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Short title: Glyphosate-resistant *Poa annua*

Confirmation of Glyphosate Resistance in Annual Bluegrass (*Poa annua*) via *EPSPS* Duplication in a Soybean and Rice Rotation

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Abstract

Annual bluegrass (*Poa annua* L.) populations in turfgrass have evolved resistance to several herbicides in the United States (US), but there has been no confirmed resistance from an agricultural field. Recently, glyphosate failed to control a *P. annua* population found in a field in a soybean [(*Glycine max* (L.) Merr.] and rice (*Oryza sativa* L.) rotation in Poinsett County, Arkansas. The present study focused on determining the sensitivity of a putatively-resistant accession (R1) to glyphosate compared with two susceptible accessions (S1 and S2). The experiments included a dose-response study, 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene copy number and expression analysis, and assessment of mutations in *EPSPS*. Based on the dose-response analysis, R1 required 1,038 g ae ha^{-1} of glyphosate to cause 50% biomass reduction, whereas S1 and S2 only required 148.2 g and 145.5 g ae ha^{-1}, respectively. The resistant index (RI) was approximately 7-fold relative to the susceptible accessions. Real-time polymerase chain reaction data revealed at least a 15-fold increase in the *EPSPS* copy number in R1, along with a higher gene expression. No mutations in *EPSPS* were found. Gene duplication was identified as the main mechanism conferring resistance in R1. The research presented here reports the first incidence of glyphosate resistance in *P. annua* from an agronomic field crop situation in the US.

Keywords: *EPSPS* duplication, glyphosate resistance, herbicide-resistant weeds, *Poa annua*

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Introduction

Annual bluegrass (*Poa annua* L.) is an allotetraploid, self-pollinating plant found mainly as a weed in turfgrass in the United States (Hutchinson and Seymore 1897). *Poa annua* evolved through the hybridization of two diploid species supine bluegrass (*Poa supina* Schrad.) and weak blugrass (*Poa infirma* Kunth.) in the Mediterranean region about 2.5 million years ago (Mao and Huff 2012; Tutin 1957). The presence of the two genomes makes it easier for the weed to adapt to varying environmental conditions (Thompson and Lumaret 1992). *Poa annua* exists in the US generally as winter annual and perennial forms in golf course fairways and roughs (Carroll et al. 2021). *Poa annua* also persists as a winter weed in vegetable crops such as lettuce (*Lactuca sativa* L.), spinach (*Spinacia oleracea* L.), artichoke (*Cynara scolymus* L.), and cole crops (Shem-Tov and Fennimore 2003). It has also been reported in rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.)-soybean [(Glycine max (L.) Merr.] double-cropping system (Jordan et al. 1999; Wilson et al. 1986). The prolific seed production of up to 200,000 seed m^{-2} makes the weed difficult to manage in turf grass (Lush 1989).

Herbicides are a common strategy for controlling *P. annua* in golf courses throughout the transition zone and the southern US. Repeated use of acetolactate synthase-inhibitors, photosystem II-inhibitors, and glyphosate have facilitated the evolution of herbicide-resistant *P. annua*, mainly on golf courses and turf (Binkholder et al. 2011; Brosnan et al. 2015; Cross et al. 2013; McElroy et al. 2013; Perry et al. 2012). *Poa annua* is effectively controlled by a burndown application of glyphosate applied alone or in mixture with other herbicides in spring prior to summer crop planting in Arkansas (Barber et al. 2024). So far, *P. annua* has been identified to have evolved resistance to 12 sites of action (SOA) (Rutland et al. 2023). Resistance to multiple herbicide classes of up to 7 SOA has been reported in a *P. annua* population in Tennessee (Heap 2024). Apart from golf courses and turfgrasses, glyphosate resistance was also reported in an almond orchard in California (Brunharo et al. 2019). The California population was highly resistant to glyphosate due to 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene duplication and mutation.

Glyphosate has been a popular herbicide option since its introduction in 1974 due to its non-toxic, non-selective, and broad-spectrum weed control properties (Powles 2008). Glyphosate inhibits EPSPS, an enzyme that facilitates the synthesis of the intermediate chorismite in the

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shikimic acid pathway (Pline-Srnic 2006). The inhibition of EPSPS leads to the depletion of chorismite and, in turn, affects the synthesis of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan, leading to plant death. The introduction of glyphosate-resistant crops increased the reliance on the herbicide in row crops, eventually leading to the evolution of glyphosate resistance in weeds (Powles 2008). The first case of glyphosate resistance in weeds was reported in rigid ryegrass (*Lolium rigidum* Gaudin) (Powles et al. 1996; Pratley et al. 1999). So far, glyphosate resistance has been identified in 59 weed species, 28 of which are dicot and 31 monocot weeds (Heap 2024). Glyphosate resistance mechanisms include target site mechanisms such as mutation of *EPSPS*, gene duplication, or increase in expression; non-target site mechanisms include reduced glyphosate absorption, reduced translocation, rapid metabolism, and vacuolar sequestration (Sammons and Gaines 2014).

In the spring of 2023, glyphosate failed to control *P. annua* in a field in a soybean and paddy rice rotation in Poinsett County, Arkansas. The goal of the research was to confirm resistance and subsequently determine the glyphosate resistance mechanism. The objectives were to i) evaluate the response of putatively resistant (R1) and susceptible (S1 and S2) accessions to glyphosate treatment via dose-response assay and ii) confirm the mechanism of glyphosate resistance in the resistant accession R1 via quantitative polymerase chain reaction (qPCR) and sequencing of *EPSPS* to detect any possible mutations.

