Characterization of the *Amaranthus palmeri* physiological response to glyphosate in susceptible and resistant populations

Manuel Fernández-Escalada, Miriam Gil-Monreal, Ana Zabalza and Mercedes Royuela*

Departamento Ciencias del Medio Natural, Universidad Pública de Navarra, Campus Arrosadía, E-31006 Pamplona, Spain.

* *To whom correspondence should be addressed*. Telephone: + 34-948169120. Fax: + 34-948168930. E-mail: royuela@unavarra.es

ABSTRACT: The herbicide glyphosate inhibits the plant enzyme 5-enolpyruvylshikimate3-phosphate synthase (EPSPS) in the aromatic amino acid (AAA) biosynthetic pathway. The physiologies of an *Amaranthus palmeri* population exhibiting resistance to glyphosate by EPSPS gene amplification (NC-R) and a susceptible population (NC-S) were compared. The EPSPS copy number of NC-R plants was 47.5-fold than the copy number of NC-S plants. Despite that the amount of EPSPS protein and activity were higher in NC-R plants than in NC-S plants, the AAA concentrations were similar in both concentrations. The increases in total free amino acid and in AAA contents induced by glyphosate were more evident in NC-S plants. In both populations, the EPSPS protein increased after glyphosate exposure, suggesting transcriptional regulation. EPSPS activity seems tightly controlled *in vivo* by coordinated transcriptional and post-transcriptional regulation. Carbohydrate accumulation and a slight induction of ethanol fermentation were detected in both populations.

KEYWORDS: Free amino acid accumulation, carbohydrate accumulation, ethanol fermentation, herbicide resistance, physiological effects

INTRODUCTION

Over the past three decades, the herbicide glyphosate has revolutionized modern agriculture. Glyphosate is a once-in-a-century herbicide that stands alone in many categories.[1] This broad-spectrum, systemic herbicide is the most widely used herbicide in the world. Glyphosate inhibits the biosynthesis of the aromatic amino acids (AAAs) tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe). The primary site of action of glyphosate is the specific inhibition of a key step in the shikimate pathway catalyzed by the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19), which converts shikimate-3-phosphate and phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate in plastids.[2]

Although chemical management has been very effective for weed control, it has also resulted in the evolution of resistant weeds. Initially, glyphosate controlled most weeds, but as early as 1997, glyphosate-resistant weeds were reported.[3] Glyphosate was widely used in non-agricultural areas, but the the introduction of genetically modified crops such as corn, cotton and soybean exacerbated the evolution of resistance to glyphosate.[4] In this context, glyphosate is used as a stand-alone weed control method on several million hectares of crop land. The intensive use of glyphosate has resulted in evolution of resistance to this herbicide in several problematic weeds to what had been a very effective herbicide. To date resistance to glyphosate has been documented in 32 species.[3] Glyphosate-resistant weeds now pose a serious challenge to modern agricultural practices and are likely to increase the cost of production.[5]

The toxic effect of glyphosate cannot be considered only in terms of its interaction at the target site. The inhibition of EPSPS results in a metabolic roadblock, with physiological consequences leading to plant death. Thus, even though the changes in physiological plant processes induced by glyphosate have not been considered as primary effects, these changes contribute to the toxic effects of glyphosate.

Despite its widespread use in global crop production, the precise mechanisms by which glyphosate kills plants remain unclear, despite studies using new molecular methods such as

transcriptional comparison,[6,7] proteomic approaches[8] and metabolomic profiling.[9,10] In general, after the target of an inhibitor has been affected, death can occur due to different causes. First, plant death could result from an accumulation or increased availability of the substrates of the inhibited enzymatic pathway. Second, death could be associated with the lack of end products generated by the inhibited pathway (mainly AAAs). Third, lethality could be associated with several side reactions triggered after the inhibition of the target because the dysregulation caused by the inhibition of this pathway can lead to effects on different metabolic pathways.

The main metabolite that accumulates upstream of EPSPS is shikimate. Massive levels of shikimate have been detected in plant tissues[11,12] because inhibition at the level of EPSPS causes feedback loops that drive an increased flow of carbon through the shikimate pathway, thereby exacerbating the accumulation of shikimate. Moreover, toxic effects of shikimate accumulation have been proposed.[13]

Some authors assume that AAA production at a level insufficient to maintain necessary protein synthesis is the main effect of glyphosate exposure, and this mechanism is consistent with the slow development of injury symptoms and the lack of essential plant compounds leading to plant death.[1] However, although a transient decrease has sometimes been reported,[14] the AAA content does not decrease significantly[14] due to an increase in protein turnover and concomitant total free amino acid accumulation[15,16] and soluble protein decrease.[10,15]

In relation to the side reactions, the blockage of the shikimate pathway by glyphosate has recently been suggested to reverberate across other biochemical pathways.[10] Deregulation at the level of PEP that is not consumed by the inhibited EPSPS in the shikimate pathway can directly affect carbon metabolism.[13,17] In this sense, glyphosate can also impair carbon metabolism by interfering with sugar metabolism and translocation. Carbohydrate accumulation has been detected in both the leaves and roots of treated pea plants, and ethanolic fermentative metabolism is enhanced by glyphosate.[18]

Amaranthus palmeri is among the three most troublesome weeds in the main crops in Georgia (cotton, peanut, and soybean) and is among the top five most troublesome weeds in most other southeastern states. This annual weed is highly problematic due to its competitiveness, aggressive growth habit and prolific seed production.[19] *A. palmeri* was initially controlled by glyphosate in glyphosate-resistant crops but become a major glyphosate-resistant weed that occurs in several states. Glyphosate-resistant *A. palmeri* was first reported in 2006,[19] and the mechanism of resistance was gene amplification.[20] This was the first reported occurrence of gene amplification in a field-evolved resistance to any herbicide.[21] The EPSPS gene was amplified from 2 to more than 100-fold compared with sensitive populations. This mechanism of resistance to glyphosate has since been reported in a number of other species, including *Amaranthus tuberculatus,* [22,23]*, Amaranthus spinosus,*[24] *Kochia escoparia,*[25] *Lolium multiflorum,*[26,27] and *Bromus diandrus.*[28]

The availability of a biotype with overexpression of the EPSPS enzyme provides an opportunity to analyze how overexpression of ESPS affects AAA synthesis and other physiological factors by comparison with a sensitive population. In addition, comparing the different effects of glyphosate on both populations will facilitate the comprehensive elucidation of the phenotypic manifestations of evolved glyphosate resistance. For this purpose, biomass, shikimate accumulation, EPSPS expression and activity, free amino acid profile, carbohydrate content and ethanol fermentation were compared in two populations of *A. palmeri* (one susceptible and the other glyphosate-resistant due to gene amplification).

MATERIALS AND METHODS

Plant material and treatment application. The seeds of the *Amaranthus palmeri* biotypes (NC-R and NC-S) were kindly provided by Dr. Gaines (Colorado State University, Fort Collins, CO, USA) and were originally collected from North Carolina (USA).[29]

Seeds were surface sterilized prior to germination.[30] For germination, seeds were incubated for 7 days at 4°C in darkness and then maintained for 48 h in a light/darkness cycle of of 16 h/8h at temperature of 30ºC in light and 8 h at 18ºC in darkness. The seeds were then transferred to aerated 2.7-L hydroponic tanks in a phytotron (day/night: 16 h/8 h; light intensity: 500 μmol s^{-1} m^{-2} PAR; temperature: 22°C/18°C; relative humidity of the air: 60/70%). Throughout the course of the experiment, the plants remained in the vegetative phenological stage. The nutrient solution[31] was supplemented with 15 mM KNO_3.

***In vivo* shikimate assay.** Shikimate accumulation following glyphosate treatment was compared between NC-R and NC-S as described previously.[32] Leaf discs 4 mm in diameter (6-10 discs) were excised from the youngest leaf of 3-6 plants of each biotype using a Harris Uni-Core™ (Healthcore, Bucks, UK).

