelled glufosinate was included as control. The plate was then incubated in a chamber at 25 °C under 150 µmol m^{-2} s^{-1} of light intensity.

After 3 or 24 h incubation, the incubation medium was decanted. The leaf discs were rapidly washed with 1 mL fresh medium containing no glufosinate and then transferred into a new well containing 1 mL fresh medium and the microtitre plate was returned to the incubation chamber. At various time intervals, an 800 µL of aliquot of the medium solution was removed from the wells and the radioactivity counted by LSS (Packard 1500, Tri-Carb®). The remaining efflux aliquot (200 µL) was withdrawn and immediately replaced by 1 mL fresh medium. The amount of [^{14}C]-glufosinate

efflux in the medium was measured at 2.5, 5, 10, 15, 30, 60, 90 and 120 min and expressed as μg g^{-1} fresh weight (FW) of leaf discs.

After completion of the efflux period, the leaf discs were washed twice with a 1 mL fresh medium and transferred into a 1.5 mL eppendorf tube and stored at −80 °C until extracted.

2.5.5 [^{14}C]-glufosinate influx (net uptake)

Plant extraction followed the protocol of Holtum *et al.*[21] and Dinelli *et al.*[22] with some modifications. Frozen leaf discs were thawed at room temperature and ground in 0.5 mL of 10% (v/v) cold methanol for 3 min with a pre-chilled mortar and pestle. The homogenate was transferred to a 1.5 mL eppendorf tube. The mortar and pestle were washed two more times with 0.5 mL 10% (v/v) cold methanol and the wash was added to the homogenate. Radioactivity in the homogenate (700 μL aliquot) in duplicate was measured by LSS. The net glufosinate uptake into the plant cell is expressed as μg g^{-1} FW of leaf discs.

2.6 Statistical analysis

Glufosinate concentration causing 50% inhibition of GS activity (IC_{50}) was estimated by non-linear regression analysis using Sigma Plot® software (version 12.0; SPSS Inc., Chicago, IL, USA). The data were fitted to the three-parameter logistic curve model:

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

where a is the upper limit, ED_{50} is the estimated dose causing 50% response and b is the slope around ED_{50}. Significant differences between S and R* populations in the IC_{50}, herbicide uptake, and herbicide translocation were determined by the *t*-test (GraphPad Prism, version 5.0; GraphPad Software Inc., La Jolla, CA, USA).

The leaf disc experiment was arranged in a completely randomised design with four replications per treatment. The data for glufosinate efflux into the medium over a period of time were plotted using a hyperbolic function:

$$y = y_0 + \frac{at}{b + t} \quad (2)$$

where y is the amount of glufosinate in the medium, y_0 is the amount of glufosinate in the medium at 0 min, a is the asymptotic value of glufosinate amount at increasing efflux times, b is the increase rate of the amount of glufosinate for a given increase in efflux time and t is the efflux time. The parameter estimates derived from this function and the net glufosinate uptake for both S and R* biotypes were subjected to the unpaired *t*-test using GraphPad Prism version 5.00 (GraphPad Software, www.graphpad.com) for mean comparisons between the S and R* biotypes.

3 RESULTS

3.1 Glutamine synthetase activity

Glutamine synthetase (GS) from S and R* plants was equally sensitive to glufosinate inhibition. At 0.1 mM glufosinate, GS activity of both S and R* samples was inhibited by less than 10%. However, GS activity was reduced to less than 50% of control values at 1 mM glufosinate. At the highest glufosinate concentration used (100 mM), GS activity was completely inhibited (Fig. 1) in both S and R* samples. The glufosinate IC_{50} for the S and R* GS was not significantly different (0.85 mM and 0.99 mM, respectively) (Table 1). Similarly,