Materials and Methods

Plant materials and experimental conditions The seed samples of the putatively resistant *P. annua* accession, R1, were collected in the spring of 2023 following the failure of glyphosate to provide effective early-season control of *P. annua* in a field before planting. The field was located in Poinsett County, Arkansas. Additionally, two susceptible accessions, S1 and S2, were used for comparison. The susceptible accessions were collected from Alabama bermudagrass settings. S1 was collected from a golf course fairway, while S2 was collected from a golf course tee box. Both sites were non-overseeded areas that had been treated annually with a preemergence herbicide, typically prodiamine, in the fall, followed by a postemergence application of ALS- and PSII-inhibitors. The experiments were conducted in greenhouses at two locations. Two trial repetitions were conducted in the greenhouses at the Milo J. Shult Agricultural Research and Extension Center, Fayetteville, Arkansas and one experimental run

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was conducted in a greenhouse at the Lonoke Extension Center, Lonoke, Arkansas. At both locations, the seeds of all the accessions were germinated in potting mix (Promix, LP15, Premier Horticulture Inc., PA, US). At Fayetteville, the greenhouse was maintained at 23/20 C day/night temperatures and a 16-h photoperiod supplemented with a light emitting diode source. Seedlings were transplanted into 50-cell plastic trays at the one-leaf stage. At Lonoke, four plants were transplanted to 15-cm diameter pots. The greenhouse was maintained at 29/18 C day/night temperature and a 10-h photoperiod.

Dose-response assay

Plants were treated with rates of glyphosate (Roundup PowerMAX® 3, 575 g ai L^{-1}, Bayer, Missouri, US) at the three- to four-leaf stage. The putatively resistant accession was treated with the following glyphosate rates: 0; 315; 630; 1,260 (1X); 2,520; 5,040; 10,080; 20,160 or 40,320 g ae ha^{-1}, and the susceptible accessions were treated with the following rates: 0; 39.37; 78.75; 157.5; 315; 630; 1260; 2520 or 5040 g ae ha^{-1}. At Fayetteville, the herbicide treatments were applied using a spray chamber equipped with two 1100067 nozzles (TeeJet® Technologies, Springfield, IL, US) spaced 50.8 cms apart, calibrated to deliver 187 L ha^{-1} at 1.6 Km h^{-1}. At Lonoke, one TP95015EVS nozzle (TeeJet® Technologies, Springfield, IL, US) was calibrated to deliver 187 L ha^{-1} at 3.6 Km h^{-1}. Each treatment in the Fayetteville trial repetitions had 5 replicates (5 plants per replicate), resulting in a total of 25 plants per treatment. The Lonoke trial had 3 replicates (4 plants per replicate), with a total of 12 plants per treatment. The experiment was performed in a completely randomized design. After the application, plants were maintained in greenhouse conditions for further analysis. At 21 d after treatment (DAT), injury was visually scored on a scale of 0% to 100% (0%= no injury and 100% = plant mortality) (Frans and Talbert 1977). Plant mortality was calculated as a percentage relative to the number of plants alive at application. The fresh aboveground biomass was collected 21 DAT and then dried at 66 C for 3 d to constant mass. The biomass was weighed and expressed relative to the nontreated biomass for each accession.

Quantification of EPSPS copy number

The gene copy number assay was conducted with the untreated plants of the resistant and susceptible accessions. About 100 mg of leaf tissue was collected from each plant and the genomic DNA was isolated using the cetyltrimethylammonium bromide (CTAB) method. After

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DNA extraction, quantification was performed using a Nanodrop spectrophotometer (Nanodrop 2000c, ThermoFisher Scientific, Waltham, MA, US), and the concentration was diluted to 10 ng μl^{-1} with molecular-grade water. A qPCR was conducted in a CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, US) to quantify the copy number of the *EPSPS*. Gene-specific primers used in the qPCR experiment are provided in Table 1. The qPCR reaction mixture consisted of 2× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, US), 0.8 µl each of 10 µM forward and reverse primers (Table 1), 5.9 µl of deionized water, and 25 ng of genomic DNA. The analysis was conducted under the following conditions: 98 C for 3 min, 40 cycles of 98 C for 10 s, and 61 C for 30 s. The dissociation curves were created by increasing the temperature from 65 C to 95 C, 0.5 C every 5 s to ensure specific amplification. The experiment was performed twice. The first run consisted of three biological and three technical replicates; the second run was performed with four biological and three technical replicates. Two housekeeping genes, Cinnamoyl-CoA reductase (*CCR*) and Peter Pan-like (*PPAN*), were used to normalize the target gene. All the qPCR primer sequences used were based on work previously completed in *Lolium spp*. (Table 1). The relative quantifications were performed using the $2^{-\Delta\Delta Ct}$ method (Gaines et al. 2010; Livak and Schmittgen 2001).

EPSPS expression

The gene expression studies were conducted with the leaf tissues collected from the same plants used in the gene copy number quantification studies. Approximately 100 mg of leaf tissues were collected, and flash-frozen immediately in liquid nitrogen and stored at -80 C. Total RNA was extracted using the TRIzol (Ambion, Life Technologies, Carlsbad, CA, US) method following the manufacturer's protocol. The quantification was performed using a nanodrop spectrophotometer (Nanodrop 2000c, Thermo Scientific, Waltham, MA, US) and agarose gel (1%). About 1 µg of total RNA was treated with DNase I (Thermo Scientific, Waltham, MA, US) and cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, US). The qPCR conditions and the primer sets used were the same as described for the copy number analysis. The experiment was performed twice. The first trial repetition consisted of three biological and three technical replicates; the second trial repetition was performed with four biological and three technical replicates. The cDNA was also used for sequencing.