Briefly, one disc was placed in a well of a 96-well microtiter plate. Each well contained 100 μL of a solution containing 169, 84.5, 42.3, 21.1, 10.6, 5.3, 2.6, 1.3 or 0 mg glyphosate L^{-1} Glyphosate was diluted from commercial formula (Glyfos, BayerGarden, Valencia, Spain). Each row of the microtiter plate contained a different glyphosate concentration. The plates were incubated at 22ºC under continuous light for 16 h. After incubation, the plants were placed in a freezer (-20ºC).

The concentration of shikimate in each cell was measured according to the procedure of Cromartie and Polge.[33] Shikimate was extracted from the freeze-thawed leaf discs by adding 25 μL of 1.25 N HCl and incubating the plates at 60 ºC for 15 min. Two 25-μL aliquots from each well were transferred to a new plate, and 100 μL of 0.25% periodic acid/0.25% metaperiodate was added to each well. After the periodic acid–metaperiodate incubation (60 min in the dark),

25 μL of 0.6 M sodium hydroxide with 0.22 M sodium sulfite solution was added. The optical density of the solution at 380 nm was determined spectrophotometrically. A shikimate standard curve was developed by adding known amounts of shikimate to wells containing leaf discs not exposed to glyphosate (3, 6, 12, 25, 50 and 100 μg mL^{-1}). One microtiter plate was used for each population. The study was repeated twice.

Dose-response studies. A dose-response study was performed to verify the previously reported resistance. The dose-response relationship was established according to Seefeld et al.[34] For each population, plants of uniform size and appearance were selected, and 3 tanks with 4-5 plants each were treated with each glyphosate dose. Both populations were treated when plants were approximately 20 days old, after reaching the growth stage defined as BBCH 14.[35] The experiment was repeated twice.

Glyphosate is recommended at 0.84 kg ha^{-1} for the control of *Amaranthus* sp. up to 46 cm in height [19]. Glyphosate herbicide (commercial formula, Glyfos, BayerGarden, Valencia, Spain) was applied using an aerograph (Mod. Definik; Sagola) connected to a compressor (Mod. Werther one, Breverrato, 60 W; 10 l m^{-1}; 2.5 bar). The herbicide was sprayed at a rate of 500 L ha^{-1}, and thus the recommended field dose resulted in 1.6 g glyphosate L^{-1}. The effect of increasing doses of glyphosate up to three times the recommended dose (0, 0.042 kg ha^{-1} (only for NC-S), 0.084 kg ha^{-1}, 0.021 kg ha^{-1}, 0.042 kg ha^{-1}, 0.084 kg ha^{-1}, 1.68 kg ha^{-1} and 2.52 kg ha^{-1}) was evaluated in terms of biomass and shikimate content, an adequate indicator of glyphosate-mediated plant injury. The control plants were treated with water because the inert proprietary ingredients could not be obtained.

For biomass evaluation, the shoot and root fresh weights of each plant were determined 5 days after treatment. The material was dried for 48 h at 75-80ºC to obtain the dry weight.

Shikimate content was evaluated at 5 days after treatment. Three to six leaf discs (4 mm diameter) were excised from the youngest leaf of each plant. Leaf discs were placed in a screw-top 2-mL Eppendorf tube, frozen and stored at -80ºC until analysis. For shikimate

141 determination, the vials were removed from the freezer, and shikimate was extracted as
142 described by Koger et al.[36] First, 100 µL of 0.2 5N HCl per disk was added to each vial. The
143 vials were incubated at 22ºC for 1.5 h and mixed by vortexing several times. The shikimate
144 content was analyzed spectrophotometrically.[33]

145 **Analytical determinations.** After the dose-response study, a physiological study was
146 performed by comparing the effect of the 0.84 kg ha^{-1} and 2.52 kg ha^{-1} doses on each population
147 to untreated-plants. The experiment for the physiological study was repeated twice in time and
148 both populations were treated when plants were approximately 20 days old. Leaf and root
149 samples were obtained 3 days after treatment, 4 h after the beginning of the photoperiod, for
150 analytical determinations. Plant material was immediately frozen in liquid nitrogen and stored at
151 –80ºC. *A. palmeri* frozen samples were ground to a fine powder under liquid N_2 using a Retsch
152 mixer mill (MM200, Retsch®, Haan, Germany) maintaining separately individual plants as
153 biological repeats. The amount of tissue needed for each analysis was separated and stored at -
154 80°C.

155 *Relative genomic EPSPS gene copy number.* Quantitative real-time PCR was performed to
156 determine the genomic copy number relative to acetolactate synthase (ALS) in untreated NC-S
157 and NC-R plants. Genomic DNA was extracted from approximately 0.1 g of previously ground
158 *A. palmeri* leaves. The plant material was homogenized in 375 µL of 2x lysis buffer (0.6 M
159 NaCl, 0.1 M Tris-HCl (pH 8.0), 40 mM EDTA (pH 8.0), 4% sarcosyl and 1% SDS) and 375 µL
160 of 2 M urea. One volume (750 µL) of phenol-chloroform-isoamyl alcohol (25:24:1) was added
161 to the mixture and mixed briefly. The homogenates were centrifuged at 20,000 *g* for 10 min at
162 room temperature. To precipitate the DNA, 0.7 volumes (525 µL) of cold isopropanol was
163 added to the supernatants, and the tubes were centrifuged at 20,000 *g* for 15 min at 4°C. The
164 DNA pellet was washed twice with 1 mL of 70% ethanol, air-dried and resuspended in 25 µL of
165 resuspension buffer (10 mM Tris-HCl (pH 8.0), containing 30 µg mL^{-1} RNase A). Samples were
166 briefly incubated at 37°C for 5 min to degrade contaminating RNAs.

167 The extracted DNA was subsequently quantified and analyzed using a NanoDrop ND-1000
168 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). OD 260 and 280 nm were
169 read for every sample. The DNA quality was also checked using a 1% agarose gel. Ten-fold
170 diluted DNA samples were loaded onto a 1% agarose gel and run at 75 mA for 35 min. The gels
171 were visualized using a Gel DocTM 2000 system (Bio-Rad Laboratories Inc., Hercules, CA,
172 USA). DNA concentrations were adjusted to 5 ng μL^{-1}.

173 Quantitative real-time PCR was used to measure EPSPS genomic copy number relative to ALS
174 as described by Gaines et al.[20] with some modifications. The following primer sets were used:
175 EPSPS-forward (5'-atgttggacgctctcagaactcttggt-3') and EPSPS-reverse (5'-
176 tgaatttcctccagcaacggcaa-3'); ALS-forward (5'-gctgctgaaggctacgct-3') and ALS-reverse (5'-
177 gcgggactgagtcaagaagtg-3')[20]. To determine the efficiency of the primers, a standard curve using
178 a 1×, 1/5×, 1/25×, 1/125×, and 1/500x dilution series of genomic DNA from NC-R was
179 conducted.

180 Real-time PCR amplifications were performed in an optical 96-well plate using an ABI PRISM
181 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each
182 reaction was performed using 10 ng of genomic DNA in a total volume of 20 µL containing 1x
183 SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Shiga, Japan), 300 nM specific forward primer
184 and 300 nM specific reverse primer. The following thermal profile was used for all PCRs: 50°C
185 for 2 min, 95°C for 1 min and 40 cycles of (95°C for 30 s and 60°C for 1 min). Melt-curve
186 analysis was conducted with a final denaturation step of 95°C for 30 s, 60°C for 15 s and 95°C
187 for 15 s.

