Research Article

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Glyphosate resistance in perennial *Sorghum halepense* (Johnsongrass), endowed by reduced glyphosate translocation and leaf uptake

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Abstract

BACKGROUND: In a large cropping area of northern Argentina, *Sorghum halepense* (Johnsongrass) has evolved towards glyphosate resistance. This study aimed to determine the molecular and biochemical basis conferring glyphosate resistance in this species. Experiments were conducted to assess target *EPSPS* gene sequences and ^{14}C-glyphosate leaf absorption and translocation to meristematic tissues.

RESULTS: Individuals of all resistant (R) accessions exhibited significantly less glyphosate translocation to root (11% versus 29%) and stem (9% versus 26%) meristems when compared with susceptible (S) plants. A notably higher proportion of the applied glyphosate remained in the treated leaves of R plants (63%) than in the treated leaves of S plants (27%). In addition, individuals of *S. halepense* accession R_2 consistently showed lower glyphosate absorption rates in both adaxial (10–20%) and abaxial (20–25%) leaf surfaces compared with S plants. No glyphosate resistance endowing mutations in the *EPSPS* gene at Pro-101–106 residues were found in any of the evaluated R accessions.

CONCLUSION: The results of the present investigation indicate that reduced glyphosate translocation to meristems is the primary mechanism endowing glyphosate resistance in *S. halepense* from cropping fields in Argentina. To a lesser extent, reduced glyphosate leaf uptake has also been shown to be involved in glyphosate-resistant *S. halepense*.

Keywords: glyphosate leaf uptake; glyphosate translocation; perennial Johnsongrass weed; non-target-site resistance mechanism

1 INTRODUCTION

Glyphosate [*N*-(phosphonomethyl)-glycine] is the most widely used herbicide in world agriculture.[1] Since 1974, glyphosate has been extensively used as a non-selective herbicide in a variety of ways,[2] and the widespread adoption of transgenic glyphosate-resistant crops has led to large increases in glyphosate use.[3] Where there has been intense glyphosate selection, glyphosate resistance has evolved in several weed species.[2,4]

Studies of the mechanistic basis of evolved glyphosate resistance reveal that there can be target-site-based resistance or non-target-site resistance. Target-site glyphosate resistance can be due to mutations of the target site for glyphosate, the *EPSPS* gene.[5] *EPSPS* gene mutations, including serine, threonine or alanine substitutions at Pro-106 (Pro-106-Ser/Thr/Ala), a highly conserved region of the *EPSPS* gene, have been reported in glyphosate-resistant *Eleusine indica* and *Lolium* spp. respectively.[6–10] In biotechnology-created glyphosate resistance, a Gly-101-Ala substitution in the *EPSPS* gene of various plant species endows glyphosate resistance.[11] Aside from these *EPSPS* gene point mutations, *EPSPS* gene amplification in glyphosate-resistant *Amaranthus palmeri*[12] and basal increase in *EPSPS* mRNA and enzyme activity in *Conyza canadensis* have been documented.[13]

Non-target-site glyphosate resistance is known in glyphosate-resistant *Conyza* and *Lolium* species.[14–16] This mechanism corresponds to an alteration in the way glyphosate is translocated such that, in resistant plants, glyphosate largely remains in treated leaves and less herbicide is translocated to other organs of the plants.[14–16] To date there is no evidence that metabolic degrada-

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tion plays a significant role in evolved glyphosate-resistant weeds, although this mechanism remains possible.[17]

Glyphosate is a water-soluble herbicide that penetrates the leaf cuticle and is symplastically translocated via phloem to apical meristems,[14,15] although acropetal glyphosate movement through the apoplast has also been reported.[18] Glyphosate movement in plants follows the photoassimilate source–sink route and, among the herbicides known to possess phloem mobility, is the most efficiently translocated to rapidly growing sink shoot and root tissues.[19,20] This is crucial for successful glyphosate control of perennial species that possess below-ground storage organs (rhizomes, tubers), which serve for asexual propagation.[21–25]

Until now the resistance mechanism studies conducted with evolved glyphosate-resistant plants have been with annual species. Perennial *Sorghum halepense* populations with glyphosate resistance have evolved in areas in northern Argentina where transgenic glyphosate-resistant soybean is grown.[26] A characteristic phenotypic response observed in glyphosate-resistant (R) *S. halepense* plants is their ability to resprout and tiller from the plant shoot meristem (crown) while other parts of the plants are severely damaged after glyphosate exposure.[26] Here, the basis of glyphosate resistance in perennial *S. halepense* is investigated. It is hypothesised that this regrowth ability in glyphosate-treated *S. halepense* plants is due to an alteration in glyphosate movement to the crown, as observed in some annual glyphosate-resistant species. In this study, the pattern of glyphosate leaf absorption and translocation and *EPSPS* gene mutations in glyphosate-resistant *S. halepense* is investigated.