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EPSPS sequencing

The cDNA was amplified using the primers (PoaF 5′-CTGTTGAACAGTGAGGATGTCCAC-3′ and PoaR 5′-CTCCCTCATTCTTGGTACTCCATC-3′) adapted from Brunharo et al (2018). Primers were designed to amplify the highly conserved Pro106 position in *EPSPS*. In another study involving glyphosate resistance in *P. annua*, primers were also designed to capture the same region (Cross et al. 2015). A polymerase chain reaction (PCR) was carried out in a total reaction volume of 60 µL using 30 µL of 2X GoTaq® G2 green master mix (Promega™, Madison, WI, US), 3 µL each of 10 µM forward and reverse primer, 100 ng of cDNA template and 21 µL of sterile water. The PCR amplification was performed with an initial denaturation of 95 °C for 5 min, followed by 34 cycles of denaturation: 94 °C for 30 s, annealing: 58 °C for 45 s, extension: 72 °C for 45 s, and final extension of 72 °C for 7 mins. Finally, the PCR products were analyzed in 1.0% agarose gel to confirm the targeted amplicon size. A PCR cleanup was performed using the PureLink™ PCR purification kit (Invitrogen, Waltham, MA, US). The purified samples were sequenced (Eurofins Genomics, Louisville, KY, US) by Sanger's method. A total of five biological replicates of resistant and susceptible accessions were sequenced. The amplicons were cloned into vectors and sequenced to confirm the sequencing accuracy. The cDNA was amplified using Q5® High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, MA, US) following the manufacturer's protocol for a total volume of 50 µL. The amplicon was then ligated into the pJET1.2/blunt cloning vector using the CloneJET PCR cloning kit (Thermo Scientific, Waltham, MA, US). After ligation, the plasmid was transformed into DH5a-competent cells (Invitrogen, Waltham, MA, US) by heat shock method. The colonies that survived ampicillin supplementation were visible the following day. The plasmids were isolated from bacterial colonies grown overnight using GeneJET plasmid miniprep kit (Thermo Scientific, Waltham, MA, US). About twelve colonies from R1 and four colonies from S2 were selected and Sanger sequencing was performed at Eurofins Genomics.

Statistical analysis

The mortality and relative biomass were averaged within bioassay location and within each accession to reduce variability since few plants were evaluated within each replication. After data were averaged, the three runs were pooled due to visually similar responses within each accession. Data were analyzed using JMP Pro Version 17 (SAS Institute, Cary, NC, US) using the Fit Curve Platform with the three *P. annua* responses as dependent variables and

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glyphosate rate (g ae ha^{-1}) as the independent variable. The model considered accession a grouping variable to fit a whole model with independent curves for each accession. The data demonstrated nonlinear relationships, so exponential growth and sigmoidal models were fitted to determine the best-fit model based on RMSE and Akaike information criterion (AIC). The relative biomass relationship was best described with an exponential 2P model and achieved an R^2 = 0.7994 (Equation 1)

$$y = a \times \text{Exp}(b \times rate) \quad (1)$$

where *y* is the relative biomass, *a* is the scale, *b* is the growth rate, and *rate* is the glyphosate rate in g ae ha^{-1}. A Weibull growth model achieved the best fit for visible injury and mortality with an R^2 = 0.8594 and 0.8976, respectively (Equation 2),

$$y = a \times (1 - \text{Exp}(-(\frac{rate}{b})^c) \quad (2)$$

where *y* is the relative visible injury or mortality, *a* is the asymptote, *b* is the inflection point, *c* is the growth rate, and *rate* is the glyphosate rate in g ae ha^{-1}. Inverse predictions were used to determine the rate required for growth reduction (GR), lethal dose (LD), and injury dose (ID) values at 50% by each accession. These values were then used to determine a resistance index (RI) to compare the fold difference between the two susceptible accessions and the putatively resistant accession. Furthermore, the predicted relative GR_{50}, LD_{50} and ID_{50} values of the resistant and susceptible accessions were also subjected to a t-test. The gene copy number and gene expression data were pooled over the experimental trial repetitions as they had similar results and were subjected to ANOVA using R program (version 4.3.1), followed by mean separation using a Tukey's honestly significant difference (HSD) test at $\alpha = 0.05$.

Results and Discussion

Glyphosate dose-response

In response to the glyphosate doses, the 5040 g ae ha^{-1} (4X) rate completely controlled the R1 accession, and the susceptible accessions (S1 and S2) were completely controlled at 630 g ha^{-1} (0.5X) rate (Figure 1). The dose-response analysis revealed that the amount of glyphosate required to reduce the aboveground biomass by 50% (GR_{50}) at 21 DAT was 1,038 g ha^{-1} for R1 compared with 148.2 and 145.5 g ha^{-1} for S1 and S2, respectively ($p < 0.0001$) (Figure 2A, Table