188 To calculate the final Ct values, ten biological replicates were performed, and each individual
189 sample was run in triplicate. The average increase in EPSPS copy number relative to ALS and
190 the standard deviation were calculated for each sample. The increase in EPSPS copy number
191 was expressed as $2\Delta Ct$, where ΔCt = (Ct, ALS – Ct, EPSPS).[20]

EPSPS extraction and activity assay. The EPSPS extraction and assay were conducted following the procedures of Gaines et al.[20] Briefly, 1 g of leaf tissue was ground to a fine powder in a chilled mortar and mixed with 10 mL of cold extraction buffer. After centrifugation, the supernatant was precipitated with ammonium sulfate (45-70%) and desalted (Zeba desalt spin columns, Pierce Biotechnology, Rockford, IL, USA). A phosphate detection kit (Molecular Probes, Eugene, OR, USA) was used for the continuous measurement of inorganic phosphate release for EPSPS activity. The total soluble protein in the reaction mixture was 12.5 μg/L for the NC-S population and 1.25 μg/mL for the NC-R population. After obtaining a background phosphate release level (10 min), the final step was the addition of shikimate-3-phosphate (up to 0.5 mM). Phosphate release was measured for an additional 10 min.

Immunoblotting of EPSPS. Extraction and electrophoresis were performed as described previously.[37] First, 0.05 g of leaf tissue was ground with 0.2 mL of extraction buffer. Proteins were separated by 12.5% SDS-PAGE. Western blots were produced according to standard techniques. The EPSPS antibody was produced by a custom peptide facility (Agrisera AB, Vännäs, Sweden) against a sequence of residues (numbers 193-206) of Palmer Amaranth EPSPS (GenBank accession number FJ861242). The antibody was raised in rabbits using the manufacturer's standard protocols. The primary antibody dilution was 1:2000. Antirabbit IgG peroxidase (Agrisera AB, Vännäs, Sweden) was used as a secondary antibody at a dilution of 1:75.000. Bands were identified using an Advanced ECL chemiluminescence detection kit (Amersham Life Science, Arlington Heights, IL, USA). Immunoblots were scanned using a GS-800 densitometer, and protein bands were quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). Membrane signals were normalized according to total soluble protein loading quantity.

Metabolite analysis and fermentative enzymes. Ground leaf and root samples (0.1 g) were homogenized in 1 M HCl for amino acid extraction. After protein precipitation, the amino acid concentrations in the supernatant were analyzed. After derivatization with FITC, amino acid

content was measured by capillary electrophoresis and a laser-induced fluorescence detector as described elsewhere.[38] Cysteine content was determined from the same acid extracts derivatized with 5-iodoacetamide fluorescein and reduced with tributylphosphine, as described previously.[39]

The soluble carbohydrate (glucose, fructose and sucrose) content was determined in ethanol-soluble extracts, and the ethanol-insoluble residue was extracted for starch analysis.[39] The starch and soluble sugar concentrations were determined by capillary electrophoresis as described by Zabalza et al.[40]

Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities were assayed in desalted extracts as described previously.[41]

Statistical analysis. Dose-response curves based on fresh/dry weight were constructed using the program Sigma Plot 12.0 to calculate the four-parameter sigmoidal log-logistic dose-response model. The doses that resulted in a 50% reduction of the fresh and dry weights were calculated for each population.

In the analytical determinations, each mean value was calculated using samples from different individual plants from the two performed experiments as replicates. The difference between untreated plants of each population was evaluated using Student's t-test and confirmed as significant when $p<0.05$.The results of each population were subjected to separate one-way ANOVA analysis (SPSS 18.0), and the means were separated using the least significant difference method ($p<0.05$). For each population, significant differences are highlighted in the figures by different letters.

RESULTS AND DISCUSSION

Population dose response to glyphosate. The dose-response experiments confirmed glyphosate resistance in the NC-R population. Figure 1A presents the effect of different doses of glyphosate on shoot dry weigh accumulation 5 days after glyphosate application, expressed as a percentage of untreated plants. Although the increase in shoot dry weight was highly variable within each dose, the effect on each population was described by a significant four-parameter log-logistic dose-response curve. The root dry weight of NC-S plants did not exhibit a dose-dependent change over the range of concentrations of glyphosate used (data not shown), so it was not possible to use this biomass parameter in the resistance characterization.

No difference in shoot dry weight accumulation was observed between the populations in the absence of glyphosate (data not shown). The glyphosate concentration that reduced the shoot dry weigth accumulation over 5 days by 50% (EC_{50}) was 0.0897 kg ha^{-1} for NC-S and 0.3310 kg ha^{-1} for NC-R, 3.7-fold greater. This difference in the EC_{50} is not really a resistance factor. When percent survival or percent reduction in fresh weight after 21 days was used, the EC_{50} estimates were 20-18-fold higher for the NC-R biotype compared with the NC-S biotype.[29,42] These differences in values reflect differences in methodology, in the parameter evaluated (i.e., percent survival versus inhibition of biomass growth) and the time point of assessment (time since treatment). However, glyphosate-resistant biotypes from North Carolina have been ereported to exhibit levels of resistance ranging from 3- to 22-fold.[43]

The accumulation of shikimate in plant tissue can be used to distinguish resistant and susceptible plants. Shikimate accumulation was observed in leaf disc tissue from NC-S plants after 5 days of treatment with glyphosate concentrations greater than 0.21 kg ha^{-1} (Figure 1B). The shikimate accumulation was maximized when the plants were sprayed with 0.84 kg ha^{-1} glyphosate, whereas accumulation was lower but still very noticeable at 1.68 and 2.52 kg ha^{-1}. No significant shikimate accumulation was observed in the glyphosate-resistant biotype NC-R at any of the doses tested and, on an expanded scale, there seems to be an effect, although much less than with the NC-S. Previous studies of the same populations have indicated that shikimate

increases in both biotypes as the glyphosate concentration increases, with a greater increase in shikimate in the NC-S biotype.[42] At glyphosate doses of 100 g ha^{-1} or greater, the shikimate concentration is always greater in the susceptible biotype.[42] Due to the increased EPSPS copy number, shikimate should not accumulate or may accumulate at lower levels compared to the susceptible biotype.

This study established the doses employed in the physiological characterization: 0.84 and 2.52 kg ha^{-1}. The 0.84 kg ha^{-1} dose was chosen because the highest shikimate accumulation in NC-S was observed at this dose, and thus the physiological status is expected to be strongly affected. The 2.52 kg ha^{-1} dose was chosen because it was the highest dose used and did not result in shikimate accumulation in NC-R plants.

***In vivo* shikimate accumulation in leaf discs.** In addition to shikimate quantification in the leaves of plants 5 days after spraying with different glyphosate concentrations (Figure 1B), plant sensitivity to glyphosate was evaluated by incubating excised leaf disc tissue for 24 h with 0.1-170 mg L^{-1}, glyphosate; shikimate accumulation in the leaf-discs submerged in different glyphosate concentrations was analyzed in both populations. This second assay has been reported to differentiate resistant from susceptible biotypes in different species.[32]

The leaf discs from the resistant biotype had less shikimate accumulation than the susceptible biotype (Figure 2). Shikimate accumulation was similar in leaf discs of NC-S plants incubated with 5 mg L^{-1} glyphosate and NC-R plants incubated with 170 mg L^{-1} glyphosate, the highest dose of glyphosate. These results confirm the resistance of the NC-R population and are consistent with previous research and with Figure 1.

EPSPS gene copy number and effects of glyphosate on protein content and enzymatic activity.

Gene amplification was first reported as a glyphosate resistance mechanism in an *A. palmeri* population from Georgia.[20] A North Carolina glyphosate-resistant population of *A. palmeri* with gene amplification of 22- to 63-fold was subsequently reported. Additional populations have

since been identified in New Mexico and Mississippi.[44,45] In this study, seeds from the North Carolina population were used. To confirm the resistance mechanism, the EPSPS relative genomic copy number was calculated by quantitative real-time PCR using ALS as an internal standard. Consistent with previous studies, the genomes of the NC-R plants exhibited a mean 47.5-fold increase in the number of copies of the EPSPS gene compared to the genomes of NC-S plants (Figure 3A).

