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A novel EPSPS Pro-106-His mutation confers the first case of glyphosate resistance in *Digitaria sanguinalis*

Running title: EPSPS Pro-106-His mutation in glyphosate-resistant *D. sanguinalis*

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Abstract

BACKGROUND: *Digitaria sanguinalis* has been identified as a species at high risk of evolving herbicide resistance, but thus far, there are no records of resistance to glyphosate. This species is one of the most common weeds of summer crops in extensive cropping areas in Argentina. This weed shows an extended period of seedling emergence with several overlapping cohorts during spring and summer, and it is commonly controlled with glyphosate. However, a *D. sanguinalis* population was implicated as a putative glyphosate-resistant biotype based on poor control at recommended glyphosate doses.

RESULTS: The field-collected *D. sanguinalis* population (Dgs R) from the Rolling Pampas has evolved glyphosate resistance. Differences in plant survival and shikimate levels after field recommended and higher glyphosate doses were evident between Dgs R and the known susceptible (Dgs S) population, and the resistance index was 5.1. No evidence of differential glyphosate absorption, translocation, metabolism, or basal EPSPS activity was found between Dgs S and Dgs R populations; however, a novel EPSPS Pro-106-His point substitution is likely the primary glyphosate resistance endowing mechanism. EPSPS in vitro enzymatic activity demonstrated that an 80-fold higher concentration of glyphosate is required in Dgs R to achieve similar EPSPS activity inhibition as in the Dgs S population.

CONCLUSION: This study reports the first global case of glyphosate resistance in *D. sanguinalis*. This yet novel transversion at the second position of the EPSPS 106 codon demonstrates the intensity of glyphosate pressure in selecting unexpected glyphosate resistance alleles if they retain EPSPS functionality.

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1. Introduction

Digitaria sanguinalis (L.) Scop. is a grass species native to the Mediterranean region and southwest Asia, but it was introduced and naturalised in tropical and temperate regions throughout the rest of the world.[1,2,3] It is an annual, C4, and facultative self-pollinated species that propagates by seeds.[4,5] In extensive cropping areas from Argentina, *D. sanguinalis* is one of the most common weeds of soybean, sunflower, maize, and sorghum crops and is similarly well adapted to both cultivated and no-tillage systems.[2,6,7] This species shows an extended period of seedling emergence with several overlapping cohorts during spring and summer and a high vegetative growth rate and fecundity.[8,9]

Chemical control of *D. sanguinalis* with glyphosate, ACCase, ALS, or VLCFA herbicides has been challenged due to the long emergence period, which enables seedling cohorts to escape from herbicide action.[9] Based on poor control at recommended glyphosate doses, a *D. sanguinalis* population was identified as a putative glyphosate-resistant biotype. This population was found to interfere with a transgenic glyphosate-resistant soybean crop in the main cropping area of the Rolling Pampas (north Buenos Aires province, Argentina). It is well known that herbicide resistance is an evolutionary process, and considering the high constancy and density of *D. sanguinalis* populations in Argentinean croplands, it is predicted that this arises as a new case of herbicide resistance.

Herbicide-resistant *D. sanguinalis* populations have been identified in Australia, Canada, China, the Czech Republic, New Zealand, and the USA, where resistance to atrazine-, ACCase-, or ALS-inhibiting herbicides has been reported, although no glyphosate-resistant populations have been documented thus far.[10,11,12,13,14,15] *Digitaria sanguinalis* has recently been pointed out as a species at high risk of evolving herbicide resistance.[16,17]

Herbicide resistance can be endowed by nontarget site mechanisms related to herbicide detoxification or exclusion processes that minimise the amount of active ingredient reaching the target enzyme or target-site mechanisms, resulting in overexpression of the target enzyme or point mutations in the herbicide target enzyme and leading to changes in the herbicide–target physical interaction.[18] Elucidating the mechanism(s) responsible for evolved herbicide resistance helps to design management strategies of resistance evolution.[19]

The aims of this study were to (1) determine the level of glyphosate resistance of a putative glyphosate-resistant *D. sanguinalis* and (2) identify the glyphosate resistance mechanisms involved.

2. Materials and methods

2.1 Plant material

In March 2019, seeds of 50 surviving *D. sanguinalis* individuals (Dgs R) were collected from a soybean field (35.15°S, 61.30°W; Lincoln district, Buenos Aires province) where glyphosate doses up to 1960 g ae ha^{-1} were applied. In the last three years, crop rotation in the sampled area involved wheat–soybean and maize–soybean rotations, where weed management has been mainly based on glyphosate treatment in fallow fields and soybean and maize crops. Seeds of a known glyphosate-susceptible *D. sanguinalis* (Dgs S) were collected from a population established as a weed in a pasture (38.19°S, 60.15°W). Inflorescences of 50 susceptible plants were collected at random in April 2019.

Seeds of both populations (Dgs R and Dgs S) were stored at room temperature and four weeks before experiments were sown in Petri dishes imbibed on moistened filter paper and incubated in the dark at 5°C for 21 days. Seed dormancy release was performed following Oreja et al.[20] Seeds were then incubated for five days in a growth chamber at fluctuating temperatures (30/20°C day/night) under an 8 h photoperiod (light (400-700 ηm) intensity of 75 µmol m^{-2} s^{-1}).

Glyphosate dose-response experiments and shikimic acid assays were conducted at Chacra Experimental Integrada Barrow (INTA-Argentina) (38.19°S, 60.14°W). Germinated seeds were transferred to 1000 cm^3 pots filled with organic substrate (soil:peat moss (1:1)) (four plants per pot) and placed in a glasshouse at 25°C (average temperature) during the spring and summer seasons.

Experiments to assess leaf spray solution retention, ^{14}C-glyphosate leaf absorption, translocation, metabolism, and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity were conducted at the Department of Agricultural Chemistry and Edaphology, University of Cordoba (Spain). For these particular experiments, Dgs R and Dgs S seedlings were transplanted into 250 cm^3 pots (one plant per pot) filled with the same substrate as above and grown in a growth chamber at fluctuating temperature (26/18°C day/night), constant relative

humidity (60%) and 12 h photoperiod (light intensity of 300 µmol m^{-2} s^{-1}). In all cases, the plants were irrigated daily.

2.2 Glyphosate dose-response assays

Plant survival of Dgs R and Dgs S in response to increasing glyphosate doses was evaluated in two repeated dose-response experiments. Glyphosate (60.8% dimethyl amine salt of N-phosphonomethyl glycine; Panzer® Gold, Argentina) was sprayed to plants at the 2–3 tiller stage at doses of 0, 240, 480, 960, 1920, and 3840 g ae ha^{-1}. Pots containing four Dgs R or Dgs S plants were used in a completely randomised design with 10 replicates per glyphosate dose. Glyphosate was applied using a laboratory belt sprayer (application rate was 200 L ha^{-1}), as described by Yanniccari et al.[21]

Twenty-one days after glyphosate treatment, plant survival was recorded, where "surviving" plants showed active growth with no apparent visual injury as opposed to "dead" plants that exhibited damaged and necrotic leaves.

2.3 Shikimic acid concentration in leaves

The difference in shikimate accumulation in glyphosate-resistant and -susceptible plants is an indicator of glyphosate sensitivity.[22] Leaf shikimate levels were estimated and compared between the Dgs S and Dgs R plants after treatment with increasing glyphosate doses. Plants at the 2–3 tiller stage were grown as described above, and quantification of shikimate was conducted 72 hours after treatment (HAT) with glyphosate at 0, 240, 480, 960, 1920, and 3840 g ae ha^{-1}. A completely randomised design with five replicates (each pot was a sampling unit) per treatment was used. A sample (50 mg of fresh biomass weight) of the youngest fully expanded leaf of one plant taken at random from each pot was analysed. Shikimic acid was quantified by a spectrophotometric method at 382 nm employing Numak 752 UV-Vis equipment following the methodology described by Perez-Jones et al.[23] A standard curve was constructed with the shikimic acid standard (3a,4a,5b-Trihydroxy-1-cyclohexene-1-carboxylic acid, 99 %. Sigma-Aldrich, Inc., USA). Shikimate concentrations in leaf tissues were quantified against the standard curve. The experiment was conducted twice.