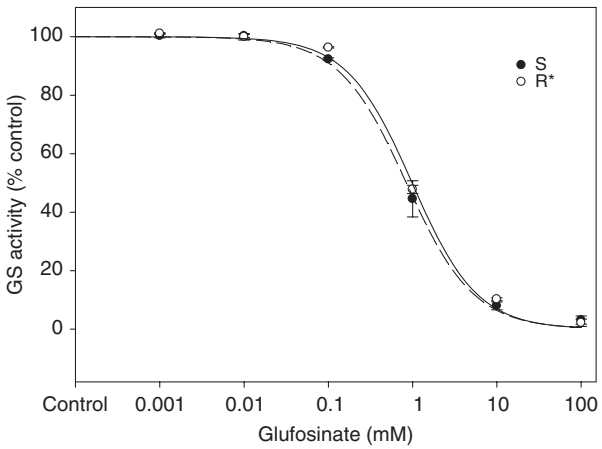

Figure 1. *In vitro* activity of glutamine synthetase (GS) from leaf extractions of the glufosinate susceptible (S) and resistant (R*) *E. indica* populations in response to increasing glufosinate concentrations. The GS specific activity without glufosinate inhibition was 0.065 and 0.068 μmol^{-1} mg^{-1} protein min^{-1} for S and R*, respectively.

there was no significant difference in the specific GS activity of the S (0.065 μmol^{-1} mg^{-1} protein min^{-1}) and R* (0.068 μmol^{-1} mg^{-1} protein min^{-1}) samples in the absence of glufosinate (Table 1). This result indicates that glufosinate resistance in this *E. indica* population is unlikely to be target-site based.

3.2 Glufosinate uptake and translocation

Leaf uptake of [^{14}C]-glufosinate was similar in both S and R* plants, with about 28% [^{14}C]-glufosinate absorbed in the first 16 HAT (Table 2), which remained stable before reaching the highest absorption of 49–57% at 72 HAT. At all sampling time points, no significant differences in [^{14}C]-glufosinate uptake rate were observed between the S and R* plants.

The S and R* plants showed typical visual symptoms of glufosinate damage such as chlorosis, with the damage extending from the leaf tip to the treated area (data not shown). At 16 and 24 HAT, no visual symptoms were present in S and R* plants, despite phosphor images clearly showing glufosinate has been absorbed and translocated throughout the plants (Fig. 2A and B). Beyond 24 HAT, symptoms of glufosinate damage were visible, with damage in R* plants less severe than in S plants, especially at 48 and 72 HAT (data not shown). In the R* plants, the damage was restricted to the glufosinate application site and extended towards the leaf tip, whereas in S plants the damage extended beyond the application site both in an acropetal and basipetal direction. Despite this, phosphor images showed similar glufosinate translocation patterns in the S and R* plants throughout the sampling time points (Fig. 2; 16 to 72 HAT). Quantification of [^{14}C]-glufosinate translocation showed that the majority of the absorbed ^{14}C activity (between 65% and 79%) was retained in the treated leaf, even at 72 HAT. A similar amount of ^{14}C was translocated to the roots and untreated shoots of the S and R* plants at all sampling time points. Translocation of ^{14}C away from the application site was reduced in the S samples after 48 HAT, likely due to self-limitation caused by glufosinate damage. Overall, except for significant but small (less than 1.8-fold) differences in ^{14}C translocation at 24 and 72 HAT, there was no major difference in [^{14}C]-glufosinate translocation outside the treated leaf of S and R* plants (Table 2). Therefore, glufosinate resistance in this *E. indica* population is unlikely to be due to differential glufosinate foliar uptake and translocation.

Table 1. Glutamine synthetase (GS) specific activity and parameters of the logistic analysis of glufosinate dose required to cause 50% inhibition of GS activity for the glufosinate susceptible (S) and resistant (R*) plants

Population	GS specific activity (μmol^{-1} mg^{-1} protein min^{-1})	*a*	*b*	$ED_{50} = IC_{50}$ (mM)	R^2 (coefficient)	IC_{50} R/S ratio
S	0.065 (0.09)	100 (0)	1.09 (0.1)	0.85 (0.06)[c]	0.99	NA
R*	0.068 (0.11)	100 (0)	1.12 (0.07)	0.99 (0.05)[c]	0.99	1.16

Standard errors are in parentheses.
Means with the same letter in a column are not significantly different ($\alpha = 0.05$) as determined by the *t*-test.
a is the upper limit, ED_{50} is the estimated dose causing 50% response (in this case, 50% inhibition in enzyme activity, IC_{50}) and *b* is the slope around ED_{50}.
NA, Not Applicable.