An increased EPSPS copy number results in elevated EPSPS expression at RNA transcript levels in *A. palmeri*,[20,45,46] *A. tuberculatus*[23] and *Kochia scoparia*[25] but not *Bromus diandrus*.[28] In addition to EPSPS expression, resistant plants with a greater EPSPS copy number have been reported to have a higher quantity of the EPSPS protein[20,25,45,46] and higher EPSPS enzyme activity.[20,23,26,45,46] In this study, the EPSPS protein was quantified. Immunoblotting with an antibody for EPSPS resulted in a single reaction band at approximately 50 kD (Figure 3B, top). Greater EPSPS protein abundance was detected in NC-R plants; the normalized signal for EPSPS was 25-fold higher in untreated NC-R plants than in NC-S plants (Figure 3B, bottom). As expected, untreated NC-R plants exhibited higher specific EPSPS activity than NC-S plants (Figure 3C) due to the increased levels of EPSPS protein in the total soluble protein. Interestingly, the activity in NC-R plants was increased 26.5-fold compared to NC-S plants, nearly identical to the 25-fold change in EPSPS protein levels.

As widely reported, the glyphosate resistance level appears to increase with higher EPSPS genomic copy number,[20,27,45–47] increased EPSPS expression, [20,26] protein content[20] and specific activity.[20] Two exceptions have been reported: cloned resistant plants of A. *palmeri* from Mississippi[48] that did not exhibit a correlation between resistance and copy number and a line of *Echinochloa colona* in which resistance could not be explained solely by higher EPSPS basal activity.[49]

In this study, the effect of glyphosate on the EPSPS protein content (Figure 3B) of NC-S and NC-R plant was assessed. In both populations, plants treated with the higher dose exhibited an increase in EPSPS protein levels (Figure 3B top, bottom). These results suggest transcriptional

regulation triggered only by a high dose of glyphosate. This study is the first to report an increase in EPSPS protein levels after glyphosate treatment. Increases in EPSPS mRNA levels after glyphosate have been reported previously, suggesting transcriptional regulation, in resistant and sensitive biotypes of *Eleusine indica,*[50] *Lolium rigidum*[51] and tobacco.[52] By contrast, in *A. tuberculatus* with multiple EPSPS copies, no increase in EPSPS expression was detected after glyphosate application.[23]

The effect of glyphosate on the specific activity of EPSPS differed between the two populations (Figure 3C). NC-R plants exhibited a dose-dependent increase in enzymatic activity with glyphosate treatment, whereas NC-S exhibited a tendency toward decreased EPSPS activity as the applied dose of glyphosate increased. The increase in the amount of protein in the NC-R population at the highest dose of herbicide was sufficient for the concomitant increase in EPSPS activity to alleviate the toxicity of the herbicide. Indeed, concomitant increases in EPSPS mRNA and activity have been reported previously.[50,51] EPSPS activity in NC-S plants decreased with glyphosate treatment (Figure 3C). This response differed from that observed in NC-R plants and demonstrates that although the amount of EPSPS protein was higher in the susceptible population, the increase was not sufficient to counteract the amount of the enzyme inhibitor (herbicide) present.

Free amino acid profile in untreated susceptible and resistant plants

If EPSPS is a bottleneck in the carbon flow through the shikimate pathway, NC-R plants should possess higher AAAs biosynthetic capacity. To test this hypothesis, the free amino acid profiles of NC-R and NC-S plants were compared before applying the herbicide. The total free amino acid (Figure 4A and 4C) and AAA contents (Figure 5A and 5C) in leaves and roots were compared. Moreover, the content of each individual amino acid in the leaves (Supporting Information Figure 1) and roots (Supporting Information Figure 2) was determined, including other groups of amino acids previously reported to be affected by glyphosate: [14] branched-chain (Val, Leu, Ile), acidic (Glu, Asp), and amide (Gln, Asn) amino acids.

346 The total free amino acid content was similar in the two populations (Figure 4A and
347 4C). Interestingly, no differences were detected in the AAA contents between untreated NC-R
348 plants and NC-S plants (nor in the leaves nor in the roots)-(Figure 5A and 5C, Supporting
349 Information Figures 1 and 2), as reported recently for two other populations of the same
350 species.[10] The contents of nearly all individual amino acids in the leaves and roots (shown in
351 Supporting Information Figure 1 and 2) were similar for untreated plants of the two populations,
352 except Val, Leu, Ala and Glu in leaves and Tyr, Val and Asp in roots.

353 NC-R plants exhibited increases in EPSPS copy number, protein amount, and activity of
354 47.5-, 23.6- and 26.5-fold, respectively, compared to NC-S plants. However, the AAA content
355 was the same in untreated plants of the two populations, indicating that the amount of AAA is
356 independent of the expression of EPSPS and suggesting post-transcriptional regulation of
357 EPSPS activity. Plants regulate carbon flux toward AAA biosynthesis at the transcriptional and
358 post-transcriptional levels.[53] Although all shikimate pathway genes have been characterized in
359 model plants,[54] information on the effect of AAA levels on the expression of these genes in
360 plants remains limited.[53] *In vitro* studies of allosteric regulation have implied that the shikimate
361 pathway in plants is mostly regulated at the gene expression level rather than the post-
362 translational level.[55] Some evidence indicates that in plants, the expression of the shikimate
363 pathway and the downstream pathway are coordinately regulated, often by the same
364 transcription factor.[53] The transcription factor EPF1 directly binds the *EPSPS* promoter and
365 controls its spatial and developmental expression.[56] Although EPSPS has been extensively
366 studied in plants due to its association with glyphosate, the significance of EPSPS activity in the
367 synthesis of AAA has not been sufficiently addressed.[57] This study of NC-R plants provides
368 insights into the tight control of the biosynthesis of AAAs by EPSPS activity *in vivo*, in which
369 transcriptional and post-transcriptional regulation are coordinated.

370 **Free amino acid profile after glyphosate treatment**

371 Changes in the free amino acid profile after glyphosate treatment have been reported,[14–
372 16,18] and thus this parameter can also be used as a physiological marker of herbicide activity. The

373 total free amino acid content was higher in NC-S plants treated with glyphosate than in
374 untreated plants (Figure 4B and 4D). In both roots and leaves, the increases after treatment with
375 0.84 or 2.52 kg ha^{-1} glyphosate were similar. Total free amino acid accumulation after
376 glyphosate treatment has been reported previously in several species, including pea,[14,15]
377 *Arabidopsis thaliana*,[16] maize[58] and soybean.[59,60] Glyphosate also increased the total free amino
378 acid content in the leaves and roots of NC-R plants in a dose-dependent manner (Figure 4B and
379 4D), although the detected increase in leaves was much lower than that in treated NC-S leaves.
380 Previous studies have observed small or no effects on this parameter in resistant soybean treated
381 with glyphosate.[59,60] The observed accumulation of free amino acids in treated plants has been
382 attributed to increased protein turnover,[15] and indeed, a decrease in the content of total soluble
383 protein was recently reported in glyphosate-treated *A. palmeri* populations.[10] However, an
384 evaluation of the effects of glyphosate on the main proteolytic systems in pea revealed that not
385 all proteolytic systems increased.[15]

386 The free amino acid profile in NC-S revealed an important increase in the content of
387 each individual amino acid after 3 days of glyphosate treatment (Supporting Information Figure
388 1 and Figure 2). In leaves, Val, Leu, Ile, Ala, Asn, Gln, Gly, Asp, Thr, Lys, Ser, Arg, His, Pro,
389 and Cys contents were higher after glyphosate treatment in a dose-dependent pattern
390 (Supporting Information Figure 1). In roots, Val, Leu, Ile, Ala, Gly, Glu, Thr, Lys, Ser, Arg, and
391 His contents were increased and in most cases the highest content was detected after treatment
392 with 0.84 kg/ha of glyphosate (Supporting Information Figure 2).. Branched-chain amino acid
393 content was greatly increased in the leaves and roots of NC-S plants after glyphosate treatment,
394 as reported recently.[10] The effect of the herbicide on the amide group of amino acids (Gln, Asn)
395 in NC-S plants varied depending on the organ, increasing and decreasing in leaves and roots,
396 respectively. GABA (γ-aminobutyric acid) is a non-protein amino acid that usually accumulates
397 under stress situations. GABA did not accumulate in glyphosate-treated NC-S plants. In NC-R
398 plants, the content of Val, Leu, Ile, Ala, Asn, Gln, Asp, Thr, Lys, Arg, His and Pro in leaves
399 and roots and the content of Gly and Cys only in leaves were increased 3 days after the

application of the highest concentration of glyphosate. However, the accumulations were not as striking as in the NC-S biotype (Supporting Information Figures 1 and 2).