2.4 Spray solution interception and retention

A mixture of glyphosate (360 g ae ha^{-1}) plus Na-fluorescein (100 mg L^{-1}) was applied to six plants (3 leaf stages) of each Dgs S and Dgs R population using the treatment equipment described in Vazquez-Garcia et al.[24] After two hours of glyphosate treatment, the plants were

cut and transferred to test tubes containing 50 mL of 5 mM NaOH. The samples were shaken for 30 s and the washed solution was used to determine fluorescein absorbance using a spectrofluorometer (Hitachi F-2500, Tokyo, Japan) involving 490 and 510 nm of excitation and absorbance wavelength, respectively. Finally, plants were weighed after drying for 48 h at 60°C and data were expressed in µL of sprayed solution per gram of dry matter. A completely randomised design was used with two repetitions; each repetition consisted of six plants per population.

2.5 ^{14}C-glyphosate absorption, translocation and visualisation

^{14}C-glyphosate plus commercial glyphosate solution was applied to Dgs S and Dgs R plants at the 3-leaf stage to compare herbicide leaf absorption and patterns of translocation. The youngest expanded leaf was marked and covered with aluminium foil before spraying the whole plant with glyphosate at 360 g ae ha^{-1} in an herbicide treatment cabinet with an output volume of 200 L ha^{-1}. Thirty minutes later, the aluminium foil was removed. ^{14}C-glyphosate (95% glycine-2-^{14}C supplied by the Institute of Isotopes Co., Ltd.; Budapest, Hungary) solution at a specific activity of 50,000 dpm µL^{-1} was used to treat five plants per population. One microliter of the solution was applied to the adaxial surface of the mentioned leaf. The plants were then maintained in a growth chamber at 26/18°C day/night with a 14 h photoperiod, 850 µmol^{-2} s^{-1} PAR, and 60% relative humidity. At 96 HAT, the non-absorbed ^{14}C-glyphosate was removed by washing the treated leaves three times with 1 mL of a water–acetone solution (1:1 v/v) each time. Subsequently, the plants were sectioned into treated leaves, the remainder of the shoot, and roots (this organ was carefully washed with distilled water, and dried with a paper towel). The plant samples were stored in cellulose cones (Perkin-Elmer, BV BioScience Packard), dried at 60°C for 96 h, and combusted in a biological oxidiser (Packard Tri Carb 307, Packard Instrument Co., Downers Grove, IL, USA). The CO_2 released from combustion was captured in 18 mL of a mix of Carbo-Sorb E and Permafluor [1:1 (v/v] (Perkin-Elmer, BV BioScience Packard). The radioactivity of each individual sample and washing solution was quantified using a scintillation counter model (LS 6500, Beckman Coulter Inc., Fullerton, CA, USA) with 10 min reading time per sample. The percentages of ^{14}C-glyphosate recovered, absorbed, and translocated to each plant section were calculated using the radioactive values at dpm following Vázquez-García et al.[24] The equipment efficiency correction factor was 90%.

The translocation of ^{14}C-glyphosate was visualised in five plants of the Dgs S and Dgs R populations. These plants were treated with the herbicide as described above and, at 96 HAT, were washed individually, fixed on filter paper, and dried at room temperature for 7 d. Finally,

the plants were pressed for 4 h under a phosphor store film and visualised using a phosphor imager (Cyclone, Perkin-Elmer, Packard BioScience BV, MA, USA).

2.6 Glyphosate metabolism

Glyphosate metabolism was measured on Dgs R and Dgs S plants. The herbicide was applied at the 3–4 leaf stage plants at a dose of 360 g ae ha^{-1} and non-treated plants were used as a control. The plants were washed with distilled water at 96 HAT and flash frozen in liquid nitrogen and stored at −40°C before being used. Glyphosate and its metabolites (amino methyl phosphonic acid (AMPA), glyoxylate, formaldehyde, and sarcosine) were determined via reversed polarity capillary electrophoresis.[25] A 3D Capillary Electrophoresis Agilent G1600A instrument (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a diode array detector (DAD, wavelength range 190–600 nm) was employed. The background electrolyte was composed of acetonitrile (10%), phthalate (7.5 mM), and hexadecyltrimethylammonium bromide (0.75 mM), and the voltage was –20 kV. The concentrations of glyphosate and metabolites were determined using standard equations.[26] A completely randomised design was employed, and three technical replications were analysed. The experiment was conducted twice.

2.7 EPSPS activity

In plants from both Dgs R and Dgs S populations, the basal EPSPS activity was compared, and the specific EPSPS activity was assayed at different concentrations of glyphosate (>99%; Sigma-Aldrich, Madrid, Spain). Five grams of young foliar tissue were taken from Dgs R and Dgs S plants and ground in liquid nitrogen using a mortar. Enzyme extraction was carried out following the methodology described by Yanniccari et al.[21] The specific EPSPS activity was measured in a reaction medium containing glyphosate at 0, 0.1, 1, 10, 100, and 1000 µM. The EPSPS enzyme reaction substrates were phosphoenolpyruvate and shikimate-3-phosphate (Sigma-Aldrich, Madrid, Spain). The EnzChek Phosphate Assay Kit (Invitrogen, Carlsbad, CA, USA) was used and the release of phosphate was measured spectrophotometrically (Beckman DU-640; Beckman Instruments Inc., Fullerton, USA) at 360 nm for 10 min. A kit for protein determination (Sigma-Aldrich, Madrid, Spain) was used to quantify the total soluble protein (TSP) in the extract. Finally, EPSPS activity was measured as µmol of inorganic phosphate released per µg of TSP per min and it was expressed as a percentage relative to the control without glyphosate.[25] A completely randomised design was used, and five technical replicates of each glyphosate treatment were analysed per population. The experiment was conducted twice.

2.8 *EPSPS* gene sequencing

Five Dgs R plants that survived a glyphosate dose of 960 g ae ha^{-1} and five glyphosate-untreated Dgs S plants were selected. The total DNA was obtained from the leaf tissue following the methodology described by Doyle and Doyle.[27] DNA quantification and quality control were spectrophotometrically determined.

The forward primer (5'-AGCTGTAGTCGTTGGCTGTG-3') and reverse primer (5'-CCCAGCTATCAGAATGCTCTGC-3') were used to amplify a conserved region encompassing 101, 102, 106, 144, and 192 codons (EPSPS numbering system used by Padgette et al.[28]) that involve low glyphosate sensitivity (Sammons & Gaines, 2014). The PCR reaction consisted of initial denaturation at 94°C for 2 min and 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 5 min. The reaction mix included 300 ng DNA template, 1X reaction buffer (Inbio Highway), 1.5 mM $MgCl_2$, 0.8 mM of dNTPs, 0.4 µM of each primer, and 1 U Taq polymerase (Inbio Highway) in a 25 µL reaction mix. An 828-bp fragment was obtained as a single PCR product. The amplicons were purified and sequenced from both ends by Macrogen Inc. (Seoul, Korea). The obtained sequence data were cleaned, aligned, and compared using Chromas v.2.6.4 (Technelysium Pty Ltd, South Brisbane, Australia) and Bioedit v.7.2 (North Carolina State University, Raleigh, USA).