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2). The accession R1 was approximately seven times more resistant than the S1 and S2 accessions. The visible injury scoring on a scale of 0 to 100% was performed on all the accessions based on the amount of chlorosis and stunting of plants at 21 DAT before the aboveground biomass was collected. The amount of glyphosate required to cause 50% visible injury (ID_{50}) in R1 was 881.5 g ha^{-1}, which was 4.8 and 3.4 times higher than for S1 and S2 ($p < 0.0001$) (Figure 2B, Table 3). Similarly, the dose required for 50% plant mortality (LD_{50}) was 6 and 4.4 times higher in R1 compared with S1 and S2, respectively ($p < 0.0001$) (Figure 2C, Table 4). In a study representing the first glyphosate resistance in *P. annua* on a golf course in Columbia, MO, the resistant biotype CCMO1 needed 5.2-times more glyphosate than the susceptible biotype S1 to reduce the growth by 50% (Binkholder et al. 2011). The resistant population of *P. annua* found in an almond (*Prunus dulcis* Mill. D. A. Webb) field in California required 18 times more glyphosate than the susceptible standard (Brunharo et al. 2019) to reduce the growth by 50%. Glyphosate-resistant populations identified in Tennessee and South Carolina had a resistance index of 12 and 4.4, respectively (Brosnan et al. 2012; Cross et al. 2015). Several studies in grass weeds such as johnsongrass [*Sorghum halepense* (L.) Pers.], annual ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot], goosegrass [*Eleusine indica* (L.) Gaertn.], and *L. rigidum* showed that a resistance index ranging from 2- to 11-fold increase was common in glyphosate-resistant biotypes (Baerson et al. 2002; Powles et al. 1998; Pratley et al. 1999b; Vila-Aiub et al. 2007).

Relative *EPSPS* copy number, expression, and sequencing

The *EPSPS* copy number and expression analyses were performed using two primer sets EPSPS-1 and EPSPS-2 along with the two housekeeping gene primers, *CCR* and *PPAN* (Figure 3). Though the primers were designed against the mRNA sequence of *Lolium spp*. the primer sets worked well with *P. annua* genomic DNA and cDNA. The relative gene copy number of R1, when normalized with *CCR,* was 20 to 25 times higher than S1 and S2 with both EPSPS-1 and EPSPS-2 primer sets ($p < 0.001$) (Figure 3A). However, normalization with *PPAN* showed a 15 to 20-fold increase in the copy number of the *EPSPS* of R1 when compared with susceptible accessions ($p < 0.033$) (Figure 3B). These findings suggest that there is at least a 15-fold increase in gene copy number in the resistant accession. The *EPSPS* gene in R1 was also severely overexpressed compared to the S1 and S2 accessions similar to the elevated copy numbers

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observed (Figure 4). When normalized with CCR, about a 15-fold increase in the relative *EPSPS* expression was observed in R1 compared with S1 and S2 ($p < 0.001$) (Figure 4A). There was a 20-fold increase of expression in R1 when normalized with PPAN compared to S1 and S2 ($p < 0.004$) (Figure 4B). Overall, the data suggests that gene duplication has resulted in the overexpression of the gene, leading to resistance in R1.

The first case of glyphosate resistance due to gene duplication in the US was found in a Palmer amaranth (*Amaranthus palmeri* S. Watson) population from Georgia (Gaines et al. 2010). In this population, the plants had up to 160 more copies of *EPSPS* compared with the resistant, and every chromosome had *EPSPS*, as revealed by the fluorescence in situ hybridization (FISH) analysis. Moreover, *EPSPS* has also been reported to be present as extrachromosomal circular copies (eccDNAs) with several confirmations (Koo et al. 2018). Gene amplification leading to glyphosate resistance has been reported in other species such as kochia [*Bassia scoparia* (L.) A.J. Scott.], waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer], and *E. indica*, (Chen et al. 2015; Sammons and Gaines 2014). In all these examples, increased glyphosate resistance was attributed to high *EPSPS* copy number. The amplification of *EPSPS* has been one of the main glyphosate resistance mechanisms in weeds. However, gene duplication of respective genes leading to glufosinate resistance and acetyl-CoA carboxylase (ACCase)-inhibitor resistance has also been reported in *A. palmeri* and large crabgrass [*Digitaria sanguinalis* (L.) Scop.], respectively (Carvalho-Moore et al. 2022; Laforest et al. 2017). Elevated enzyme activity caused by gene amplification leading to xenobiotic resistance has also been observed in other species, such as fungi, bacteria, and insects (Brochet et al. 2008; Marichal et al. 1997; Puinean et al. 2010).

Glyphosate resistance in a *P. annua* population from South Carolina was attributed to a Pro106 to Ala mutation in the coding sequence of the target gene (Cross et al. 2015). However, the relative copy number of the resistant population was not determined in that case. Several SNP mutations at position 106 endowing glyphosate resistance in weeds have been reported (Sammons and Gains 2014). These mutations confer about 3- to 15-fold resistance compared with susceptible biotypes. A mutation in the first or second base of codon would result in the substitution of Pro106 to Ser, Ala, Thr, or Leu (Bostamam et al. 2012; Collavo and Sattin 2012; González-Torralva et al. 2012; Jasieniuk et al. 2008). The glyphosate-resistant population

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obtained from the almond field in California had a 7-fold increase in the copy number along with a missense mutation at coding position 106 of *EPSPS* (Brunharo et al. 2019). The current study suggested that the resistant accession had at least a 15-fold increase in copy number; however, no mutation was found at position 106 of *EPSPS* in R1 (Figure 5). Double mutations involving positions Thr102 and Pro106 have been reported to cause high levels of glyphosate resistance in weeds and transgenic crops (Funke et al. 2006; Yu et al. 2015). A triple amino acid substitution involving positions Thr102, Ala103, and Pro106 of *EPSPS* g, leading to glyphosate resistance, was also identified in smooth pigweed (*Amaranthus hybridus* L.) (Perotti et al. 2019). Therefore, the region encompassing *EPSPS* was selected for sequencing. The Sanger sequencing of the amplified PCR products showed no mutations in R1. The result was further validated by sequencing the amplicons that were cloned into vectors. As the gene expression positively associates with the relative *EPSPS* copy number, gene duplication can be considered the main mechanism for conferring glyphosate resistance in R1. Further studies, such as non-target site resistance mechanism analysis, including metabolism, absorption, and translocation of glyphosate in the resistant and susceptible accessions, should provide further insight into the glyphosate-resistant mechanisms in the R1 accession.