Table 2. Uptake and translocation of [^{14}C]-glufosinate (from treated leaf to root and untreated shoots) in the glufosinate susceptible (S) and resistant (R*) *E. indica* plants at 16, 24, 48 and 72 h after treatment

		Translocation (% of absorbed [^{14}C]-glufosinate)		
Population	Foliar uptake (% of [^{14}C]-glufosinate recovered)	Root	Untreated shoots	Treated leaf
16 h				
S	29.3[a]	26.8 (2.44)[a]	17.4 (1.09)[a]	55.8(4.7)[a]
R*	27.6[a]	24.8 (3.72)[a]	14.5 (1.93)[a]	60.7 (4.7)[a]
24 h				
S	25.9 (2.25)[a]	26.8 (1.87)[a]	20.5 (1.76)[a]	52.7 (2.33)[a]
R*	26.8 (0.98)[a]	28.7 (3.24)[a]	15.5 (1.18)[b]	55. 9 (4.36)[a]
48 h				
S	31.2 (2.20)[a]	16.7 (1.47)[a]	13.4 (1.35)[a]	69.9 (1.94)[a]
R*	29.1 (3.87)[a]	18.6 (1.92)[a]	15.3 (1.82)[a]	65.9 (2.91)[a]
72 h				
S	56.7 (4.05)[a]	10.5 (1)[a]	10.8 (0.69)[a]	78.7 (1.35)[a]
R*	49.9 (1.15)[a]	18.8 (3.49)[b]	15.3 (2.37)[a]	65.9 (5.77)[b]

Standard errors are in parentheses.
Means with the same letter in a column for each paired S and R* sample at each time point are not significantly different ($\alpha = 0.05$) as determined by the *t*-test.

3.3 Glufosinate metabolism

The retention time of glufosinate was resolved by HPLC (with UV–visible detection) at 10.76 min for glufosinate-ammonium (GA), and the retention times for possible glufosinate metabolites were resolved at 16.64 min for MPB, 20.19 min for MPP, 22.39 min for MPA, 55.42 min for NAG and 72.88 min for PPO (Fig. 3A). The [^{14}C]-glufosinate-HCl reference standard was resolved by HPLC at 10.78 min (with radioactive detection) (data not shown). Transgenic glufosinate-tolerant tobacco is known to metabolise glufosinate to non-toxic NAG which resolved by [^{14}C]-HPLC at 56.38 min (Fig. 3B), close to the non-radioactive NAG reference standard peak. Leaf extracts of [^{14}C]-glufosinate treated S and

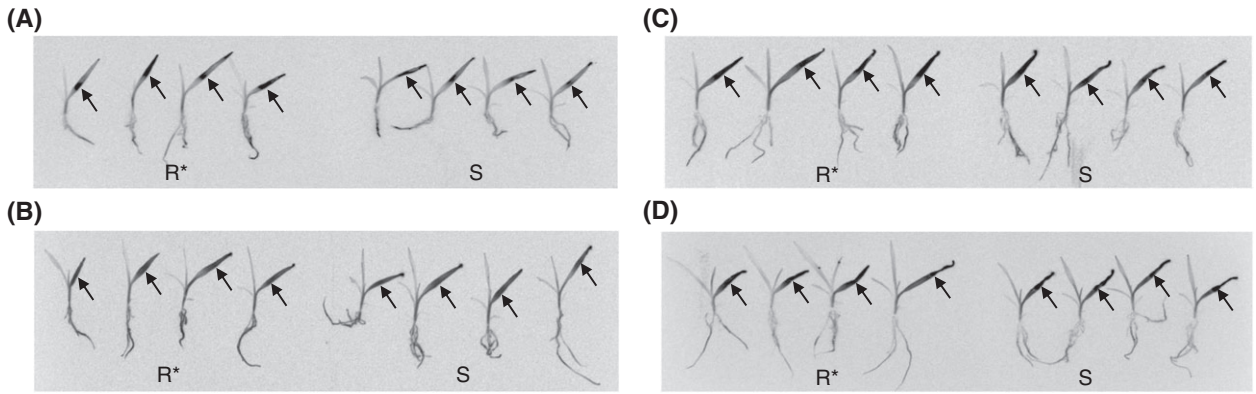

Figure 2. Phosphor images of glufosinate susceptible (S, four plants to the right of each panel) and resistant (R*, four plants to the left of each panel) *E. indica* plants following [^{14}C]-glufosinate treatment at: (A) 16 HAT, (B) 24 HAT, (C) 48 HAT and (D) 72 HAT. The arrows indicate the herbicide application site.