2.9 *EPSPS* gene relative copy

Genomic DNA obtained from Dgs S and Dgr R plants (n = 5) was employed as a template for qPCR reactions using a Real-Time PCR System (Applied Biosystems QuantStudio3) and FastStart Universal SYBR Green Master (Roche). The reaction mix (25 ul of final volume) included a 125 ng DNA template and was performed following the methodology described by Yanniccari et al.[29] A 246-bp fragment of the constitutive gene *18S rRNA* was used for normalisation (forward primer: 5'-TGCAACAAACCCCGACTTCT-3' and reverse primer: 5'-CCTTGGATGTGGTAGCCGTT-3'). Primers specific to *EPSPS* were employed (forward: 5'-CTTGAGTTCCTTGCTGATG-3' and reverse: 5'-GTACTTCTGTCCTCCTTTAATG-3') and the following programme was used: one cycle at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Negative controls consisting of primers with no templates were included. Primer efficiency was 92.35% for *EPSPS* (R^2=0.99) and 90.91% for *18S rRNA* (R^2=0.98). Each measurement was performed in triplicate.

2.10 Statistical analysis

A nonlinear log-logistic regression model was fitted on plant survival and EPSPS activity data over increasing glyphosate doses:[30]

$$y= c+\{(d-c)/[(1+(x/I_{50})^b]\}$$

where *y* is the variable response at the glyphosate rate *x*; *c* and *d* are the lower and upper asymptote, respectively; *b* is the slope of the line at I_{50}; and I_{50} or is the glyphosate concentration required to reduce the maximum response for EPSPS enzyme activity (I_{50}) to 50% or the herbicide dose causing 50% mortality (LD_{50}). F-tests were performed to assess the accuracy of the models, and residual variance analysis and the coefficient of determination (R^2) were calculated. Finally, I_{50} and LD_{50} parameters calculated on the Dgs R and Dgs S models were compared with the F-test ($P < 0.05$) (GraphPad Prism 6 Software, San Diego, USA), and a resistance index (RI) was determined as the LD_{50} of the Dgs R population divided by LD_{50} of the Dgs S population.

Analysis of variance (ANOVA) was performed to evaluate the effect of Dgs R and Dgs S populations on glyphosate leaf retention, uptake, translocation, and metabolism, EPSPS activity, and *EPSPS* gene relative copy number. Differences in the mean values of shikimic acid concentration and glyphosate spray retention between Dgs R and Dgs S were compared with Fisher's test ($P < 0.05$) (Statistica® v7.1. Stat Soft, Córdoba, Argentina).

3. Results

3.1 Differential plant survival and shikimate accumulation in the Dgs S and Dgs R populations

Around 85% of Dgs R plants survived the recommended field dose of glyphosate (960 g ae ha^{-1}), while all Dgs S individuals were controlled at this glyphosate dose (Figure 1). Dose-response models fitted to plant survival of the Dgs R and Dgs S populations were compared, and LD_{50} parameters differed significantly between populations ($P < 0.01$). This parameter calculated on Dgs S and Dgs R plants was 393.7 and 2010.0 g ae ha^{-1}, respectively, and the estimated RI was 5.1. The LD_{50} associated with Dgs R was approximately two fold the recommended dose of glyphosate.

The levels of shikimate accumulation in response to glyphosate were significantly different between the Dgs S and Dgs R populations ($P < 0.01$) (Figure 2). The basal shikimate content was similar for both Dgs S and Dgs R plants (i.e., without glyphosate treatment, both populations showed the same shikimate level in leaves). At the lowest glyphosate dose, the

Dgs S plants accumulated 8.0-fold more shikimate than the control without herbicide, and the shikimate content increased 15.0-fold when this population was treated at glyphosate doses ≥480 g ae ha^{-1}. In contrast, the Dgs R plants showed no significant changes in shikimate concentration with respect to the basal level in response to increasing glyphosate doses ≤960 g ae ha^{-1}. At 1920 or 3840 g ae ha^{-1} glyphosate doses, the shikimate content of Dgr R plants increased 5.8-fold compared to their controls (Figure 2).

3.2 Spray solution interception, glyphosate absorption and translocation

The spray solution containing glyphosate showed different levels of leaf retention between the Dgs R and Dgs S populations. Plants from Dgs R retained less spray solution in the leaves compared to Dgs S plants (544 versus 596.78 µL g^{-1} of dry weight, respectively; Figure 3).

At 96 HAT, both Dgs S and Dgs R populations absorbed 92.1% ^{14}C-glyphosate, and no significant differences were detected between them (Figure 4). The ^{14}C-glyphosate translocation pattern was similar in Dgs S and Dgs R plants; around 43% of ^{14}C-glyphosate absorbed was found in the treated leaf, whereas the remaining glyphosate accumulated was detected in the shoots (34% of ^{14}C-glyphosate absorbed) and roots (23% of ^{14}C-glyphosate absorbed) (Figure 4). The Phosphor Imager images confirmed these results, which suggests that no differences in ^{14}C-glyphosate translocation were evident between Dgs S and Dgs R (Figure 5).

3.3 No major glyphosate metabolism

No evidence of differential glyphosate metabolism was found between the Dgs S and Dgs R populations. At 96 HAT, only glyoxylate was detected in plants, but at a level lower than 5%. Given that the AMPA metabolite was not detected in glyphosate-treated plants, the estimated glyoxylate concentration is unlikely to be associated with glyphosate metabolism. Glyphosate recovery was higher than 95%, regardless of the population analysed (Table 1).

3.4 Higher EPSPS activity associated with Dgs R plants

In the absence of glyphosate treatment, EPSPS activity was similar between the Dgs R and Dgs S populations, at 0.044 and 0.042 µmol Pi μg^{-1} TSP min^{-1}, respectively (Figure 6). Regression models, fitted to EPSPS activity in response to glyphosate, estimated that the concentration of the herbicide required to inhibit EPSPS activity by 50% (I_{50}) differed significantly between populations ($P < 0.001$). The I_{50} calculated for the Dgs R and Dgs S populations was 47.97 and

0.60 µM, respectively, which is an 80-fold higher concentration of glyphosate required in Dgs R to achieve similar EPSPS activity inhibition as in the Dgs S population (Figure 6).

3.5 A target-site *EPSPS* gene mutation is identified in Dgs R plants

A DNA fragment of the *EPSPS* gene was sequenced (OM311258 and OM311259), and potential target-site glyphosate resistance mechanisms were explored. A transversion mutation from cytosine to adenine was detected, indicating a Pro-106-His substitution in the glyphosate EPSPS target. The resistant plants were homozygous for the Pro-106-His allele (Figure 7). No amino acid changes were identified at Gly-101, Thr-102, Gly-144, or Ala-192 residues, which have also been shown to endow glyphosate resistance in plants. No differences in *EPSPS* gene relative copy were detected between the Dgs S and Dgs R plants (Figure 8).

4. Discussion

The present study aimed to comprehensively characterise the level of glyphosate resistance at the molecular (*EPSPS* target site mutations and *EPSPS* gene amplification), physiological (shikimic acid content, glyphosate leaf uptake, translocation, and metabolism), enzymatic (EPSPS activity), and whole plant (survival) levels in a *D. sanguinalis* population recurrently exposed for several years to glyphosate selection in extensive cropping conditions. The results show that the field-collected *D. sanguinalis* population from the Rolling Pampas has evolved glyphosate resistance. Differences in plant survival and shikimate levels after field recommended and higher glyphosate doses were evident between Dgs R and the known susceptible (Dgs S) population. This finding adds a novel species to the ever-increasing list of weeds evolving resistance to glyphosate in global agriculture.