In conclusion, the results indicate that glyphosate resistance in the *P. annua* accession (R1) is likely due to the increased genomic copy number and elevated expression. Annual preplant glyphosate use in agricultural fields in Arkansas has caused selection pressure on *P. annua*, ultimately leading to the evolution of glyphosate resistance in the population. Gene duplication is a common adaptive mechanism observed in plants under selection (Jugulam et al. 2014; Perry et al. 2007). The copy number and gene expression will likely increase if the population is exposed to additional glyphosate treatments. Therefore, revising the herbicide strategies that have been a common practice in weed control is necessary. Herbicide resistance management programs must consider appropriate cultural, mechanical, and alternate herbicide options to reduce the spread of herbicide-resistant genes (Norsworthy et al. 2012). The present study exposes a new incidence of glyphosate resistance in *P. annua*, the first ever document from a field crop situation in the US for this species. The P. *annua* population will add to the growing list of glyphosate-resistant weeds already found in the US.

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Competing Interests

The authors declare none.

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Table 1. Primer pairs used to quantify the *EPSPS* copy number and expression by real-time polymerase chain reaction in *Poa annua* accessions.

Target gene	Primer name	Primer sequence
EPSPS	EPSPS-1[a]	F 5'- CTGATGGCTGCTCCTTTAGCTC-3'
		R 5'- CCCAGCTATCAGAATGCTCTGC-3'
EPSPS	EPSPS-2[b]	F 5'- TTGAGTTCCTTGCTGATGGC-3'
		R 5'- GCCAAAACGCTCCATCAATC-3'
CCR	CCR[a]	F 5'- GATGTCGAACCAGAAGCTCCA-3'
		R 5'- GCAGCTAGGGTTTCCTTGTCC-3'
PPAN	PPAN[b]	F 5'- CCGTCATTACTCCATCAAGCTC-3'
		R 5'- CCTAAGGTCTGGCACTTGATTG-3'

[a]The primer information was obtained from Salas et al. (2012).

[b]The primer information was obtained from Dr. González-Torralva, co-author of this study.

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Table 2. The regression parameters describing the biomass reduction of *Poa annua* accessions to glyphosate treatment under greenhouse conditions.

Accessions	Model parameters[a,b]		Biomass reduction[c,d]		
	Scale (a) (SE)	Growth rate (b) (SE)	GR_{50} (SE)	RI	
			g ae ha^{-1}	R/S	
S1	97.7 (9.2)	-0.004 (0.001)	148.2(26.6)	1	-
S2	117.5 (13.2)	-0.005 (0.001)	145.5(27.4)	-	1
R1	97.1 (8.4)	-0.0006 (0.0001)	1038(188.4)	7	7.1

[a]All model parameters were significant at $p < 0.0001$.

[b]Exponential 2P model (Equation 1; $R^2 = 0.7994$) and parameters determined using JMP pro 17 fit curve platform.

[c]GR_{50} is the dose required for 50% plant growth reduction.

[d]RI is the resistance index; ratio of R/S GR_{50} values, resistant/susceptible.

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Table 3. The regression parameters describing the visible injury of *Poa annua* accessions to glyphosate treatment under greenhouse conditions.

	Model parameters[a,b]			Visible injury[c,d]		
Accessions	Asymptote (a) (SE)	Inflection point (b) (SE)	Growth rate (c) (SE)	ID_{50} (SE)	RI	
				g ae ha^{-1}	(R/S)	
S1	99.0 (2.5)	216.9 (15.1)	2.0 (0.3)	183.2(14.9)	1	-
S2	96.6 (3.2)	302.0 (28.7)	1.7 (0.3)	252.1(28.4)	-	1
R1	96.9 (2.4)	1112.3 (101.9)	1.3 (0.2)	881.5(100)	4.8	3.4

[a]All model parameters were significant at $p < 0.0001$.

[b]Weibull growth model (Equation 2; $R^2 = 0.8594$) and parameters determined using JMP pro 17 fit curve platform.

[c]ID_{50} is the dose required for 50% visible injury.

[d]RI is the resistance index; ratio of R/S ID_{50} values, resistant/susceptible.

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Table 4. The regression parameters describing the mortality rate of *Poa annua* accessions to glyphosate treatment under greenhouse conditions.

	Model parameters[a,b]			Plant mortality[c,d]		
Accessions	Asymptote (a) (SE)	Inflection point (b) (SE)	Growth rate (c) (SE)	LD_{50} (SE)	RI	
				g ae ha^{-1}	(R/S)	
S1	99.2 (4.6)	278.7 (30.2)	2.2 (0.6)	238.2(29.9)	1	-
S2	98.5 (5.5)	344.4 (57.0)	4.9 (8.1)	321.3(56.5)	-	1
R1	96.0 (4.5)	1635 (215.1)	2.3 (0.7)	1437(212.8)	6	4.4

[a]All model parameters were significant at $p < 0.05$.

[b]Weibull growth model (Equation 2; $R^2 = 0.8976$) and parameters determined using JMP pro 17 fit curve platform.

[c]LD_{50} is the dose required for 50% plant mortality.

[d]RI is the resistance index; ratio of R/S LD_{50} values, resistant/susceptible.