2 MATERIALS AND METHODS

2.1 Plant material

Four evolved glyphosate-resistant (R) *S. halepense* accessions (R_1, R_2, R_3 and R_4) from transgenic glyphosate-resistant soybean fields in the province of Salta, Argentina, exhibit a glyphosate resistance index of 2–4.5, whether evaluating plants grown from rhizomes or seeds.[26] The nomenclature of the present study (R_1, R_2, R_3 and R_4) corresponds, respectively, to the S1B, S2A, S1A and S2B populations described in that study.[26] A glyphosate-susceptible (S_1) accession never exposed to glyphosate selection and collected from the Pampas (Province of Buenos Aires, Argentina) was used as reference material in all experiments. For the *EPSPS* gene sequence analyses, and in addition to the S_1 accession, a glyphosate-susceptible accession (S_2) collected from the same cropping area as the resistant ones was also employed.

2.2 Isolation of *EPSPS* mature protein coding sequence

Total RNA was isolated from leaf tissue of individual plants originating from field-collected rhizomes of S_1, S_2, R_1 and R_2 accessions using RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's specifications. Synthesis of cDNA from 5 µg samples of total RNA was carried out using SuperScript III reverse transcriptase and random primers (Invitrogen, USA). The *EPSPS* coding sequence was amplified by RT-PCR using primer pairs designed by comparing published plant *EPSPS* sequences: Sa up 5′-GAGGAGATCGTGCTSCAGCC-3′ and Sa low 5′-CACATCACCCTGCAAACTGG-3′. Amplification was performed using Platinum Pfx DNA polymerase (Invitrogen) followed by incubation with Platinum Taq polymerase in the presence of dATP. The amplified products were purified using Gel Extraction kit (Qiagen, Germany), cloned into pGEMt easy vector (Life Technologies, USA) and sequenced in both directions using an ABI 3730 XL automated sequencer. In all cases, 2–4 clones from each amplicon were analysed to determine the consensus sequence. To obtain and analyse 5′ and 3′ termini of *EPSPS* coding regions, 5′ and 3′ rapid amplification of cDNA ends (RACE) was performed using kits from Invitrogen.[27] The gene-specific primer pairs were: 5′R 5′-CAACTTTGTCTGCTTCGACA-3′ and 5′Rn 5′-CTGTTCAACAGGTTATCA AC-3′ for 5′RACE, and 3′R 5′-GGTG ATGCCTCAAGTGCAAG-3′ and 3′Rn 5′-CTGCAATTACTGGAGG GACT-3′ for 3′RACE. The resulting 5′ and 3′ RACE products were then gel purified, cloned into pGEMt easy and sequenced. The complete nucleotide sequence (1335 kb) was assembled for the mature *S. halepense EPSPS* protein and compared among the R and S plants using NTI Suite 8.0 software (InforMax Inc., USA). Databases were searched using the BLAST program.[28] The *EPSPS* sequences used for comparison were: *Sorghum bicolor* (XP002436424), *Zea mays* (X63374), *Oryza sativa* (G101A and P106L), *Eleusine indica* (AJ417033), *Lolium rigidum* (ACB05442) and *Lolium multiflorum*.[10,11,29] The nucleotide sequences reported in this paper were submitted to GenBank under accession numbers HQ436351 (R_1), HQ436352 (R_2), HQ436353 (S_1) and HQ436354 (S_2).

2.3 [^{14}C]-Glyphosate leaf absorption and translocation

Individuals from R_1, R_2, R_3 and R_4 and S_1 accessions were employed in this study. Seedlings were grown from seed. For radiolabelled glyphosate uptake and translocation experiments, seedlings of 2 cm height were transferred into small plastic cups (60 × 60 mm) (one seedling per cup) containing potting mix and kept in a growth chamber at constant 25 °C, 12 h photoperiod and 250 µmol photons m^{-2} s^{-1} irradiance.