4.1 A novel target site EPSPS Pro-106-His mutation endows glyphosate resistance in *D. sanguinalis*

Various EPSPS target site point mutations have been shown to endow low to moderate glyphosate resistance levels in several weed species.[18,31,32] In particular, amino acid substitutions at Pro-106 in the *EPSPS* gene generally confer 2- to 7-fold glyphosate resistance in many weed species.[18,33] This is likely due to resistance mutations at Pro-106 residue located outside the EPSPS active site and not directly involved in glyphosate binding and therefore leading to slight structural alterations in the glyphosate/PEP binding site.[34,35]

Resistance mutations in Pro-106 have been frequently associated with substitutions to Ser, Ala, or Thr through changes at the first base of the 106 codon, but cases of *EPSPS* mutations at the second base of the codon are infrequent or unknown.[18] For several glyphosate-resistant weeds, it has been shown that a transition mutation at the second base of the 106 codon produces Leu,[36,37,38,39] but transversion mutations at this position (i.e., Pro-106-Arg, -Gln, or -His) have not been reported to date.[40] Proline is a non-polar amino acid, and substitution by a polar residue, such as Thr and Ser, is the most common in target-site glyphosate-resistant weeds.[41]

The results presented here show that a novel EPSPS Pro-106-His mutation endows glyphosate resistance in the Dgs R population. The Pro-106-His substitution involves the modification of a non-polar amino acid by a positively charged one, and it could be disruptive to the active site.[18] Interestingly, this is the first report of a transversion mutation at the second base of the Pro-106 codon involving a novel Pro-106-His substitution in a weed. This *EPSPS* target-site mutation implies no differences in EPSPS basal activity, and it is associated with an 80- and 5-fold higher resistance at the EPSPS enzyme and plant levels, respectively. The 80-fold resistance index at the EPSPS enzyme level endowed by this novel Pro-106-His mutation is higher than those associated with other glyphosate resistance *EPSPS* mutations at Pro-106, such as 106-Leu and 106-Ser, documented in plants (reviewed in Vila-Aiub et al.[43]). Despite the low affinity of EPSPS Pro-106-His variant for glyphosate, the difference between RI calculated on EPSPS activity, and whole-plant assays, suggest that other factors such as EPSPS expression in S and R Dgs plants in response to glyphosate treatment, the minimum level of EPSPS inhibition that triggers toxic effects and other metabolic deregulations, can be involved in the reduced glyphosate sensitivity of resistant plants.[37,42] The EPSPS Pro-106-His mutation was found at the homozygous state in R Dgs plants, which is expected in self-pollinated species as *D. sanguinalis*. However, the inheritance pattern of this nuclear gene in this tetraploid species would be elucidated in future studies.

Considering that glyphosate is the main agronomic tool used to control *D. sanguinalis* under no-tillage systems in glyphosate-tolerant soybean and corn crops,[8] glyphosate resistance evolution in *D. sanguinalis* emerges as a challenge for farmers in the Rolling Pampas agricultural region. Nevertheless, other herbicides have been shown to be effective in controlling Dgs R plants in different crops, as no evidence of multiple herbicide resistance was found (data not shown).

Other target-site resistance mechanisms, such as *EPSPS* gene amplification (duplication) or *EPSPS* gene overexpression, have been reported to endow glyphosate resistance in several weed species.[18,43] However, the results of the present study reveal that *EPSPS* gene expression, estimated as EPSPS specific activity, and gene amplification do not contribute to glyphosate resistance in the Dgs R population (Figure 8).

4.2 Non-target site resistance mechanisms do not contribute to glyphosate resistance in Dgs R

Among the nontarget site glyphosate resistance mechanisms studied in the Dgs R population, a minor but significant 8% lower foliar retention was recorded when compared to Dgs S. The lower spray retention, however, is unlikely to account for the low level of shikimate or high plant survival exhibited by Dgs R plants when treated with 2–4 times higher glyphosate field doses. Thus, the differential glyphosate spray retention found in Dgs R plants is likely associated with variations in leaf surface or plant architecture that may contribute to an increase in the variability in the amount of glyphosate spray solution interception and retention.[44]

Reduced glyphosate translocation has often been reported as a mechanism for endowing glyphosate resistance in plants.[43,45,46] In addition to impaired glyphosate translocation, reduced glyphosate foliar uptake has also been observed in some weeds.[46,47,48] Until the recent identification of the specific *EcAKR4-1* gene endowing enhanced glyphosate metabolism in *Echinochloa colona*,[49] glyphosate metabolism in field-evolved glyphosate-resistant weeds has been rarely documented (but see de Carvalho et al.[25] and Gonzalez-Torralva et al.[50]). Two glyphosate-resistant *D. insularis* populations from Brazil have shown a large amount of glyphosate metabolites (AMPA, glyoxylate, sarcosine) produced 48 h after glyphosate treatment.[26] In contrast to this evidence, results from the present study showed that ^{14}C-glyphosate foliar uptake and long-distance translocation and enhanced glyphosate metabolism are not responsible for glyphosate resistance in the Dgs R population.

5. Conclusion

This study reports the first global case of glyphosate resistance in *D. sanguinalis*, where a novel EPSPS Pro-106-His point substitution is likely the primary glyphosate endowing mechanism. This unlikely yet novel transversion at the second position of the EPSPS 106 codon demonstrates the intensity of glyphosate pressure in selecting unexpected glyphosate resistance alleles if they retain EPSPS functionality. The effects of the reported target-site

EPSPS glyphosate resistance Pro-106-His mutation on EPSPS catalytic activity, the inheritance pattern and adaptive fitness of Dgs R plants should be addressed in future studies.

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References

1 Mitich L, 1988. Crabgrass. *Weed Tech* **2**:114-115.

2 de la Fuente EB, Suárez SA and Ghersa CM, Soybean weed community composition and richness between 1995 and 2003 in the Rolling Pampas (Argentina). *Agric Ecosyst Environ* **115**:229-236 (2006).

3 Kim T, Neal JC, Ditomaso JM and Rossi FS, A survey of weed scientists' perceptions on the significance of Crabgrasses (*Digitaria* spp.) in the United States. *Weed Tech* **16**:239-242 (2002).

4 Fuentes N, Pauchard A, Sánchez P, Esquivel J and Marticorena A, A new comprehensive database of alien plant species in Chile based on herbarium records. *Biol Invasions* **15**:847-858 (2013).

5 Jones EAL, Contreras DJ and Everman WJ, *Digitaria ciliaris*, *Digitaria ischaemum*, and *Digitaria sanguinalis*, in Biology and Management of Problematic Crop Weed Species, ed. by Chauhan BS, Academic Press, pp. 173-195 (2021).

6 Puricelli E and Tuesca D, Weed density and diversity under glyphosate-resistant crop sequences. *Crop Prot* **24**:533-542 (2005).

7 Oreja FH, Batlla D and de la Fuente EB, Effect of soybean crop structure on large crabgrass (*Digitaria sanguinalis*) growth and seed dormancy. Weed Sci **69**:372-378 (2021).

8 Oreja, FH, Bastida F and Gonzalez-Andujar JL, Simulation of control strategies for decision-making regarding *Digitaria sanguinalis* in glyphosate-resistant soybeans. *Cienc Inv Agr* **39**:299-308 (2012).

9 Oreja FH, Batlla D and de la Fuente EB, *Digitaria sanguinalis* seed dormancy release and seedling emergence are affected by crop canopy and stubble. *Weed Res* **60**:111-120 (2020).

10 Buddenhagen CE, James TK, Ngow Z, Hackell DL, Rolston MP, Chynoweth RJ, Gunnarsson M, Li F, Harrington KC and Ghanizadeh H, Resistance to post-emergent herbicides is becoming common for grass weeds on New Zealand wheat and barley farms. *PLoS One* **16**:e0258685 (2021).

11 Hidayat I and Preston C, Cross-resistance to imazethapyr in a fluazifop-P-butyl-resistant population of *Digitaria sanguinalis*. *Pestic Biochem Phys* **71**:190-195 (2001).

12 Laforest M, Soufiane B, Simard MJ, Obeid K, Page E and Nurse RE, Acetyl-CoA carboxylase overexpression in herbicide-resistant large crabgrass (*Digitaria sanguinalis*). *Pest Manag Sci* **73**:2227-2235 (2017).

13 Mei Y, Si C, Liu M, Qiu L and Zheng M, Investigation of resistance levels and mechanisms to nicosulfuron conferred by non-target-site mechanisms in large crabgrass (*Digitaria sanguinalis* L.) from China. *Pestic Biochem Physiol* **141**:84-89 (2017).

14 Volenberg D and Stoltenberg D, Altered acetyl-coenzyme A carboxylase confers resistance to clethodim, fluazifop and sethoxydim in *Setaria faberi* and *Digitaria sanguinalis*. *Weed Res* **42**:342-350 (2002).