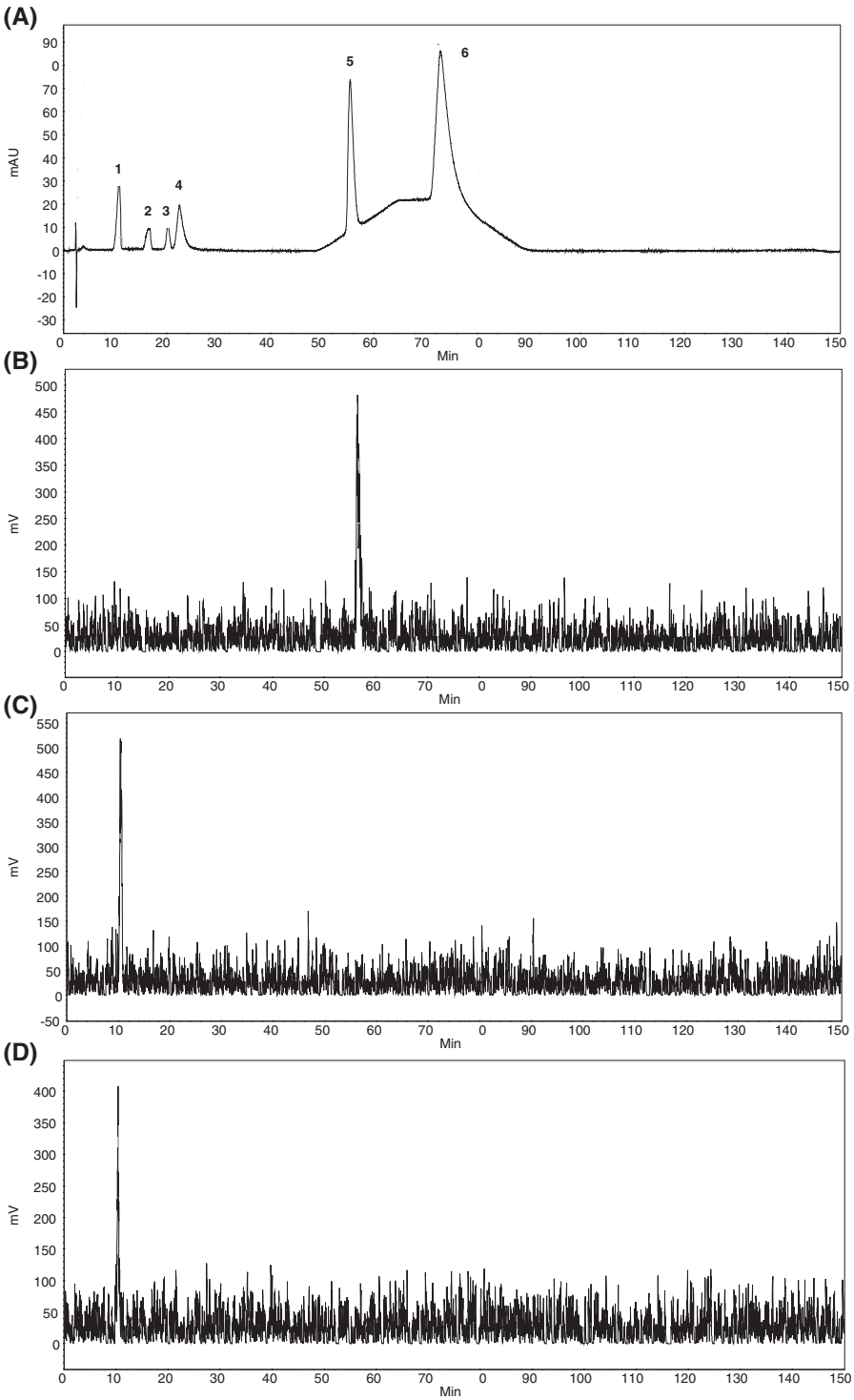

Figure 3. (A) HPLC chromatogram of glufosinate (1) and its metabolite standards 4-methylphosphinylbutanoic acid (MPB) (2), 3-methylphosphinylpropionic acid (MPP) (3), 2-methylphosphinylacetic acid (MPA) (4), 2-acetamido-4-methylbutanoic acid (NAG) (5) and 4-methylphospinyl-2-oxobutanoic acid (PPO) (6). (B) The ^{14}C-HPLC chromatogram of transgenic glufosinate-tolerant tobacco leaf extracts, showing resolution of the metabolite NAG. The ^{14}C-HPLC chromatograms of S (C) and R* (D) *E. indica* leaf extracts at 24 HAT.