At the four-leaf stage, one droplet (1 µL) of ^{14}C-labelled glyphosate solution was applied to the midpoint of the adaxial surface of plants of similar size. The treatment glyphosate solution (1 µL) consisted of 25 mM of glyphosate containing 0.53 kBq of [^{14}C]-glyphosate ([^{14}C]-phosphonomethyl; Sigma-Aldrich) in a diluted commercial glyphosate formulation (RoundUp Power Max as potassium salt, 540 g L^{-1} AE SL; Nufarm, Laverton North, Victoria, Australia) plus 0.06% (v/v) non-ionic surfactant BS1000 (1000 g L^{-1} of alcohol alkoxylate). The single foliar-applied droplet of glyphosate solution, which contained 52 Bq µg^{-1} of glyphosate, had a spray concentration equivalent to the field rate of 450 g AE ha^{-1}. Both single-drop (1 µL) and spray volume applications proved to be very effective in discriminating between glyphosate-resistant and glyphosate-susceptible plants. Preliminary experiments were performed with or without plant pretreatment with commercial (unlabelled) glyphosate at 450 g ha^{-1} (Roundup Power Max) to mimic the field situation. Unlabelled glyphosate was applied 30 min prior to application of the ^{14}C-glyphosate (labelled glyphosate application sites on the leaves were covered with aluminium foil) using a laboratory moving boom sprayer equipped with two Teejet (Teejet Australasia, Victoria, Australia) fan nozzles with a total output volume of 112 L ha^{-1} of water at a pressure of 200 kPa. As the glyphosate translocation patterns were similar with these two methodological approaches (with versus without unlabelled glyphosate pretreatment), the results reported here are from experiments that only used labelled glyphosate applied as a single drop on a leaf.

Glyphosate-treated plants were returned to the controlled-environment chamber. At different time intervals after glyphosate treatment (48, 72, 96 and 120 h), plants were harvested, the treated leaf of each plant was rinsed with 20 mL of 0.1% (v/v) Triton X-100

and the [^{14}C] present in the rinse solution was quantified by liquid scintillation to determine glyphosate remaining in the leaf surface. Glyphosate leaf absorption (uptake) was calculated from the difference between the [^{14}C] applied and that recovered in the leaf wash solution. The plants were then pressed between paper towels and oven dried at 70 °C for 3 days. Glyphosate translocation within the plant was visualised using a phosphor imager (BS 2500, FujiFilm, Japan). After imaging, each plant was divided into different sections: treated leaf, untreated leaves, crown and roots. The treated leaf was further divided into sections above and below the treatment zone (1 cm long and full leaf width in the middle of the fourth leaf). The plant sections were combusted in a biological oxidiser (RJ Harvey Instrument Corporation, Hillsdale, NJ). Released [$^{14}CO_2$] was trapped in the cocktail solution and measured in a liquid scintillation counter. Herbicide translocation was expressed as a percentage of the total absorbed (total [$^{14}CO_2$] recovered minus [$^{14}CO_2$] in the leaf wash solution). Mean total recovery of applied [$^{14}CO_2$] was higher than 96% for all accessions and experiments conducted. An additional experiment evaluated [^{14}C]-glyphosate uptake by abaxial and adaxial leaf surfaces of glyphosate-resistant plants (R_2) in comparison with susceptible plants (S_1). [^{14}C]-Glyphosate was applied to both abaxial and adaxial leaf surfaces, as described above, and assessments were conducted 24, 72 and 120 h after glyphosate treatment. Ten replicates were used per treatment.

2.4 Leaf blade anatomy and surface features

Leaf blade gross anatomy (mesophyll and bundle sheath chloroplast distribution) and surface morphology were examined in plants of the R_2 and S_1 *S. halepense* accessions. To reveal mesophyll and bundle sheath chloroplasts, leaf cross-sections (2.5 µm thick) were examined in fluorescent (365 nm UV; emission 397 nm) and green light (546/42 nm; emission 590 nm) excitation and by scanning electron microscopy (Zeiss 1555 SUPRA variable-pressure scanning electron microscope operating at 15 kV). Leaf segments were fixed following standard procedures.[30,31]

2.5 Statistical analysis

Results were represented by the average of three experiments, each with five replicates per treatment. A completely randomised design was used in all experiments. Two-way analysis of variance (ANOVA) was performed to assess the main effects of genotype (resistant and susceptible) and time course (48, 72, 96 and 120 h) on [^{14}C]-glyphosate leaf absorption and translocation. Similarly, for the additional glyphosate leaf absorption experiment, a three-way ANOVA was performed to determine the effect of genotype, leaf surface (abaxial versus adaxial) and time (24 versus 72 versus 120 h) on [^{14}C]-glyphosate leaf absorption. When necessary, percentage values were angular transformed ($y = \arcsin\sqrt{x}$) to increase normality and variance homogeneity. Where appropriate, treatment means were separated using Tukey's honestly significant difference (HSD) ($\alpha = 5\%$).