15 Heap I, The International Survey of Herbicide Resistant Weeds. http://www.weedscience.org [accessed 10 January 2022].

16 Ngow Z, Chynoweth RJ, Gunnarsson M, Rolston P and Buddenhagen CE. A herbicide resistance risk assessment for weeds in wheat and barley crops in New Zealand. *PLoS One* **15**:e0234771 (2020).

17 Ngow Z, James TK and Buddenhagen CE, A herbicide resistance risk assessment for weeds in maize in New Zealand. *N Z Plant Prot* **74**:78-86 (2021).

18 Sammons RD and Gaines TA, Glyphosate resistance: state of knowledge. *Pest Manag Sci* **70**:1367-1377 (2014).

19 Powles SB and Yu Q, Evolution in action: plants resistant to herbicides. *Annu Rev Plant Biol* **61**:317-347 (2010).

20 Oreja FH, de la Fuente EB and Batlla D, Role of seed environment and covering structures on large crabgrass germination. *S Afr J Bot* **111**:170-175 (2017).

21 Yanniccari M, Vázquez-García JG, Gómez-Lobato ME, Rojano-Delgado AM, Alves PL and De Prado R, First case of glyphosate resistance in *Bromus catharticus* Vahl.: examination of endowing resistance mechanisms. *Front Plant Sci* **12**:617945 (2021).

22 Singh BK and Shaner DL, Rapid determination of glyphosate injury to plants and identification of glyphosate-resistant plants. *Weed Technol* **12**:527-530 (1998).

23 Perez-Jones A, Park KW, Polge N, Colquhoun J and Mallory-Smith CA, Investigating the mechanisms of glyphosate resistance in *Lolium multiflorum*. *Planta* **226**:395-404 (2007).

24 Vázquez-García JG, Rojano-Delgado AM, Alcántara-de la Cruz R, Torra J, Dellaferrera I, Portugal J and De Prado R, Distribution of glyphosate-resistance in *Echinochloa crus-galli* across agriculture areas in the Iberian Peninsula. *Front Plant Sci* **12**:617040 (2021).

25 de Carvalho LB, da Costa Aguiar Alves PL, González-Torralva F, Cruz-Hipolito HE, Rojano-Delgado AM, De Prado R, Gil-Humanes J, Barro F and Luque de Castro MD, Pool of resistance mechanisms to glyphosate in *Digitaria insularis*. *J Agric Food Chem* **60**:615-622 (2012).

26 Rojano-Delgado AM, Ruiz-Jiménez J, de Castro MDL, and De Prado R, Determination of glyphosate and its metabolites in plant material by reversed-polarity CE with indirect absorptiometric detection. *Electrophoresis* **31**:1423-1430 (2010).

27 Doyle J and Doyle L, Isolation of plant DNA from fresh tissue. *Focus* **12**:13-15 (1990).

28 Padgette SR, Re RD, Barry GF, Eichholtz DE, Delannay X, Fuchs RL, Kishore GM and Fraley RT, New weed control opportunities: development of soybeans with a roundup ready gene, in Herbicide-resistant Crops: Agricultural, Economic, Environmental, Regulatory, and Technological Aspects. ed. by Duke SO, CRC Press, Boca Raton, pp. 53-84 (1996).

29 Yanniccari M, Gomez-Lobato M, Istilart C, Natalucci C, Giménez D and Castro AM, Mechanism of resistance to glyphosate in *Lolium perenne* from Argentina. *Front Ecol Evol* **5**:123 (2017).

30 Streibig JC, Rudemo M and Jensen JE, Dose-response curves and statistical models, in Herbicide Bioassays, ed. by Streibig JS and Kudsk P, CRC Press, Boca Raton, pp. 29-55 (1993).

31 Bostamam Y, Malone J, Dolman F, Boutsalis P and Preston C, Rigid ryegrass (*Lolium rigidum*) populations containing a target site mutation in EPSPS and reduced glyphosate translocation are more resistant to glyphosate. *Weed Sci* **60**:474-479 (2012).

32 Wakelin AM and Preston C, A target-site mutation is present in a glyphosate-resistant *Lolium rigidum* population. *Weed Res* **46**:432-440 (2006).

33 Kaundun SS, Zelaya IA, Dale RP, Lycett AJ, Carter P, Sharples KR and McIndoe E, Importance of the P106S target-site mutation in conferring resistance to glyphosate in a goosegrass (*Eleusine indica*) population from the Philippines. *Weed Sci* **56**:637-646 (2008).

34 Healy-Fried ML, Funke T, Priestman MA, Han H and Schonbrunn E, Structural basis of glyphosate tolerance resulting from mutations of Pro(101) in *Escherichia coli* 5-enolpyruvylshikimate-3-phosphate synthase. *J Biol Chem* **282**:32949-32955 (2007).

35 Sammons R, Heering D, DiNicola N, Glick H and Elmore G, Sustainability and stewardship of glyphosate and glyphosate-resistant crops. *Weed Technol* **21**:347-354 (2007).

36 Chen J, Huang H, Zhang C, Wei S, Huang Z, Chen J and Wang X, Mutations and amplification of EPSPS gene confer resistance to glyphosate in goosegrass (*Eleusine indica*). *Planta* **242**:859-868 (2015).

37 Han H, Yu Q, Widderick MJ and Powles SB, Target-site EPSPS Pro-106 mutations: sufficient to endow glyphosate resistance in polyploid *Echinochloa colona*?. *Pest Manag Sci* **72**:264-271 (2016).

38 Ngo TD, Krishnan M, Boutsalis P, Gill G and Preston C, Target-site mutations conferring resistance to glyphosate in feathertop Rhodes grass (*Chloris virgata*) populations in Australia. *Pest Manag Sci* **74**:1094-1100 (2016).

39 Page E, Grainger C, Laforest M, Nurse R, Rajcan I, Bae J and Tardif F, Target and non–target site mechanisms confer resistance to glyphosate in Canadian accessions of *Conyza canadensis*. *Weed Sci* **66**:234-245 (2018).

40 Gaines TA and Heap IM, Mutations in herbicide-resistant weeds to inhibition of enolpyruvyl shikimate phosphate synthase. http://www.weedscience.com. [accessed 3 January 2022].

41 Gaines TA, Duke SO, Morran S, Rigon CAG, Tranel PJ, Küpper A and Dayan FE, Mechanisms of evolved herbicide resistance. *J Biol Chem* **295**:10307-10330 (2020).

42 Alarcón-Reverte R, García A, Watson SB, Abdallah I, Sabaté S, Hernández MJ, Dayan FE and Fischer AJ, Concerted action of target-site mutations and high EPSPS activity in glyphosate-resistant junglerice (*Echinochloa colona*) from California. *Pest Manag Sci* **71**:996-1007 (2015).

43 Vila-Aiub MM, Yu Q and Powles SB, Do plants pay a fitness cost to be resistant to glyphosate?. *New Phytol* **223**:532-547 (2019).

44 Fernández-Moreno P, Alcántara-de la Cruz R, Smeda R and De Prado R. Differential resistance mechanisms to glyphosate result in fitness cost for *Lolium perenne* and *L. multiflorum*. *Front Plant Sci* **8**:1796 (2017).

45 Preston C and Wakelin AM, Resistance to glyphosate from altered herbicide translocation patterns. *Pest Manag Sci* **64**:372–376 (2008).

46 Vila-Aiub M, Balbi M, Distéfano A, Fernández L, Hopp E, Yu Q and Powles SB, Glyphosate resistance in perennial *Sorghum halepense* (Johnsongrass), endowed by reduced glyphosate translocation and leaf uptake. *Pest Manag Sci* **68**:430-436 (2012).

47 Michitte P, De Prado R, Espinoza N, Ruiz-Santaella JP and Gauvrit C, Mechanisms of resistance to glyphosate in a ryegrass (*Lolium multiflorum*) biotype from Chile. *Weed Sci* **55**:435-440 (2007).