The general increase in the content of each free amino acid due to protein turnover could mask the specific change in each AAA. Thus, each individual AAA and their sum are presented as a percentage of the total free amino acids instead of as absolute values (Figures 5B and 5D). In general, in the NC-S population, the relative content of each AAA and the sum of the three AAA contents were increased after glyphosate application. The detected increase was more striking at the highest dose of glyphosate, following the same pattern as other free amino acids. The absolute contents of Trp, Tyr and Phe were increased in the leaves and roots of the susceptible plants after glyphosate application (Supporting Information Figures 1 and 2). Other studies have shown no clear pattern in AAA content in response to glyphosate exposure. In sensitive *A. palmeri* and soybean, only Trp accumulated after glyphosate treatment,[10,59] whereas rapeseed exhibited an increase in Phe concentration in response to low glyphosate concentrations and no change in response to higher glyphosate concentrations.[61] The AAA content in NC-R plants was largely unaffected by glyphosate treatment: it was not affected in roots and was only increased in leaves at a dose of 2.52 kg ha^{-1}. By contrast, metabolic profiling of a resistant biotype of *A. palmeri* revealed perturbations in AAA levels after glyphosate treatment.[10]

It is difficult to predict how greatly an increase in one enzyme of the shikimate pathway may affect the AAA content. Transgenic *Arabidopsis* plants expressing a feedback-insensitive bacterial DHAPS exhibit higher Phe and Trp contents.[62] Thus, the *in vivo* roles of two enzymes of the shikimate pathway that are feedback regulated by AAA, chorismate mutase and anthranilate synthase, remain unclear.[55] In our study, the comparison of untreated plants of both populations demonstrated that resistant plants with much higher EPSPS protein levels and activity exhibited the same AAA content as susceptible plants. This result suggests a regulatory mechanism after EPSPS expression that controls AAA content. The free amino acid content reflects the biosynthesis, catabolism, use rate in protein synthesis and proteolysis of amino

acids. AAAs are used not only in protein synthesis, but also in the biosynthesis of many aromatic metabolites, such as phenylpropanoids.

Glyphosate treatment of the NC-R population revealed that inhibition of EPSPS activity by the herbicide (even a small amount, as resistant plants possess a large amount of EPSPS protein) induces an increase in EPSPS protein levels (Figure. 3B), demonstrating transcriptional control of EPSPS. In these resistant glyphosate-treated plants, with higher EPSPS protein amount, the AAA content was largely unaffected (Figure 5B and 5D), supporting that transcriptional and post-transcriptional mechanisms interplay to control EPSPS *in vivo*. Although the signal eliciting the increase in EPSPS protein synthesis has not been elucidated, our results indicate that AAA content is not involved because AAA levels remained nearly constant after glyphosate treatment. The response-triggering signal is proposed to be related to minor changes in other intermediate metabolites, such as shikimate or chorismate.

The results obtained after glyphosate treatment of the susceptible population confirmed the physiological effects described above for the resistant plants (an increase in EPSPS protein). However, the increase in EPSPS protein in susceptible plants (Figure 3B) was insufficient to overcome the effect of the herbicide, as free amino acid content was clearly increased (Figure 4). Indeed, the AAA content was also increased, most likely due to increased protein turnover, thus confirming that AAA levels are not the signal triggering the increase in EPSPS synthesis.

Carbon allocation and ethanol fermentation after glyphosate treatment

Carbohydrate accumulation is induced by the application of glyphosate[10,16,18] and thus can be used as a physiological marker of herbicide toxicity. Total soluble sugars (the sum of glucose, fructose and sucrose) and starch contents were measured in the roots and leaves of both populations of *A. palmeri* 3 days after treatment with glyphosate (Figure 6). Comparison of the control plants (untreated) of the two populations revealed similar carbohydrate levels, although several differences were detected: the control leaves and roots of the resistant biotype exhibited significantly higher contents of starch and total soluble sugars, respectively, than the susceptible

plants. In NC-S plants treated with the lowest concentration of glyphosate, accumulation of total soluble sugars and starch in the leaves and of starch in roots was detected. Sugar accumulation in NC-S plants exhibited a trend to decline to control values at the highest concentration, but this behavior does not indicate recovery. On the contrary, the severity of the treatment of these plants makes it difficult to maintain carbohydrate accumulation, corresponding to a possible decline in carbon assimilation.[14,40] This pattern was not detected in NC-R plants, where a general increase in carbohydrate content was detected after both concentrations were applied. Carbohydrate accumulation in the leaves and roots of pea and *A. thaliana* plants supplied with glyphosate through the nutrient solution has been described.[16,18] Moreover, it has been described recently the same pattern when glyphosate was sprayed onto the leaves, evidencing that the plant response was similar after foliar or residual applications.[63] Glyphosate is a systemic herbicide that is translocated through all the plant body independently of the site of application and the detected physiological effects were similar. In these studies, accumulation in sinks and sources was attributed to growth arrest. The accumulation of unused carbohydrates in sinks suggests that sucrose is transported from the leaves to the roots at a higher rate than it is used in the sinks. Under these conditions, the sugar gradient required for long-distance transport is abolished, and carbohydrates accumulate in the leaves of treated plants because of a decrease in sink strength.[18]

To evaluate another key parameter of carbon metabolism in the roots, ethanol fermentation was assessed in both populations (Figure 7). Previous studies have reported the induction of aerobic fermentation after EPSPS inhibition in pea and *A. thaliana*.[16,18]

The roots of untreated plants of both populations exhibited similar PDC activity, whereas ADH activity was higher in NC-R roots. The roots of the NC-S plants exhibited an increase in PDC and ADH activities after treatment with 0.84 kg ha^{-1} and a trend to decline to the control values when the highest dose of glyphosate was applied. Resistant plants only exhibited induction of ADH activity at the highest treatment. Fermentative induction after

treatment with glyphosate cannot be easily explained, and the fermentative response can likely be considered a physiological effect induced under stress.[18]

Collectively, these results indicate that *A. palmeri* exhibits the physiological markers typical of the toxic consequences of glyphosate and reported previously in other species (with some deviations): total free amino acid accumulation, carbohydrate accumulation and ethanol fermentation induction (only at the lowest dose applied). Although both populations exhibited these symptoms, the pattern was not similar for all effects detected. Carbohydrate accumulation and ethanol fermentation were detected in NC-S and NC-R plants to a similar extent, although a trend to decline to control values was detected in NC-S plants treated with 2.52 kg ha^{-1}, most likely due to the severity of the dose. By contrast, individual and total free amino accumulation were less pronounced in NC-R plants than in NC-S plants. Free amino acid accumulation induced by glyphosate was alleviated in the resistant plants due to their reduced susceptibility to herbicide phytotoxicity, and thus this physiological marker was more directly related to the severity of the treatment and lethality. One important exception in the free amino acid content is that AAA content remained constant after glyphosate treatment, suggesting tight control of EPSPS activity *in vivo* at the transcriptional and post-transcriptional levels. Different physiological patterns of sensitive and resistant biotypes after glyphosate treatment have recently been described by metabolic profiling.[10] As proposed in that study, resistance to glyphosate in NC-R plants, although primarily conferred by the EPSPS gene amplification, may be complemented by other physiological responses, such as an anti-oxidative protective mechanism (detected in [10]) or the maintenance of a constant AAA content level detected in our study.