3 RESULTS

3.1 Resistance not due to target-site *EPSPS* change

The full *S. halepense EPSPS* coding sequence was determined to be 1335 kb, encoding 444 amino acids. When the deduced amino acid sequences of R_1, R_2, S_1 and S_2 were analysed, identities of 99 and 96% were observed with the *Sorghum bicolor* and *Oryza sativa EPSPS* genes respectively. Identities of 98–99% were observed with *Zea mays*, and identities of 94–95% with *Triticum aestivum*. *EPSPS* gene sequence comparison of glyphosate-resistant versus glyphosate-susceptible *S. halepense* plants revealed polymorphisms in both nucleotides and deduced amino acid sequences, but there were no amino acid changes in the known resistance mutation sites from amino acids 101 to 106 (Table 1). Although other nucleotide polymorphisms were detected within the *EPSPS* coding and non-coding sequences, none of them showed association with glyphosate resistance (data not shown). Therefore, the mutations in the *EPSPS* gene known to confer glyphosate resistance in some annual weeds[5] are not present in the assessed R *S. halepense* accessions.

Table 1. Amino acid sequences in the conserved region of *EPSPS* cDNA isolated from glyphosate-resistant (R_1 and R_2) and glyphosate-susceptible (S_1 and S_2) *S. halepense* accessions, and from glyphosate-resistant (R) and glyphosate-susceptible (S) species. The *EPSPS* sequences used for comparison were: *Sorghum bicolor* (XP002436424), *Zea mays* (X63374), *Oryza sativa*[29] (AF413081), *Eleusine indica* (AJ417034, AJ417033), *Lolium rigidum* (AF349754, ACB05442, A) and *Lolium multiflorum* (AAZ79230)[10]

Amino acid position[a]	101	102	103	104	105	106	107	108
Sorghum halepense (R_1)	D	A	K	E	E	V	Q	L
Sorghum halepense (R_2)	D	A	K	E	E	V	Q	L
Sorghum halepense (S_1)	D	A	K	E	E	V	Q	L
Sorghum halepense (S_2)	D	A	K	E	E	V	Q	L
Sorghum bicolor (S)	D	A	K	E	E	V	Q	L
Zea mays (S)	D	A	K	E	E	V	Q	L
Oryza sativa (S)	D	A	K	E	E	V	Q	L
Oryza sativa (R)	D	A	K	E	E	V	Q	L
Eleusine indica (S)	D	A	K	E	E	V	Q	L
Eleusine indica (R)	D	A	K	E	E	V	Q	L
Lolium rigidum (S)	D	A	K	E	E	V	K	L
Lolium rigidum (R)	D	A	K	E	E	V	K	L
Lolium multiflorum (S)	D	A	K	E	E	V	K	L
Lolium multiflorum (R)	D	A	K	E	E	V	K	L

Amino acid position[a]	109	110	111	112	113	114	115	116
Sorghum halepense (R_1)	G	N	A	G	T	A	M	R
Sorghum halepense (R_2)	G	N	A	G	T	A	M	R
Sorghum halepense (S_1)	G	N	A	G	T	A	M	R
Sorghum halepense (S_2)	G	N	A	G	T	A	M	R
Sorghum bicolor (S)	G	N	A	G	T	A	M	R
Zea mays (S)	G	N	A	G	T	A	M	R
Oryza sativa (S)	G	N	A	G	T	A	M	R
Oryza sativa (R)	G	N	A	G	T	A	M	R
Eleusine indica (S)	G	N	A	G	T	A	M	R
Eleusine indica (R)	G	N	A	G	T	A	M	R
Lolium rigidum (S)	G	N	A	G	T	A	M	R
Lolium rigidum (R)	G	N	A	G	T	A	M	R
Lolium multiflorum (S)	G	N	A	G	T	A	M	R
Lolium multiflorum (R)	G	N	A	G	T	A	M	R