R* samples had only a single peak with the same retention time at 10.65 min (Fig. 3C and D). This peak was believed to be un-metabolised glufosinate, as it corresponded to the retention time of [^{14}C]-glufosinate standard and the non-radiolabelled GA peak (peak 1; Fig. 3A). None of the above glufosinate metabolites were detected from the S or R* leaf extracts at 24, 48 or 72 HAT.

Glufosinate metabolism in S and R* plants was also analysed using non-radioactive LC-MS. The detection times and mass for glufosinate metabolite standards resolved by LC-MS were 11.14 min and 181.032 for PPO, 11.34 min and 223.968 for NAG, 11.47 and 138.904 for MPA, 11.62 min and 152.968 for MPP and 11.77 min and 167.032 for MPB, respectively (Fig. 4A). However,

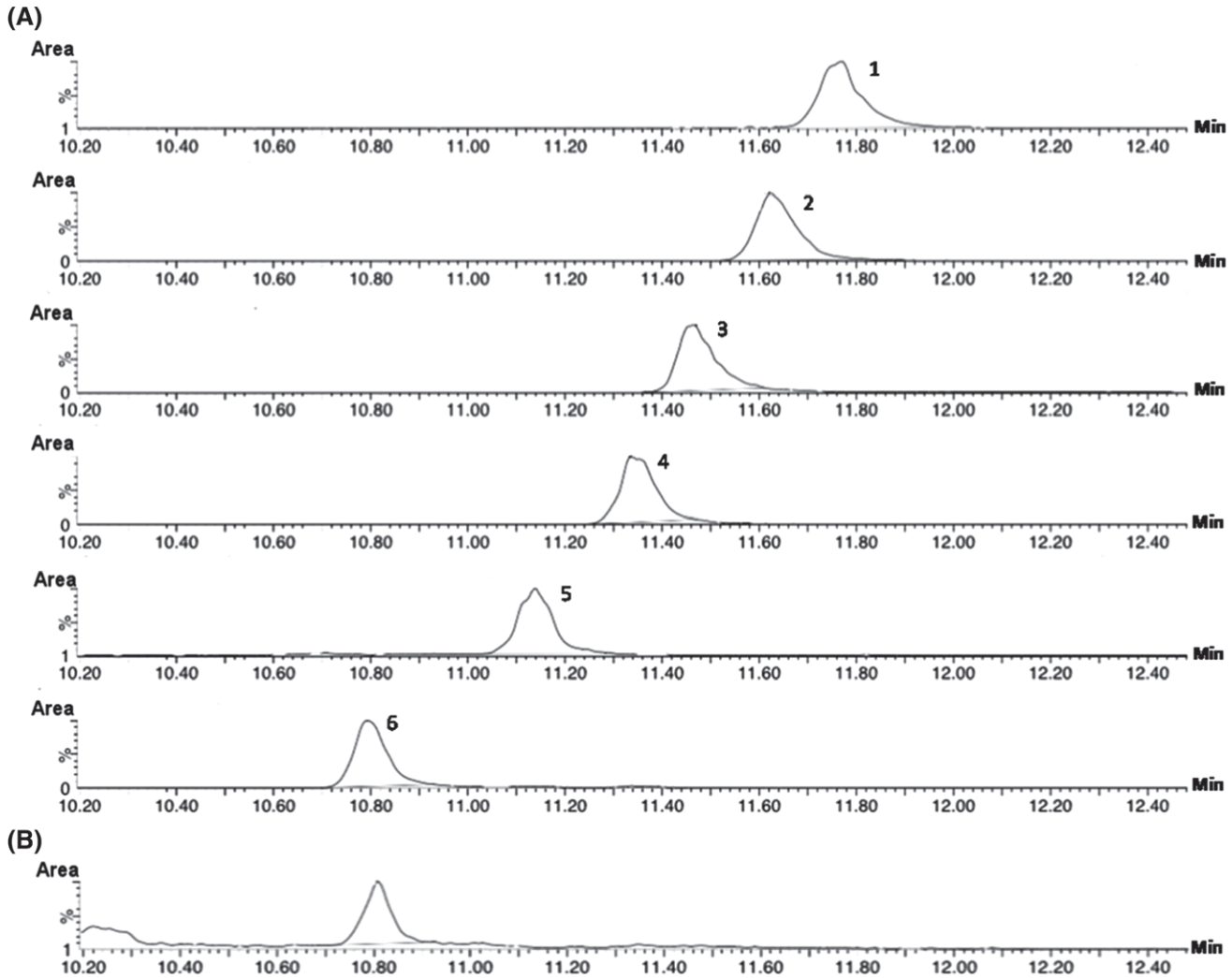

Figure 4. (A) LC-MS chromatogram of glufosinate and its metabolite standards 4-methylphosphinylbutanoic acid (MPB) (1), 3-methylphosphinylpropionic acid (MPP) (2), 2-methylphosphinylacetic acid (MPA) (3), 2-acetamido-4-methylbutanoic acid (NAG) (4), 4-methylphospinyl-2-oxobutanoic acid (PPO) (5) and glufosinate-ammonium (6). (B) The LC-MS chromatogram of S (R* the same as the S, hence not shown) *E. indica* leaf extracts treated with non-radiolabelled glufosinate at 24 HAT.

only a single peak corresponding to the detection time and mass of glufosinate (10.79 min, 182.032) was detected from the S or R* (10.81 min, 182.032) leaf extracts (Fig. 4B). No major metabolites were detected from the leaf extracts of S and R* plants at 24, 48 and 72 HAT. These results confirm that there is virtually no major glufosinate metabolism in leaves of *E. indica*, and therefore, glufosinate metabolism is unlikely to play a role in resistance, at least in this resistant population.

3.4 Glufosinate leaf efflux and influx