48 Nandula VK, Reddy KN, Poston DH, Rimando AM and Duke SO, Glyphosate-tolerance mechanisms in Italian ryegrass (*Lolium multiflorum*) from Mississippi. *Weed Sci* **56**:344-349 (2008).

49 Pan L, Yu Q, Han H, Mao L, Nyporko A, Fan L, Bai L and Powles S, Aldo-keto reductase metabolizes glyphosate and confers glyphosate resistance in *Echinochloa colona*. *Plant Physiol* **181**:1519-1534 (2019).

50 González-Torralva F, Rojano-Delgado AM, Luque de Castro MD, Mülleder N and De Prado R, Two non-target mechanisms are involved in glyphosate-resistant horseweed (*Conyza canadensis* L. Cronq.) biotypes. *J Plant Physiol* **169**:1673-1679 (2012).

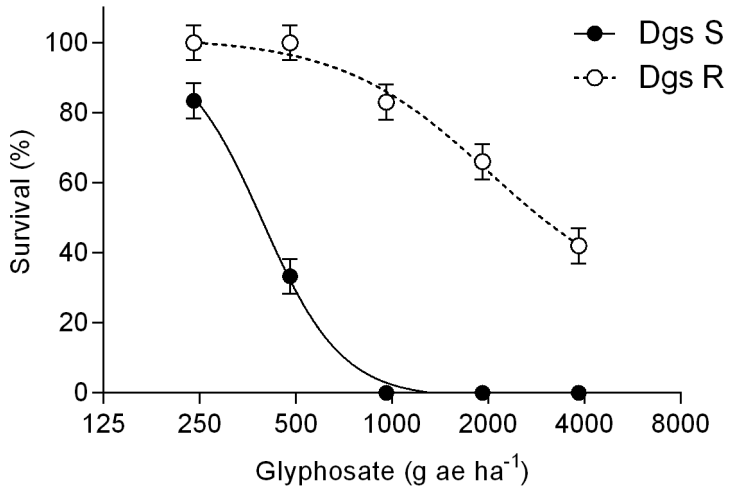


Figure 1. Effects of glyphosate doses on plant survival for the glyphosate-susceptible (Dgs S) and -resistant (Dgs R) *Digitaria sanguinalis* 21 days after treatment. Symbols represent mean values, and bars indicate ± 1 standard error of the mean. The predicted responses are shown by lines according to the adjusted models: (Dgs S) $y = -1.3+\{(99.4+1.3)/[(1+(x/393.7)^{3.49}]\}$ ($P < 0.01$; $R^2 = 0.99$) and (Dgs R) $y = 24.6+\{(101.5+24.6)/[(1+(x/2010.0)^{1.86}]\}$ ($P < 0.01$; $R^2 = 0.99$).

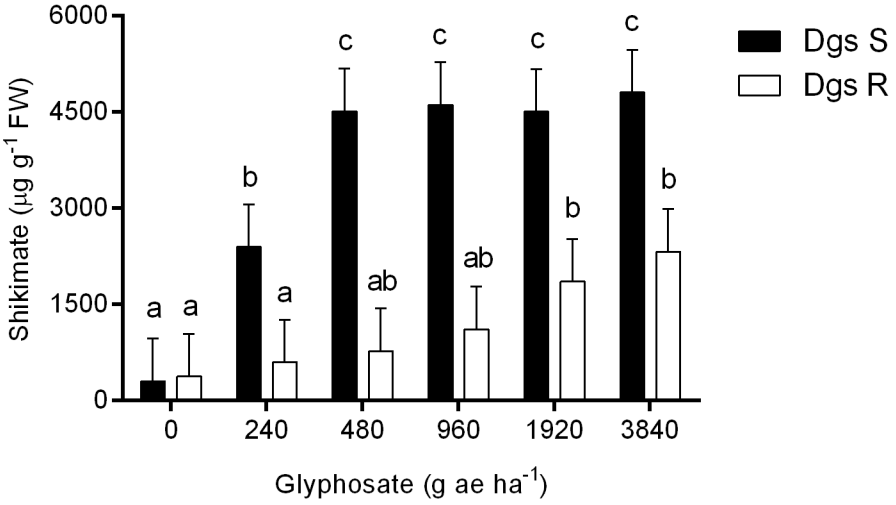


Figure 2. Accumulation of shikimic acid in the youngest fully expanded leaf of glyphosate-susceptible (Dgs S) and -resistant (Dgs R) *Digitaria sanguinalis* 72 h after glyphosate treatment. Columns represent mean values, and vertical bars indicate the standard error of the mean. Letters above the bars indicate statistical significance ($p < 0.05$).

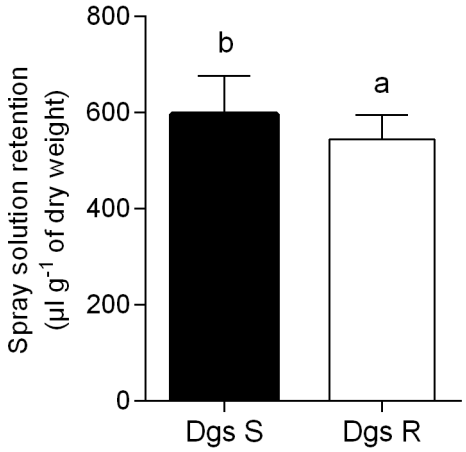


Figure 3. Spray solution interception and retention by resistant (Dgs R) and susceptible (Dgs S) *Digitaria sanguinalis* plants. Columns represent mean values, and vertical bars indicate the standard error of the mean. Different letters above the bars indicate significant differences between populations ($P < 0.01$).