Amino acid position[a]	117	118	119	120	121	122	123	124
Sorghum halepense (R_1)	P	L	T	G	N	L	S	I
Sorghum halepense (R_2)	P	L	T	G	N	L	S	I
Sorghum halepense (S_1)	P	L	T	G	N	L	S	I
Sorghum halepense (S_2)	P	L	T	G	N	L	S	I
Sorghum bicolor (S)	P	L	T	G	N	L	S	I
Zea mays (S)	P	L	T	G	N	L	S	I
Oryza sativa (S)	P	L	T	G	N	L	S	I
Oryza sativa (R)	L	L	T	G	N	L	S	I
Eleusine indica (S)	P	L	T	G	N	L	S	I
Eleusine indica (R)	S	L	T	G	N	L	S	I
Lolium rigidum (S)	P	L	T	G	N	L	S	I
Lolium rigidum (R)	A	L	T	G	N	L	S	I
Lolium multiflorum (S)	P	L	T	G	N	L	S	I
Lolium multiflorum (R)	S	L	T	G	N	L	S	I

[a] Amino acids are numbered according to the plant EPSPS numbering used by Padgette *et al.*[43]

3.2 Reduced glyphosate leaf absorption in R_2 accession

Only for the R_2 accession, quantitative determination of [^{14}C]-glyphosate leaf absorption rates revealed less glyphosate leaf uptake in R than in S plants ($P < 0.0001$) (Fig. 1). Averaged over three time intervals, [^{14}C]-glyphosate adaxial leaf surface absorption rates were lower (26%) for R_2 than in S individuals (42%) (Fig. 1). If R_2 plants were pretreated with unlabelled commercial glyphosate and then exposed to [^{14}C]-glyphosate, individuals of the R_2 accession always displayed lower adaxial leaf surface absorption rates (14%) compared with S plants (32%) over a 72 h period after glyphosate treatment (data not shown). Notably, there were no differences in [^{14}C]-glyphosate leaf absorption rates for the other three R accessions (R_1, R_3 and R_4) when compared with S plants (Fig. 1).

In order to confirm the reduced glyphosate leaf absorption in the R_2 accession, [^{14}C]-glyphosate absorption rates for both abaxial and adaxial leaf surfaces of R_2 versus S_1 plants were determined. Differences in [^{14}C]-glyphosate leaf absorption rates were again observed between these two genotypes ($P < 0.0001$) (Table 2). Abaxial leaf surfaces of the R_2 plants absorbed approximately 10–20% less [^{14}C]-glyphosate than those of S_1 plants when measured 24, 72 and 120 h after glyphosate treatment (Table 2).

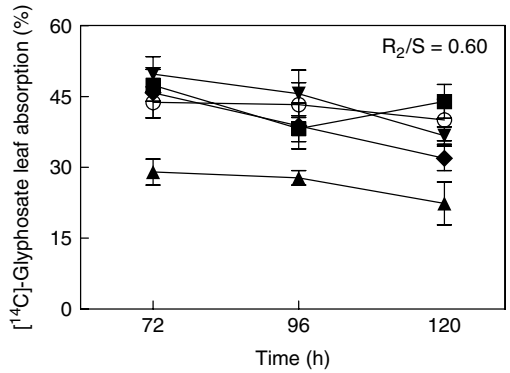

Figure 1. Quantification (%) of [^{14}C]-glyphosate absorption rates in the adaxial leaf surface of the glyphosate-susceptible (S_1, ○) and glyphosate-resistant (R_1, ■; R_2, ▲; R_3, ▼; R_4, ♦) *Sorghum halepense* plants 72, 96 and 120 h after [^{14}C]-glyphosate foliar treatment. Values are the mean [^{14}C]-glyphosate ($n = 15$) ± standard error. R_2/S_1 represents the ratio of R_2 over S_1 absorption estimates averaged over the three time intervals.

Table 2. Quantification of [^{14}C]-glyphosate abaxial and adaxial leaf surface absorption rates between glyphosate-susceptible (S_1) and glyphosate-resistant (R_2) *Sorghum halepense* individuals 24, 72 and 120 h after [^{14}C]-glyphosate foliar treatment. Values are mean ^{14}C-glyphosate leaf absorption expressed as percentage of [^{14}C]-glyphosate leaf applied. Values in parentheses are the standard error of the mean ($n = 6–10$). The glyphosate absorption index (AI) is the ratio of R_2 over S_1 glyphosate leaf absorption estimates. Asterisks (*) indicate significant differences between absorption estimates within each time interval according to Tukey's HSD test ($\alpha = 5\%$)