Glufosinate efflux from the leaf discs into incubation medium was rapid in the first 20 min before slowing towards the end of the experiment (120 min; Fig. 5). Glufosinate efflux in R* leaf discs was slightly but not significantly higher than that from S leaf discs after 24 h of incubation (Fig. 5, Table 3). Similarly, no differences were observed in glufosinate efflux in S and R* leaf discs after 3 h of incubation (data not shown). Leaf disc net uptake (glufosinate remaining in the leaf disc after efflux) of glufosinate was higher after 3 h than 24 h incubation time (Table 4) likely due to higher [^{14}C]-glufosinate loading in the 3 h experiment. However, no significant difference in the amount of glufosinate net uptake was observed between the S and R* leaf discs, regardless of the incubation time (Table 4). This result indicates a similar amount of glufosinate was absorbed inside cells of S and R* plants, but caused differential damage (quantified as ammonia accumulation).

4 DISCUSSION

Compared to field-evolved resistance to other major herbicides, resistance to glufosinate is still rare in crop weeds, involving only in a few populations of two weed species.[23] Here working with a glufosinate resistant (R*) *E. indica* population,[13] we found that resistance in this population is unlikely to be due to a target-site based mechanism. Firstly, the total extractable enzyme activity (specific GS activity) was similar between the S and R* populations, indicating that resistance is not due to enhanced GS activity. In contrast, previous studies with laboratory selected glufosinate-resistant plant cell lines have shown that resistance can be due to higher constitutive GS activity conferred by gene amplification.[24] Secondly, the glufosinate *in vitro* inhibition assay showed that GS of S and R* populations is equally sensitive to glufosinate (Table 1,