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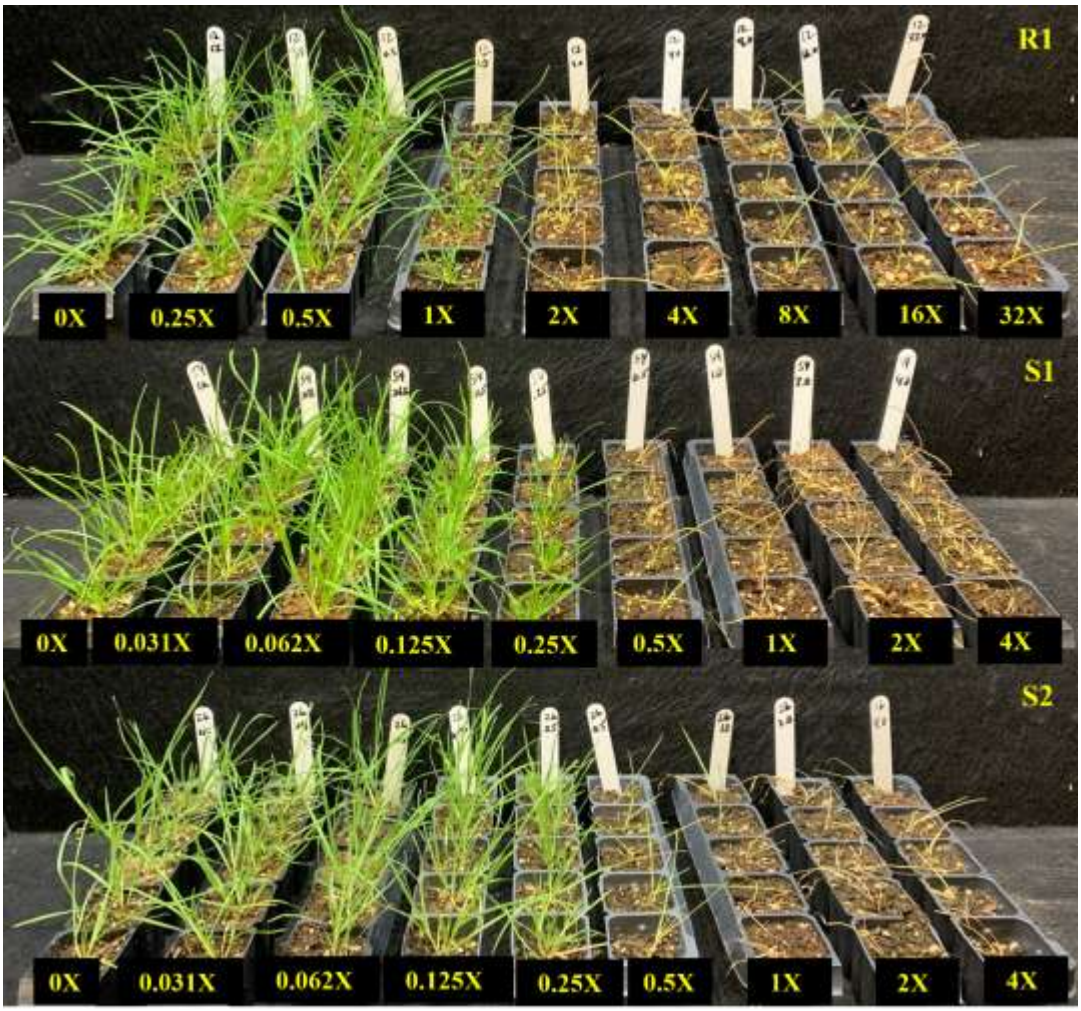

Figure 1. The response of *Poa annua* resistant accession (R1) to 0.25, 0.5, 1, 2, 4, 8, 16 and 32X (1X = 1260 g ae ha^{-1}) dose of glyphosate treatment and susceptible accessions (S1 and S2) to 0.031, 0.062, 0.125, 0.25, 0.5, 1, 2, and 4X dose at 14 days after treatment.

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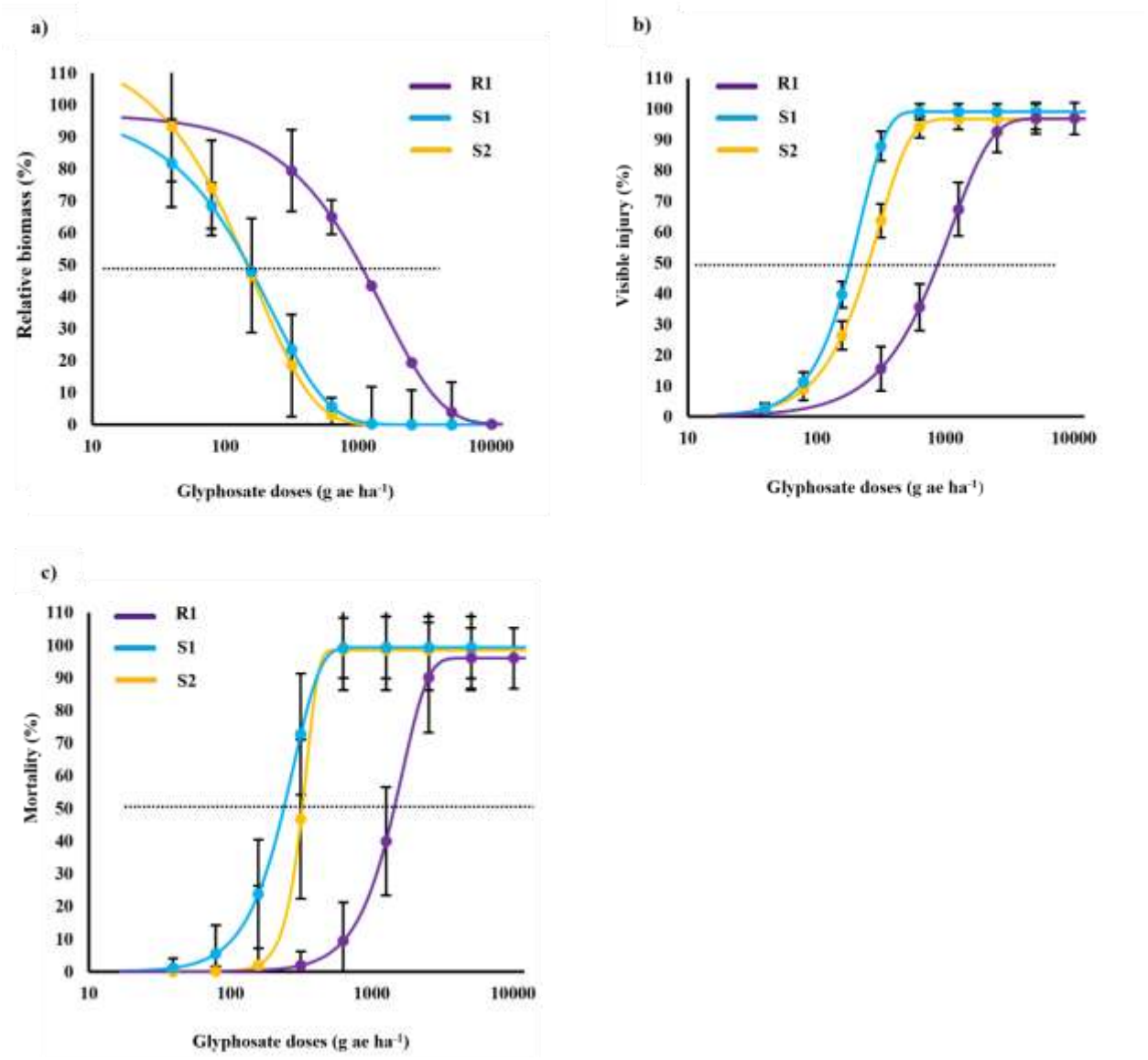