	Adaxial leaf surface			Abaxial leaf surface		
Time	S_1	R_2	AI	S_1	R_2	AI
24	51.9 (2)	54.8 (3)	1.05	71.7 (6)	56.5 (4)	0.80*
72	58.3 (3)	46.3 (2)	0.80*	66.1 (2)	58.5 (1)	0.90*
120	57.7 (4)	43.4 (3)	0.75*	65.9 (2)	57.8 (2)	0.90*
Total	56.4 (2)	47.6 (2)	0.80*	67.5 (2)	57.8 (1)	0.85*

* Tukey's HSD differences ($P < 5\%$) within each time interval

With the exception of the treatment at 24 h, adaxial leaf surfaces of the R_2 plants absorbed less [^{14}C]-glyphosate (20–25%) compared with S_1 plants (Table 2). Regardless of the accession, [^{14}C]-glyphosate absorption rates through the adaxial surface were approximately 10% greater ($P < 0.001$) than for the abaxial leaf surface – a consistent result across all the assessed time intervals (time effect: $P = 0.53$).

No differences in leaf structure and anatomy were evident between the R_2 and S_1 individuals that could account for differences in glyphosate leaf absorption. The leaf blade anatomy in both accessions displayed similar epidermal cells, and mesophyll tissues contained a similar amount of chloroplasts and vascular bundles. SEM revealed no obvious differences in stomata distribution or leaf surface features (waxy materials) in the abaxial and adaxial leaf surfaces of individuals of R_2 versus S_1 accessions (data not shown).

3.3 Reduced glyphosate translocation from treated leaf to other plant organs

For all four R accessions, clear differences were observed in glyphosate translocation from the treated leaf to the crowns and roots between R and S plants, whereas only 9% of the absorbed glyphosate was on average translocated from the treated leaf to the crown of R plants (120 h after treatment), and 26% translocation occurred to the basal crown of S plants (Fig. 2B). The amount of [^{14}C]-glyphosate translocated to the basal crown area of all R plants was therefore nearly threefold less than in S plants ($P < 0.0001$) (Fig. 2B). There were no major differences in the amount of absorbed [^{14}C]-glyphosate that was translocated to the untreated leaves in the R versus S accessions (Fig. 2A).

On average, while 11% of the absorbed glyphosate was translocated to roots of the R plants, 29% translocated to the roots of the S plants ($P < 0.0001$). These values indicate that nearly threefold less glyphosate was translocated to roots of individuals of all R accessions than to roots of S plants (Fig. 2C). Phosphor imaging confirmed these differences in [^{14}C]-glyphosate translocation patterns to both roots and crowns between the R and S plants (Fig. 3). Correspondingly, a significantly greater amount of the total absorbed [^{14}C]-glyphosate remained in the treated leaves of R versus S plants ($P < 0.0001$) (Fig. 2D). On average, across all time intervals (time effect: $P = 0.20$), 63% versus 27% of absorbed [^{14}C]-glyphosate was found in treated R versus S leaves respectively. Thus, R plants translocated 2.4-fold less glyphosate out of the treated leaves relative to S plants (Fig. 2D), and this translated into threefold less glyphosate translocated to the crown and roots of R plants (Figs 2A and B).

Further dissection of the glyphosate-treated leaves showed that, in R leaves, approximately 60% of the leaf-absorbed [^{14}C]-glyphosate remained in the treatment zone (1 cm long), whereas only 33% remained in the same area of the S leaves (Table 3). Only 7% of the total [^{14}C]-glyphosate within treated leaves was found in the base of R leaves, as opposed to 16% in S leaves (Table 3). However, the amount of [^{14}C]-glyphosate that moved to the leaf tip was not significantly different in the R versus S plants (Table 3).

4 DISCUSSION

4.1 No target-site *EPSPS* gene changes in the resistant accessions

Target-site glyphosate resistance endowed by amino acid substitution at amino acid position Gly-101, Thr-102 or Pro-106 of the *EPSPS* gene is well known.[5,11,32] The present full *EPSPS* coding sequence analysis indicated the absence of any target-site resistance point mutations in the studied glyphosate-resistant *S. halepense* accessions. In order to determine the independence of glyphosate resistance events in the *S. halepense* accessions studied here, DNA polymorphism analyses are currently in progress. Other possible target-site mechanisms of glyphosate resistance, such as *EPSPS* gene amplification[12] and basal increase in $EPSPS_mRNA$,[13] remain to be investigated.