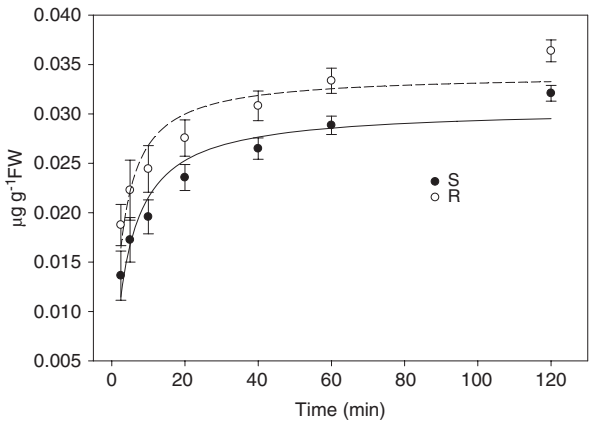

Figure 5. Efflux (*y* axis) of [^{14}C]-glufosinate from leaf discs of glufosinate susceptible (S) and resistant (R*) *E. indica* plants after 24 h incubation. Each point is the mean ± standard error of four replicates.

Table 3. Parameter estimates of glufosinate efflux after 24 h of incubation for leaf discs from the glufosinate susceptible (S) and resistant (R*) plants of *E. indica* derived from the fitted hyperbolic regression model, Eqn (2)

	Estimated parameter			
Biotype	y_0	*a*	*b*	R^2
S	NA	0.023 (0.0011)	19.9 (4.63)	0.98
R*	NA	0.022 (0.0012)	23.6 (5.96)	0.98
t-value	NA	0.55	0.49	–
df	NA	38	38	–
P	NA	0.58	0.63	–

Standard errors are shown in parentheses.
Means within a column followed by the same letter are not significantly different according to the *t*-test ($\alpha = 0.05$).
y is the amount of glufosinate in the medium, y_0 is the amount of glufosinate in the medium at 0 min, *a* is the asymptotic value of glufosinate amount at increasing efflux times, *b* is the increase rate of the amount of glufosinate for a given increase in efflux time, *t*.
NA, Not Applicable.

Table 4. Net [^{14}C]-glufosinate uptake in the leaf discs of the glufosinate susceptible (S) and resistant (R*) *E. indica* plants following 120 min efflux after incubation for 3 and 24 h

Phenotype	Incubation period (h)	Amount of [^{14}C]-glufosinate (µg g^{-1} FW)
S	3	0.349 (0.04)[a]
R*		0.362 (0.03)[a]
S	24	0.292 (0.013)[a]
R*		0.278 (0.004)[a]

Standard errors are in parentheses.
Means within a column in a time point (3 or 24 h) followed by the same letter are not significantly different according to the *t*-test ($\alpha = 0.05$).

Fig. 1). Thus, it is unlikely that the resistance is due to altered GS sensitivity caused by gene mutation(s). In a study comparing the GS sequence in glufosinate-susceptible and glufosinate-resistant cultured soybean cells, the resistant line showed eight amino acid substitutions.[25–27] Only one of these, the His-249-Tyr mutation, occurs at the GS substrate/inhibitor binding site.[27] The role of the other seven amino acid substitutions in glufosinate resistance remains unclear. In one glufosinate-resistant *Lolium* biotype the GS activity was less sensitive to glufosinate inhibition than that in susceptible plants, with an IC_{50} R/S ratio of 18 to 79 (depending on the susceptible population used), and the Asp-171-Asn mutation in the GS gene was proposed to be responsible for resistance.[12]

Differential glufosinate uptake and translocation can contribute to plant species-dependent variations in glufosinate sensitivity.[4,5,28–30] However, in the current study, foliar [^{14}C]-glufosinate uptake was similar between the S and R* plants, with about 50% leaf uptake by 72 HAT.

Glufosinate has the physico-chemical characteristics for phloem mobility, although glufosinate translocation from the site of application is somewhat limited,[5,28–30] due to the rapid phytotoxicity of glufosinate in the source leaf tissue.[1] In our experiments, nearly half of the absorbed [^{14}C]-glufosinate had already been translocated out of the treated leaf in both S and R* plants at 24 HAT. Translocation through the phloem was evident, with more than 20% of absorbed ^{14}C activity detected in roots at 16 and 24 HAT, indicating no primary alterations in long-distance glufosinate translocation in R* plants. At 48 and 72 HAT, however, translocation from the treated leaf became restricted in both S and R* plants, likely due to necrosis-induced self-limitation causing a higher amount of ^{14}C activity to be retained in the treated leaf. Due to this restricted translocation, phloem movement (downwards; basipetal) of glufosinate was affected more than the upwards (acropetal) xylem-mediated translocation of glufosinate, as evidenced by the decreased ^{14}C activity detected in the root. It was noticed that at 72 HAT, the S plants were more damaged than the R plants; correspondingly, significantly less basipetal [^{14}C]-glufosinate translocation occurred in the S than R* plants at this time point (Table 2).