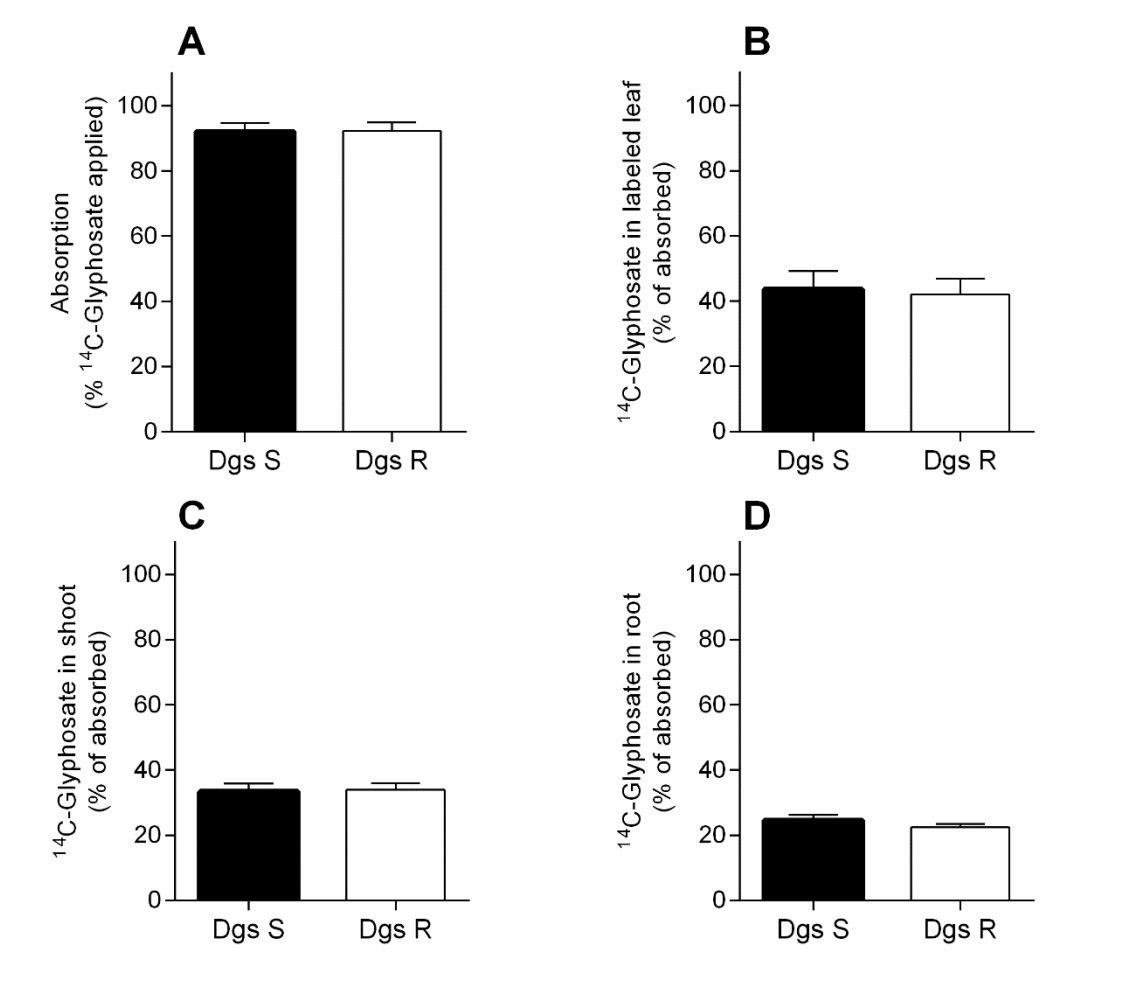


Figure 4. Glyphosate absorption and translocation in glyphosate-susceptible (Dgs S) and -resistant (Dgs R) *Digitaria sanguinalis* plants 96 h after glyphosate treatment. (A) Absorption of ^{14}C-glyphosate (P = 0.90). (B) ^{14}C-glyphosate detected in the labelled leaf (P = 0.23). (C) Translocation of ^{14}C-glyphosate from the treated leaf to the rest of the shoot (P = 0.12). (D) ^{14}C-glyphosate detected in the root system (P = 0.08). Columns represent mean values, and vertical bars indicate the standard error of the mean.

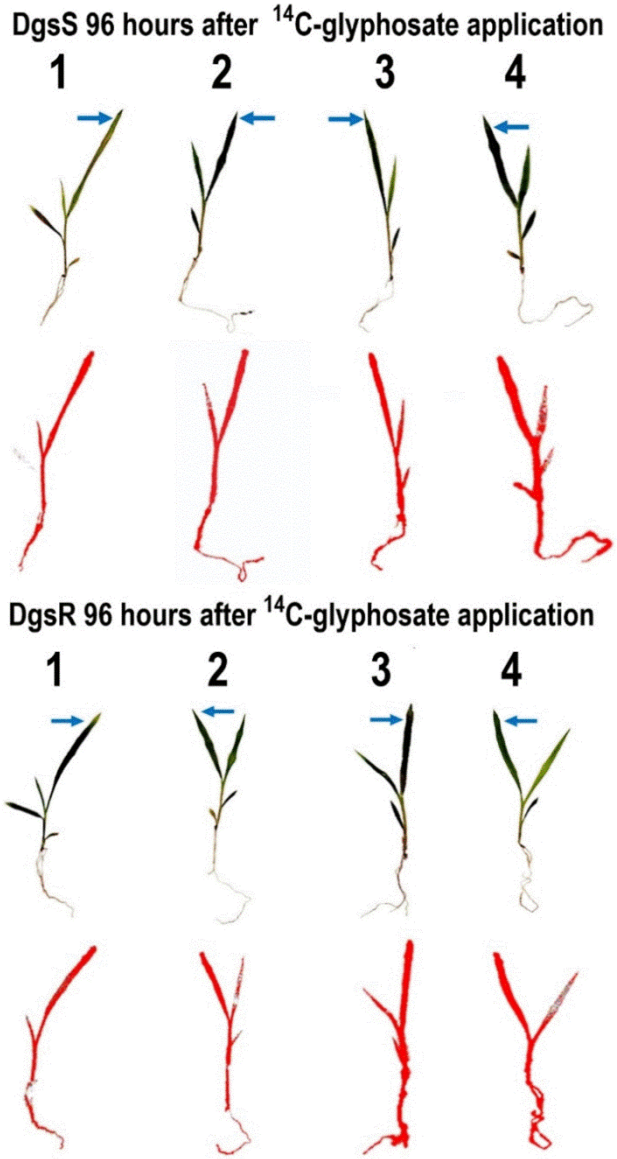


Figure 5. Camera image (upper panel) and the ^{14}C-glyphosate radioactive image (lower panel) of the glyphosate-susceptible (Dgs S) and -resistant (Dgs R) *Digitaria sanguinalis* seedlings 96 h after glyphosate treatment. ^{14}C-glyphosate was applied as a droplet to the midpoint of the youngest leaf of each 4-leaf stage plant (arrowed).

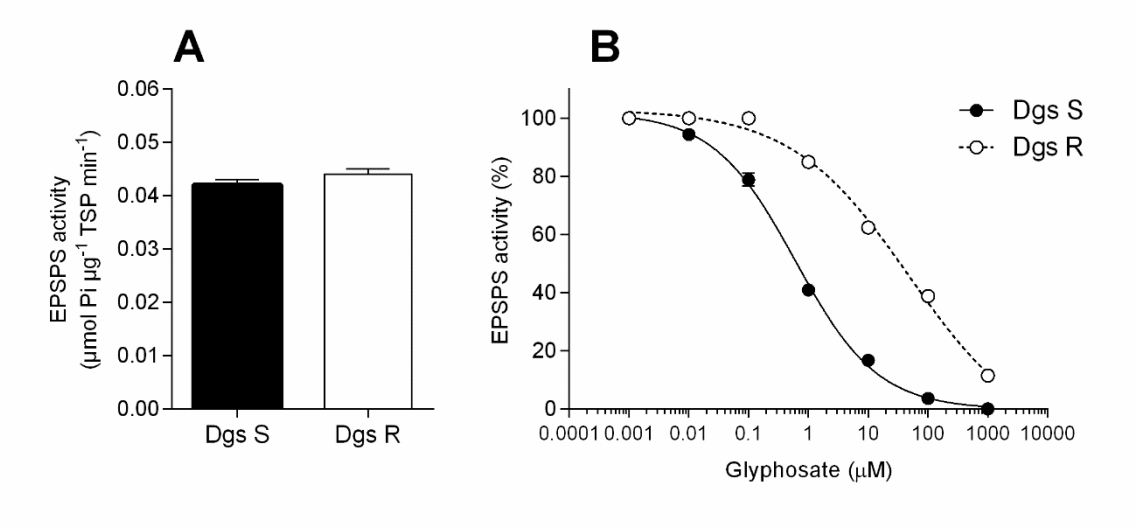


Figure 6. EPSPS enzyme activity of glyphosate-susceptible (Dgs S) and -resistant (Dgs R) *Digitaria sanguinalis* populations. (A) Basal EPSPS activity without herbicide; columns represent mean values and vertical bars indicate the standard error of the mean (P = 0.51). (B) Relative EPSPS activity exposed to different glyphosate concentrations (µM), expressed as a percentage of the untreated control. Predict dose-response curves are shown according to the adjusted models: (Dgs S) $y = -0.1+\{(101.9+0.1)/[(1+(x/0.6)^{0.63}]\}$ ($P < 0.01$; $R^2 = 0.99$) and (Dgs R) $y = -11.4+\{(103.0+11.4)/[(1+(x/47.9)^{0.44}]\}$ ($P < 0.01$; $R^2 = 0.99$).