4.2 Reduced glyphosate leaf absorption

Reduced foliage absorption of herbicides has thus far been rarely documented as a herbicide resistance mechanism in plants.[5] However, in some biotypes of glyphosate-resistant *Lolium multiflorum*, reduced glyphosate leaf absorption has been reported.[33,34] In the present work it is shown that reduced glyphosate leaf uptake is evident in one of the R *S. halepense* accessions (R_2) but not in the other three resistant accessions studied. With R_2 plants, both adaxial and abaxial leaf surfaces absorbed up to 25% less glyphosate than the S plants (Table 2; Fig. 1). Microscopic examination revealed no distinct difference

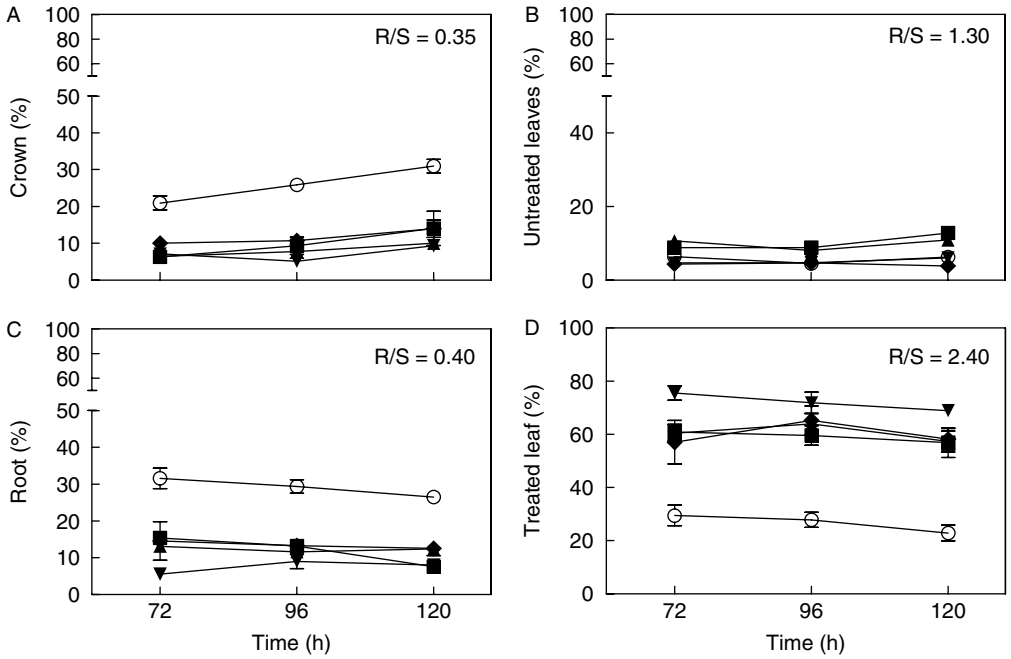

Figure 2. Distribution (%) of leaf-absorbed [^{14}C]-glyphosate in crown (A), untreated (B), root (C) and treated leaves (D) of plants of the glyphosate-susceptible (S_1, ○) and glyphosate-resistant (R_1, ■; R_2, ▲; R_3, ▼; R_4, ♦) *Sorghum halepense* accessions. Quantitative estimations occurred at 72, 96 and 120 h after [^{14}C]-glyphosate foliar treatment. Values are mean [^{14}C]-glyphosate ($n = 15$) ± standard error. R/S represents the ratio of R over S_1 [^{14}C]-glyphosate estimations in each plant organ averaged over all resistant accessions and time intervals.

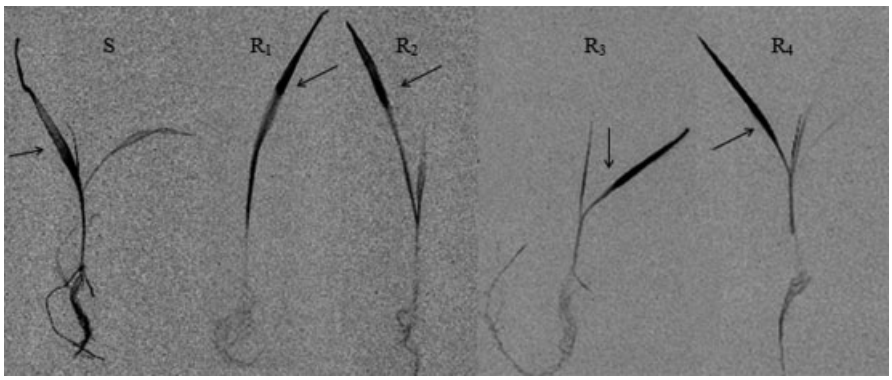

Figure 3. Phosphor imaging comparing the translocation pattern of [^{14}C]-glyphosate in representative plants of the susceptible (S_1) and resistant (R_1, R_2, R_3, R_4) *Sorghum halepense* accessions. Plants were treated with one droplet (1 µL) of [^{14}C]-glyphosate at the midpoint (arrowed) of the fourth leaf stage, and plants were imaged 72 h after treatment.