Compared to the whole plant foliar [^{14}C]-glufosinate uptake approach, the leaf disc herbicide uptake system can provide information on the net glufosinate uptake inside plant cells when equilibrium between the apoplastic (efflux) and symplastic (influx) glufosinate is established (20 min). In this experiment, at predetermined discriminating glufosinate concentration (5 µM), both the efflux and net uptake of glufosinate in S and R* leaf discs were similar. This result, together with the lack of differences in translocation pattern and ammonia content in S and R* plants, suggests that it is possible that cytoplasmic non-target-site mechanisms are operating in the R* cells to protect the target GS activity.

In most herbicide resistance cases where reduced translocation is evident, it is indicative of (1) impaired herbicide long-distance translocation via phloem loading/unloading,[31] (2) restricted herbicide cellular uptake, i.e. less entry into cytoplasm,[32] and (3) enhanced herbicide cellular sequestration, e.g. into vacuole, hence less available herbicide in the cytosol.[33] Nevertheless, lack of difference in long-distance herbicide translocation does not necessarily exclude the possibility of cellular mechanisms extruding/sequestrating the herbicide, especially in C4 plants. In our experiment, reduced herbicide long-distance translocation or net cellular uptake was not evident by the various ^{14}C methods. However, we cannot exclude the possible involvement of cellular mechanisms for glufosinate compartmentation, particularly in C4 plants like *E. indica*, due to its more complex assimilate loading/unloading and phloem translocation than in C3 plants.[34] Similarly in glyphosate resistance studies, populations of some species with evolved glyphosate resistance (such as *Desmanthus illinoensis*, *Amaranthus palmeri* and *Amaranthus*

tuberculatus) demonstrated similar glyphosate uptake and translocation between the susceptible and resistant biotypes with no evidence of target-site and enhanced glyphosate metabolism as resistance mechanisms. It was hypothesised that restricted glyphosate chloroplast entry could be the resistance mechanism for these species.[32]

One common non-target-site mechanism of herbicide resistance is detoxification via herbicide metabolism. However, glufosinate metabolism in plants is low to non-existent, depending on the species.[18,29] In plants that metabolise glufosinate, the final stable metabolic products were identified as MPP, MHB or both.[8,18,35,36] Transgenic glufosinate-tolerant crops detoxify glufosinate by acetylating glufosinate into non-toxic *N*-acetyl-glufosinate (NAG).[8] In treated leaves of both S and R* *E. indica*, glufosinate metabolism was not detected, and hence is unlikely to be responsible for resistance in this R* population. In contrast, transgenic glufosinate-tolerant tobacco completely metabolises glufosinate into NAG (Fig. 3). Our result contradicts the finding of Everman *et al.*[30] where two glufosinate metabolites were detected in *E. indica*. It should be noted that Everman *et al.*[30] used thin layer chromatography (TLC), and the nature of the two metabolites was not identified. In our preliminary TLC study using a solvent system similar to that of Everman *et al.*,[30] we obtained two bands, one co-migrating with [^{14}C]-glufosinate, and the other of an unknown nature (data not shown). However, HPLC analysis of the same sample only displayed a single radioactive peak corresponding to glufosinate. Efforts to extract the unknown TLC band for further analysis were unsuccessful (data not shown). The LC-MS analysis further confirmed that glufosinate metabolism in *E. indica* is negligible. Nevertheless, as we only examined glufosinate metabolism in herbicide treated leaves, the possibility of glufosinate metabolism in stem or root tissues cannot be excluded.

5 CONCLUSION

In this research with this *E. indica* glufosinate resistant biotype we exclude the possibility of target-site-based resistance mechanism, altered long-distance herbicide translocation or enhanced metabolism. Rather, cytoplasmic mechanisms reducing herbicide concentration reaching the target GS, such as vacuole sequestration (as has been demonstrated for glyphosate resistance[32]) need to be examined to explain glufosinate resistance.

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