In conclusion, this study shows a complex regulation of EPSPS activity by transcriptional and post-transcriptional mechanisms. In both populations, the herbicide induced increase of the EPSPS protein, indicating a transcriptional regulation that can be useful in the new weed management strategy based on RNA interference technology (branded BioDirect™) to overcome glyphosate resistance in weeds.[64] No inherent differences in AAA content between

the biotypes in the absence of glyphosate were found, despite the massive amount of EPSPS enzyme detected in NC-R plants. Moreover, AAA content was maintained constant in resistant plants, even with the glyphosate-induced increase of EPSPS enzyme. These results indicate a post-transcriptional regulation at the level of EPSPS, whose signal remains unknown but can not be AAA content. On the other hand, it has been possible to describe new insights of the physiological manifestations of the evolved glyphosate resistance. The physiological markers that have been reported before after glyphosate treatment were detected in susceptible and resistant plants: Carbohydrate accumulation, induction of ethanol fermentation and free amino acid accumulation. Resistant plants accumulate less amino acids than susceptible plants and the effect of glyphosate on AAA content was almost abolished in resistant plants, suggesting that a constant free amino acid pool and AAA content are key parameters in complementing the resistance in NC-R population.

Funding

This work was financially supported by a grant from the Ministerio Español de Economía y Competitividad (AGL-2013-40567R). M. F-E and M. G-M received funding from fellowships through the Universidad Pública de Navarra.

ABBREVIATIONS USED

AAAs, aromatic amino acids; DHAPS, 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; PDC, pyruvate decarboxylase; PCR, polymerase chain reaction; ADH, alcohol dehydrogenase; ALS, acetolactate synthase

ACKNOWLEDGEMENTS

We thank Gustavo Garijo, Oscar Armendáriz and Amaia Crespo for technical assistance. Dr. Todd A. Gaines (Colorado State University, Fort Collins, CO, USA) is acknowledged for providing seeds of both populations.

REFERENCES

(1) Duke, S. O.; Powles, S. B. Glyphosate: a once-in-a-century herbicide. *Pest Manag. Sci.* **2008**, *64* (4), 319–325.

(2) Steinrucken, H. C.; Amrhein, N. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. *Biochem. Biophys. Res. Commun.* **1980**, *94* (4), 1207–1212.

(3) Heap, I. The International Survey of Herbicide Resistant Weeds. Online. Internet.

(4) Nandula, V. K. *Glyphosate Resistance in Crops and Weeds. History, Development, and Management*; Nandula, V. K., Ed.; John Wiley & Sons, Inc. Hoboken, New Jersey, **2010**.

(5) Livingston, M.; Fernandez-Cornejo, J.;Unger, J.; Osteen, C.; Schimmelpfennig, D.; Park, T.; Lambert, D. *The economics of glyphosate resistance management in corn and soybean production*. Economic Research Report No. (ERR-184), United States Department of Agriculture, **2015**

(6) Yu, W. C.; Zhang, R.; Li, R. Z.; Guo, S. D. Isolation and characterization of glyphosate-regulated genes in soybean seedlings. *Plant Sci.* **2007**, *172* (3), 497–504.

(7) Zhu, J.; Patzoldt, W.; Shealy, R. Transcriptome response to glyphosate in sensitive and resistant soybean. *J. Agric. Food Chem.* **2008**, *56* (15), 6355–6363.

(8) Ahsan, N.; Lee, D.-G.; Lee, K.-W.; Alam, I.; Lee, S.-H.; Bahk, J. D.; Lee, B.-H. Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. *Plant Physiol. Biochem.* **2008**, *46* (12), 1062–1070.

(9) Trenkamp, S.; Eckes, P.; Busch, M.; Fernie, A. R. Temporally resolved GC-MS-based metabolic profiling of herbicide treated plants treated reveals that changes in polar primary metabolites alone can distinguish herbicides of differing mode of action. *Metabolomics* **2009**, *5* (3*)*, 277–291.

(10) Maroli, A. S.; Nandula, V. K.; Dayan, F. E.; Duke, S. O.; Gerard, P.; Tharayil, N. Metabolic profiling and enzyme analyses indicate a potential role of antioxidant systems in complementing glyphosate resistance in an *Amaranthus palmeri* biotype. *J. Agric. Food Chem.* **2015**, *63* (41), 9199–9209.

(11) Lydon, J.; Duke, S. O. Glyphosate induction of elevated levels of hydroxybenzoic acids in higher-plants. *J. Agric. Food Chem.* **1988**, *36* (4), 813–818.

(12) Becerril, J. M.; Duke, S. O.; Lydon, J. Glyphosate effects on shikimate pathway products in leaves and flowers of velvetleaf. *Phytochemistry* **1989**, *28* (3), 695–699.

(13) De María, N.; Becerril, J. M.; García-Plazaola, J. I.; Hernández, A.; De Felipe, M. R.; Fernández-Pascual, M. New insights on glyphosate mode of action in nodular metabolism: Role of shikimate accumulation. *J. Agric. Food Chem.* **2006**, *54* (7), 2621–2628.

(14) Orcaray, L.; Igal, M.; Marino, D.; Zabalza, A.; Royuela, M. The possible role of quinate in the mode of action of glyphosate and acetolactate synthase inhibitors. *Pest Manag. Sci.* **2010**, *66* (3), 262–269.

(15) Zulet, A.; Gil-Monreal, M.; Villamor, J. G.; Zabalza, A.; van der Hoorn, R. A. L.; Royuela,

M. Proteolytic pathways induced by herbicides that inhibit amino acid biosynthesis. *PLoS One* **2013,** *8* (9),e73847.

(16) Zulet, A.; Gil-Monreal, M.; Zabalza, A.; van Dongen, J. T.; Royuela, M. Fermentation and alternative oxidase contribute to the action of amino acid biosynthesis-inhibiting herbicides. *J. Plant Physiol.* **2015**, *175* (1*)*, 102–112.

(17) Colombo, S. L.; Andreo, C. S.; Chollet, R. The interaction of shikimic acid and protein phosphorylation with PEP- carboxylase from the C_4 dicot *Amaranthus viridis*. *Phytochemistry* **2000**, *48* (I), 55–59.

(18) Orcaray, L.; Zulet, A.; Zabalza, A.; Royuela, M. Impairment of carbon metabolism induced by the herbicide glyphosate. *J. Plant Physiol.* **2012**, *169* (1), 27–33.

(19) Culpepper, A. S.; Grey, T.; Vencill, W. Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) confirmed in Georgia. *Weed Sci.* **2006**, *54* (4*)*, 620–626.

(20) Gaines, T. A; Zhang, W.; Wang, D.; Bukun, B.; Chisholm, S. T.; Shaner, D. L.; Nissen, S. J.; Patzoldt, W. L.; Tranel, P. J.; Culpepper, A. S.; Grey T.L.; Webster, T.M.; Vencill, W.K.; Sammons, R.D.; Jiang, J.; Preston, C.; Leach, J.E.; Westra, P. Gene amplification confers glyphosate resistance in *Amaranthus palmeri. Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (3), 1029–1034.