Figure 2. Dose–response curves describing the response of *Poa annua* accessions S1 (susceptible), S2 (susceptible), and R1 (resistant) to glyphosate treatment. (A) The relative biomass was analyzed using an exponential 2P model [Equation 1; $y = a \times \text{Exp}(b \times rate)$] at 21 days after treatment (DAT), (B) visible injury % and (C) mortality % was analyzed using a Weibull growth model [Equation 2; $y = a \times (1 - \text{Exp}(-(\frac{rate}{b})^c)$] at 21 DAT. Error bars represent standard error of the means.

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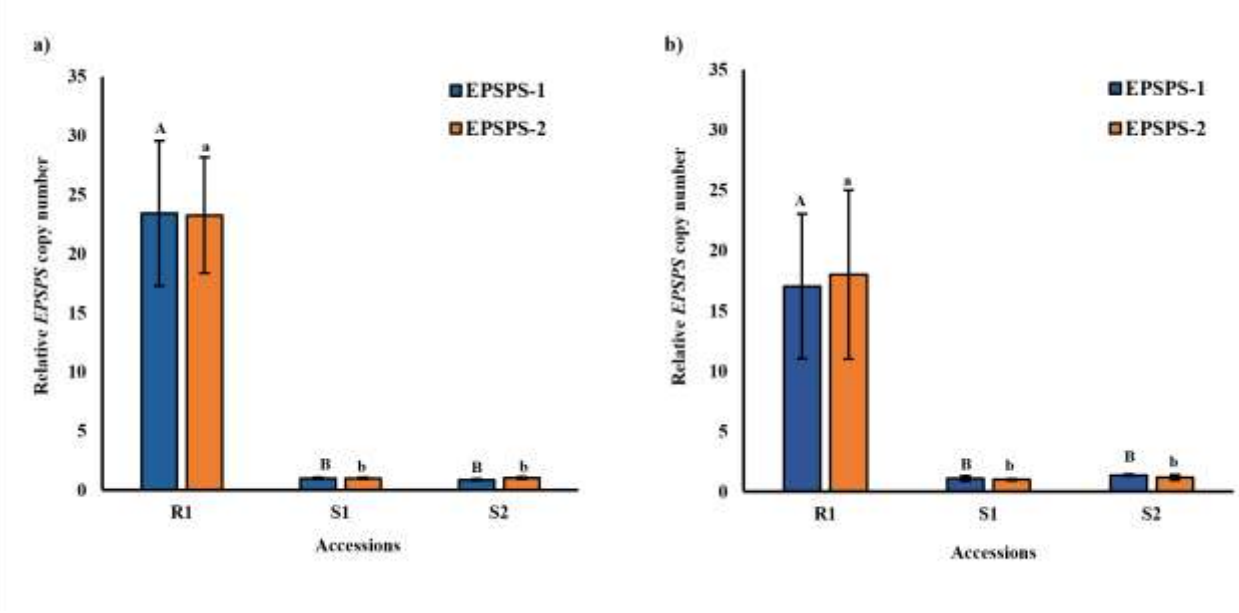

Figure 3. 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) copy number (A) relative to *Cinnamoyl-CoA reductase* (*CCR*) and (B) *peter Pan-like* (*PPAN*) reference genes in glyphosate resistant accession R1 and susceptible accessions S1 & S2. EPSPS-1 and EPSPS-2 represent the two sets of primers used. Error bars represent standard errors of the means (n = 7), and upper and lowercase letters represent differences identified by separation of means within a primer set using Tukey's HSD (α=5%).