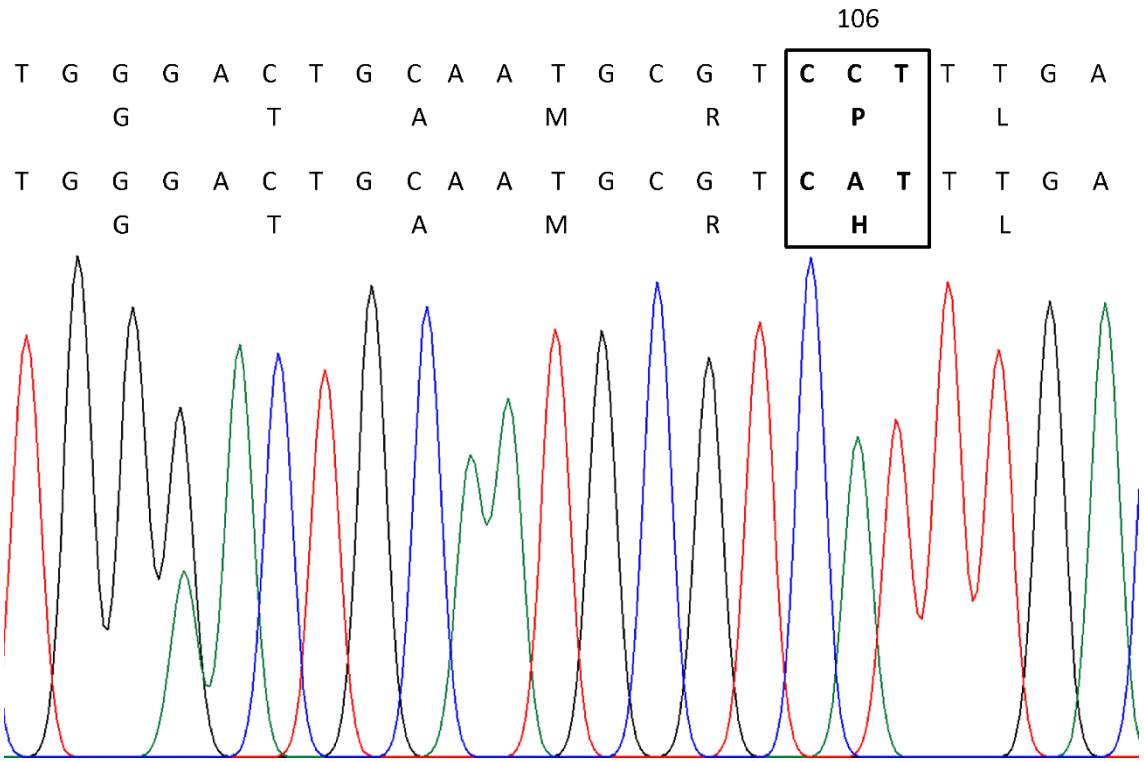


Figure 7. Partial sequence and chromatogram of the *EPSPS* gene obtained from the Dgs R *Digitaria sanguinalis* plants aligned to the EPSPS sequence of the Dgs S counterpart and the conceptual translation of the amino acid sequences. Codon corresponding to EPSPS amino acid position 106 is shown in the box.

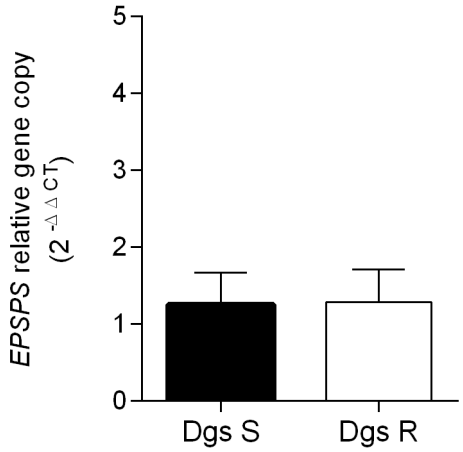
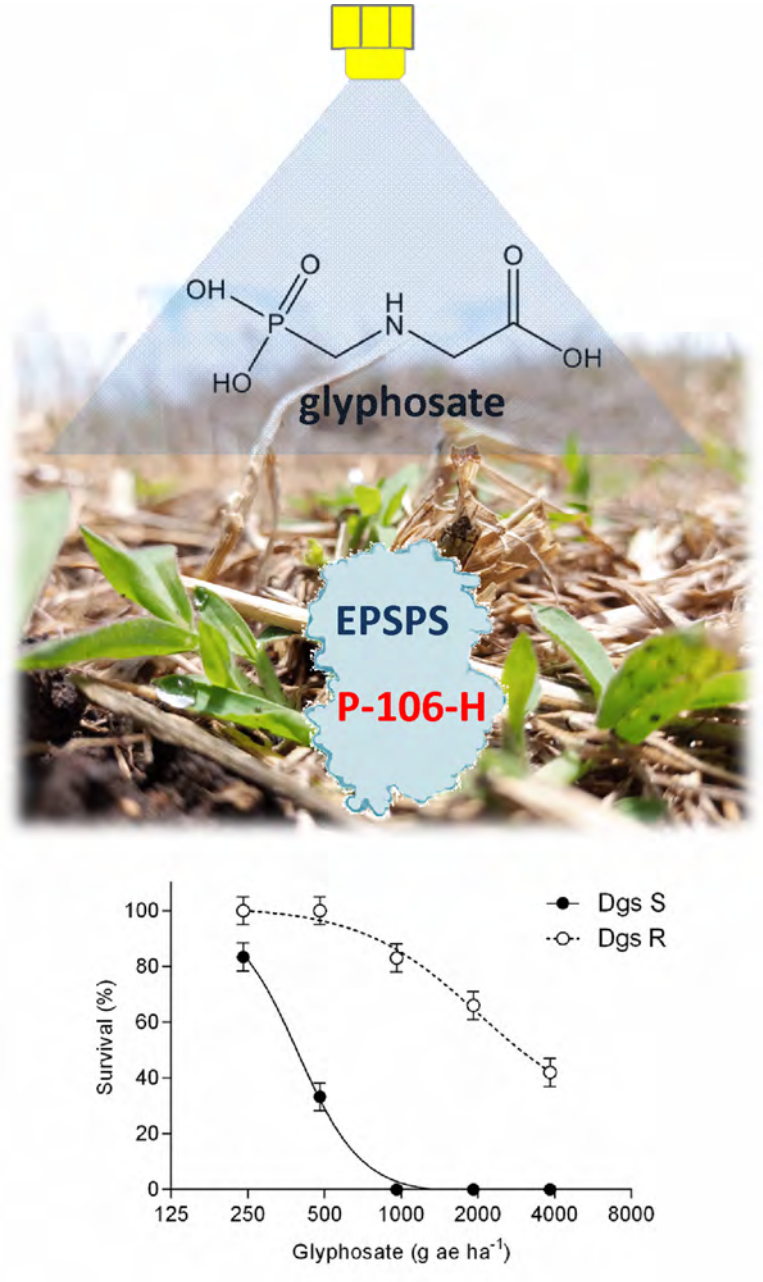


Figure 8. *EPSPS* relative gene copy number of glyphosate-susceptible (Dgs S) and -resistant (Dgs R) *Digitaria sanguinalis* plants. All EPSPS relative copy numbers were estimated against an internal reference gene (*18S rRNA*). Columns represent mean values, and vertical bars indicate the standard error of the mean (P = 0.96).

Table 1. Glyphosate metabolism (%) 96 h after treatment (HAT) in glyphosate-susceptible (Dgs S) and -resistant (Dgs R) *Digitaria sanguinalis* populations. Mean values ± 1 standard error of the mean are shown (n = 6).

	Metabolism (%) at 96 HAT		
Population	**Glyphosate**	**AMPA**	**Glyoxylate**
Dgs S	95.7 ± 2.0	–	4.3 ± 0.9
Dgs R	96.0 ± 1.9	–	4.0 ± 0.6



PS_6940_Graph abs.tif

A novel EPSPS Pro-106-His point substitution is likely the primary glyphosate resistance mechanism detected in *Digitaria sanguinalis*. This unlikely yet novel transversion at the second position of the EPSPS 106 codon demonstrates the intensity of glyphosate pressure in selecting unexpected glyphosate resistance alleles if they retain EPSPS functionality.