(21) Sammons, R. D.; Gaines, T. A. Glyphosate resistance: State of knowledge. *Pest Manag. Sci.* **2014**, *70* (9), 1367–1377.

(22) Lorentz, L.; Gaines, T. A; Nissen, S. J.; Westra, P.; Strek, H. J.; Dehne, H. W.; Ruiz-Santaella, J. P.; Beffa, R. S. Characterization of glyphosate resistance in *Amaranthus tuberculatus* populations. *J. Agric. Food Chem.* **2014**, *62* (32), 8134–8142.

(23) Chatham, L. A.; Wu, C.; Riggins, C. W.; Hager, A. G.; Young, B. G.; Gordon, K.; Tranel, P. J. EPSPS Gene amplification is present in the majority of glyphosate-resistant illinois waterhemp (*Amaranthus tuberculatus*) populations. *Weed Technol.* **2015**, *29* (1), 48–55.

(24) Nandula, V. K.; Wright, A. A.; Bond, J. a.; Ray, J. D.; Eubank, T. W.; Molin, W. T. EPSPS amplification in glyphosate-resistant spiny amaranth (*Amaranthus spinosus*): A case of gene transfer via interspecific hybridization from glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*). *Pest Manag. Sci.* **2014**, *70* (12), 1902–1909.

(25) Wiersma, A. T.; Gaines, T. A.; Preston, C.; Hamilton, J. P.; Giacomini, D.; Robin Buell, C.; Leach, J. E.; Westra, P. Gene amplification of 5-enol-pyruvylshikimate-3-phosphate synthase in glyphosate-resistant *Kochia scoparia. Planta* **2014**, *241* (2), 463–474.

(26) Salas, R. A.; Dayan, F. E.; Pan, Z.; Watson, S. B.; Dickson, J. W.; Scott, R. C.; Burgos, N. R. EPSPS gene amplification in glyphosate-resistant Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) from Arkansas. *Pest Manag. Sci.* **2012**, *68* (9), 1223–1230.

(27) Salas, R. A; Scott, R. C.; Dayan, F. E.; Burgos, N. R. EPSPS gene amplification in glyphosate-resistant italian ryegrass (*Lolium perenne* ssp. *multiflorum*) populations from arkansas (United States). *J. Agric. Food Chem.* **2015**, *63* (25), 5885–5893.

(28) Malone, J. M.; Morran, S.; Shirley, N.; Boutsalis, P.; Preston, C. EPSPS gene amplification in glyphosate-resistant *Bromus diandrus*. *Pest Manag. Sci.* **2015**, Epub ahead of print, doi:

10.1002/ps.4019.

(29) Chandi, A.; Milla-Lewis, S. R.; Giacomini, D.; Westra, P.; Preston, C.; Jordan, D. L.; York, A. C.; Burton, J. D.; Whitaker, J. R. Inheritance of evolved glyphosate resistance in a North Carolina Palmer amaranth (*Amaranthus palmeri*) Biotype. *Int. J. Agron.* **2012**, *2012*, 1–7.

(30) Labhilili, M.; Joudrier, P.; Gautier, M. F. Characterization of cDNAs encoding T*riticum durum* dehydrins and their expression patterns in cultivars that differ in drought tolerance. *Plant Sci.* **1995**, *112* (2), 219–230.

(31) Hoagland, D. R.; Arnon, D. I. The water-culture method for growing plants without soil. *Calif. Agric. Exp. Stn. Circ.* **1950**, *347* (347), 1–32.

(32) Koger, C. H.; Shaner, D. L.; Henry, W. B.; Nadler-Hassar, T.; Thomas, W. E.; Wilcut, J. W. Assessment of two nondestructive assays for detecting glyphosate resistance in horseweed (*Conyza canadensis*). *Weed Sci.* **2005**, *53* (5), 559–566.

(33) Cromartie, T. H.; Polge, N. D. An improved assay for shikimic acid and its use as monitor for the activity of sulfosate. In *Weed Science Society of America Proceedings*; 2000; pp 4, 121.

(34) Seefeldt, S.S.; Jensen,J.E.; Fuerst, P.Log -logistic analysis of herbicide dose-response relationships. *Weed Technol.* **1995**, *9* (2), 218–227.

(35) Hess, M.; Barralis, G.; Bleiholder, H. Use of the extended BBCH scale—general for the descriptions of the growth stages of mono- and dicotyledonous weed species. *Weed Res.* **1997**, *37* (6), 433–441.

(36) Koger, C. H.; Shaner, D. L.; Krutz, L. J.; Walker, T. W.; Buehring, N.; Henry, W. B.; Thomas, W. E.; Wilcut, J. W. Rice (*Oryza sativa*) response to drift rates of glyphosate. *Pest Manag. Sci.* **2005**, *61* (12), 1161–1167.

(37) Hoagland, R. E.; Jordan, R. H.; Teaster, N. D. Bioassay and characterization of several palmer amaranth (*Amaranthus palmeri*) biotypes with varying tolerances to glyphosate. *Am. J. Plant Sci.* **2013**, *4* (05), 1029–1037.

(38) Zulet, A.; Zabalza, A.; Royuela, M. Phytotoxic and metabolic effects of exogenous quinate on *Pisum sativum* L. *J. Plant Growth Regul.* **2013**, *37*, 779–788.

(39) Zinellu, A.; Sotgia, S.; Posadino, A. M.; Pasciu, V.; Perino, M. G.; Tadolini, B.; Deiana, L.; Carru, C. Highly sensitive simultaneous detection of cultured cellular thiols by laser induced fluorescence-capillary electrophoresis. *Electrophoresis* **2005**, *26* (6), 1063–1070.

(40) Zabalza, A.; Orcaray, L.; Gaston, S.; Royuela, M. Carbohydrate accumulation in leaves of plants treated with the herbicide chlorsulfuron or imazethapyr is due to a decrease in sink strength. *J. Agric. Food Chem.* **2004**, *52* (25), 7601–7606.

(41) Gaston, S.; Zabalza, A.; González, E. M.; Arrese-Igor, C.; Aparicio-Tejo, P. M.; Royuela, M. Imazethapyr, an inhibitor of the branched-chain amino acid biosynthesis, induces aerobic fermentation in pea plants. *Physiol. Plant.* **2002**, *114* (4), 524–532.

(42) Whitaker, J. R.; Burton, J. D.; York, A. C.; Jordan, D. L.; Chandi, A. Physiology of glyphosate-resistant and glyphosate-susceptible palmer amaranth (*Amaranthus palmeri*)

biotypes collected from North Carolina. *Int. J. Agron.* **2013**, *2013*, 1–6.

(43) Culpepper, A S.; Whitaker, J. R.; MacRae, A. W.; York, A. C. Distribution of glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) in Georgia and North Carolina during 2005 and 2006. *J. Cotton Sci.* **2008**, *12* (3), 306–310.

(44) Mohseni-Moghadam, M.; Schroeder, J.; Ashigh, J. Mechanism of resistance and inheritance in glyphosate resistant Palmer amaranth (*Amaranthus palmeri*) populations from New Mexico, USA. *Weed Sci.* **2013**, *61* (4), 517–525.

(45) Ribeiro, D. N.; Pan, Z.; Duke, S. O.; Nandula, V. K.; Baldwin, B. S.; Shaw, D. R.; Dayan, F. E. Involvement of facultative apomixis in inheritance of EPSPS gene amplification in glyphosate-resistant *Amaranthus palmeri*. *Planta* **2014**, *239* (1), 199–212.

(46) Gaines, T. A; Shaner, D. L.; Ward, S. M.; Leach, J. E.; Preston, C.; Westra, P. Mechanism of resistance of evolved glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*). *J. Agric. Food Chem.* **2011**, *59* (11), 5886–5889.