in leaf anatomy and morphology in R_2 versus S plants, so the basis for reduced glyphosate leaf uptake observed in this *S. halepense* accession is unknown. As the cuticle is the main barrier for glyphosate diffusion, analyses of chemical composition and thickness of cuticle and the presence and type of epicuticular waxes are required. In particular, the presence of Ca^{2+} in foliage has been shown to antagonise and reduce glyphosate leaf absorption.[35,36]

4.3 Reduced glyphosate translocation

Effective glyphosate translocation from the foliage to the roots, rhizomes and apical meristems is key to the herbicidal effect of glyphosate in its ability to control perennial species, including *S. halepense*.[22,37,38] Thus, any mechanism reducing glyphosate translocation to growing meristems should reduce glyphosate efficacy. The results of this study provide evidence that glyphosate resistance in four independent *S. halepense* accessions is associated with substantially reduced glyphosate translocation to sink tissues. Importantly and additionally, reduced glyphosate leaf uptake was observed in one of these R accessions, which likely also contribute to resistance to glyphosate.

Results show that glyphosate-resistant *S. halepense* plants exhibit reduced rates of glyphosate translocation from leaf to basal crown and root tissues, as compared with the S plants (Figs 2A and B). This reduced basipetal glyphosate translocation in R plants corresponds to higher retention of absorbed glyphosate in the treated leaves relative to susceptible plants (Fig. 2D). This result is consistent with the reduced glyphosate translocation found in glyphosate-resistant annual weed genera such as *Conyza* and *Lolium*.[5]

In these glyphosate-resistant species, about 11–22% more glyphosate is retained in the treated leaves, and 3–13% less glyphosate is transported to roots when compared with

Table 3. Quantification of leaf-absorbed [^{14}C]-glyphosate in treated leaves of glyphosate-susceptible (S_1) and glyphosate-resistant (R_1, R_2, R_3, R_4) *Sorghum halepense* individuals. Assessment was conducted 72 h after [^{14}C]-glyphosate treatment in the centre (1 cm) of the youngest fully expanded leaf (fourth). Treated leaves were then subdivided in sections above (leaf tip) and below (leaf base) the centre treatment zone (leaf centre). Values are the mean [^{14}C]-glyphosate ($n = 8$) estimated for plants of all R accessions ± the standard error of the mean. Different letters indicate significant differences between accessions within each leaf section according to Tukey's HSD test ($\alpha = 5\%$)

	[^{14}C]-Glyphosate (% of total recovered)		
Accession	Leaf base	Leaf centre	Leaf tip
S_1	16 (3) a	33 (4) a	51 (5) a
R	7 (1) b	61 (2) b	32 (3) a

susceptible reference populations.[14,15] However, differences in glyphosate leaf retention and translocation to roots between R and S *S. halepense* are notably higher. About 35–50% more glyphosate is retained in the leaf, and 15–25% less glyphosate is translocated to the roots. Similarly, absorbed glyphosate in glyphosate *S. halepense* R plants does not tend to accumulate in the leaf tips (unlike *Conyza and Lolium* species[15,39,40]) but rather remains at the droplet application site (Table 3; Fig. 3). A recent study with a single population from Arkansas (USA) has also identified reduced glyphosate translocation as the resistance mechanism in *S. halepense*.[41]

It is emphasised that the four field-evolved glyphosate-resistant *S. halepense* accessions from Argentinian cropping systems have likely evolved independently, yet all four exhibit the same glyphosate resistance mechanism, evident as reduced glyphosate translocation to the basal crown and roots. A glyphosate resistance mechanism of reduced translocation to the roots is known in several glyphosate-resistant annual plant species[5] and now in the perennial *S. halepense*. Recent evidence with glyphosate-resistant *Conyza canadensis* with this mechanism is that the glyphosate is loaded into the vacuole in leaves of R plants, thereby reducing the potential for translocation throughout the plant and thus reducing the toxic effect of glyphosate.[42] This could be the case in these four glyphosate-resistant *S. halepense* populations, but it remains to be investigated.

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