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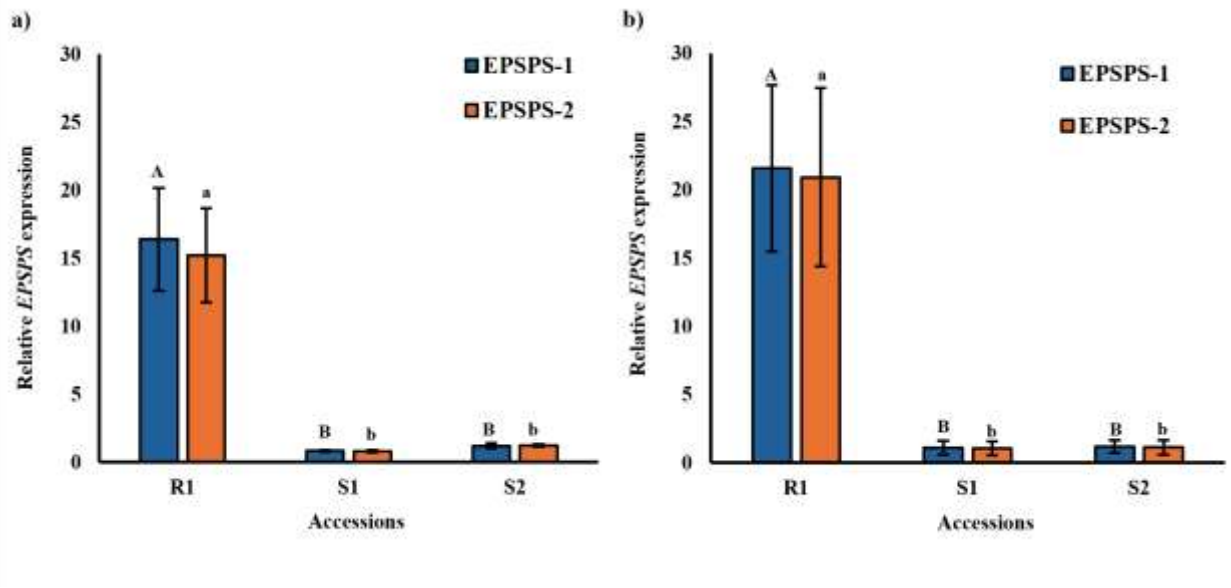

Figure 4. 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene expression (A) relative to Cinnamoyl-*CoA reductase (CCR)* and (B) *peter Pan-like (PPAN)* in glyphosate-resistant accession R1 and susceptible accessions S1 & S2. EPSPS-1 and EPSPS-2 represent the two sets of primers used. Error bars represent standard errors of the means (n = 7), and upper and lowercase letters represent differences identified by separation of means within a primer set using Tukey's HSD (α=5%).

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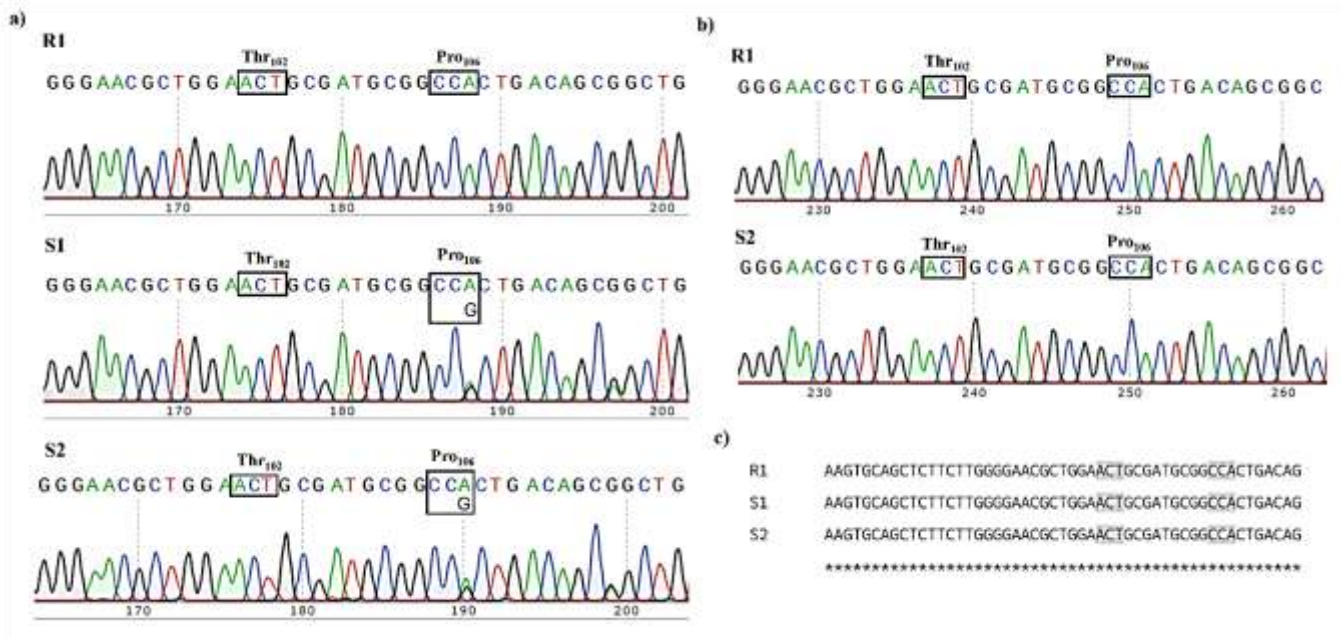

Figure 5. Sanger sequencing result of the nucleotide region surrounding the highly conserved Pro106 position in resistant (R1) and susceptible (S1 & S2) accessions. Chromatogram of (A) the PCR amplicons, (B) amplicons cloned into vectors and (C) nucleotide sequence showing no mutations at positions 102 and 106 of *EPSPS*.

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