(47) Vila-Aiub, M. M.; Goh, S. S.; Gaines, T. A; Han, H.; Busi, R.; Yu, Q.; Powles, S. B. No fitness cost of glyphosate resistance endowed by massive EPSPS gene amplification in *Amaranthus palmeri*. *Planta* **2014**, *239* (4), 793–801.

(48) Teaster, N. D.; Hoagland, R. E. Characterization of glyphosate resistance in cloned *Amaranthus palmeri* plants. *Weed Biol. Manag.* **2014**, *14* (1), 1–10.

(49) Alarcón-Reverte, R.; García, A.; Watson, S. B.; Abdallah, I.; Sabaté, S.; Hernández, M. J.; Dayan, F. E.; Fischer, A. Concerted action of target-site mutations and high EPSPS activity in glyphosate-resistant junglerice (*Echinochloa colona)* from California. *Pest Manag. Sci.* **2015**, *71* (530), 996–1007.

(50) Baerson, S. R.; Rodriguez, D. J.; Tran, M.; Feng, Y.; Biest, N. a; Dill, G. M. Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. *Plant Physiol.* **2002**, *129* (3), 1265–1275.

(51) Baerson, S. R.; Rodriguez, D. J.; Biest, N. a.; Tran, M.; You, J.; Kreuger, R. W.; Dill, G. M.; Pratley, J. E.; Gruys, K. J. Investigating the mechanism of glyphosate resistance in rigid ryegrass (*Lolium ridigum*). *Weed Sci.* **2002**, *50* (6), 721–730.

(52) Garg, B.; Vaid, N.; Tuteja, N. In-silico analysis and expression profiling implicate diverse role of EPSPS family genes in regulating developmental and metabolic processes. *BMC Res. Notes* **2014**, *7* (1), 58.

(53) Maeda, H.; Dudareva, N. The shikimate pathway and aromatic amino acid biosynthesis in plants. *Annu. Rev. Plant Biol.* **2012**, *63* (1), 73–105.

(54) Tohge, T.; Watanabe, M.; Hoefgen, R.; Fernie, A. R. Shikimate and phenylalanine biosynthesis in the green lineage. *Front. Plant Sci.* **2013**, *4*, Article 62.

(55) Tzin, V.; Galili, G. New Insights into the shikimate and aromatic amino acids biosynthesis pathways in plants. *Mol. Plant* **2010**, *3* (6), 956–972.

(56) Takatsuji, H.; Mori, M.; Benfey, P. N.; Ren, L.; Chua, N. Characterization of a zinc finger DNA-binding protein expressed specifically in *Petunia* petals and seedlings. *EMBO J.* **1988**,

1 (1), 241–249.

(57) Galili, G.; Hofgen, R. Metabolic engineering of amino acids and storage proteins in plants. *Metab. Eng.* **2002**, *4* (1), 3–11.

(58) Liu, Y.; Zhang, Y.; Liu, Y.; Lu, W.; Wang, G. Metabolic effects of glyphosate on transgenic maize expressing a G2-EPSPS gene from *Pseudomonas fluorescens*. *J. Plant Biochem. Biotec.* **2015**, *24* (2), 233–241.

(59) Vivancos, P. D.; Driscoll, S. P.; Bulman, C. A.; Ying, L.; Emami, K.; Treumann, A.; Mauve, C.; Noctor, G.; Foyer, C. H. Perturbations of amino acid metabolism associated with glyphosate-dependent inhibition of shikimic acid metabolism affect cellular redox homeostasis and alter the abundance of proteins involved in photosynthesis and photorespiration. *Plant Physiol.* **2011**, *157* (1), 256–268.

(60) Moldes, C. A.; Medici, L. O.; Abrahao, O. S.; Tsai, S. M.; Azevedo, R. A. Biochemical responses of glyphosate resistant and susceptible soybean plants exposed to glyphosate. *Acta Physiol. Plant.* **2008**, *30* (4), 469–479.

(61) Petersen, I. L.; Hansen, H. C. B.; Ravn, H. W.; Sørensen, J. C.; Sørensen, H. Metabolic effects in rapeseed (*Brassica napus* L.) seedlings after root exposure to glyphosate. *Pestic. Biochem. Physiol.* **2007**, *89* (3), 220–229.

(62) Tzin, V.; Malitsky, S.; Zvi, M. M. Ben; Bedair, M.; Sumner, L.; Aharoni, A.; Galili, G. Expression of a bacterial feedback-insensitive 3-deoxy-d-arabino-heptulosonate 7-phosphate synthase of the shikimate pathway in Arabidopsis elucidates potential metabolic bottlenecks between primary and secondary metabolism. *New Phytol.* **2012**, *194* (2), 430–439.

(63) Armendáriz, O.; Miriam, G.-M.; Zulet, A.; Zabalza, A.; Royuela, M. Both foliar and residual applications of herbicides that inhibit amino acid biosynthesis induce alternative respiration and aerobic fermentation in pea roots. *Plant Biol.* **2015**, E-pub ahead of print. doi:100.1111/plb.12412.

(64) Shaner, D. L.; Beckie, H. J. The future for weed control and technology. *Pest Manag. Sci.* **2014**, *70* (9), 1329–1339.

FIGURE CAPTIONS

Figure 1.

A. Dose-response of the shoot dry weight accumulation in glyphosate-susceptible (NC-S) and –resistant (NC-R) *Amaranthus palmeri* plants over the 5 days following glyphosate application, as a percentage of the untreated plants. Log-logistic dose-response curves.

B. Shikimate content in the leaves of glyphosate-susceptible (NC-S) and -resistant (NC-R) *Amaranthus palmeri* plants 5 days after spraying with glyphosate. Means ± SE (n=4-7).

Figure 2. Effect of glyphosate concentration on shikimate levels in excised leaf discs from glyphosate-susceptible ((NC-S) and –resistant (NC-R) *Amaranthus palmeri* biotypes. Means ± SE (n=16).

Figure 3.

A: *Amaranthus palmeri* genomic copy number of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) relative to acetolactate synthase (ALS) in glyphosate-susceptible (NC-S) and -resistant (NC-R) biotypes. Mean ± SE (n=9).

B: EPSPS protein levels in the leaves of glyphosate-susceptible (NC-S) and -resistant (NC-R) *Amaranthus palmeri* populations untreated (0) or treated with 0.84 or 2.52 kg ha^{-1} glyphosate, 3 days after application. Top: A representative sample blot is shown, and μg protein loaded/well is indicated. Bottom: Normalized EPSPS quantity. Mean ± SE (n=3).

C: Effect of glyphosate on EPSPS activity in leaves of glyphosate-susceptible (NC-S) and -resistant (NC-R) *Amaranthus palmeri* populations (3 days after application). Means ± SE (n=4).

Figure 4.

Total amino acid content in leaves (A) and roots (C) of untreated plants of glyphosate-susceptible (NC-S) and -resistant (NC-R) *Amaranthus palmeri* populations. The effect of glyphosate on the total amino acid content in leaves (B) and roots (D) 3 days after treatment. Means ± SE (n=6-9). The symbol * indicates significant differences between control plants (without herbicide) of each population. Different capital letters in the NC-R population and different lowercase letters in the NC-S population indicate significant differences between treatments (p value≤0.05).

Figure 5

Aromatic amino acid (Phe, Tyr and Trp) content in leaves (A) and roots (C) of untreated plants of glyphosate-susceptible (NC-S) and -resistant (NC-R) *Amaranthus palmeri* populations.

Effect of glyphosate on aromatic amino acid (Phe, Tyr, Trp) content with respect to the total free amino acids (percent of the total free amino acids) in leaves (B) and roots (D) 3 days after treatment. Means ± SE (n=6-9). Different capital letters for the NC-R population and different lowercase letters for the NC-S population indicate significant differences between treatments (p value≤0.05). The letters embedded in the columns indicate differences for each individual amino acid, and letters above the columns indicate differences between the sums of aromatic amino acids.

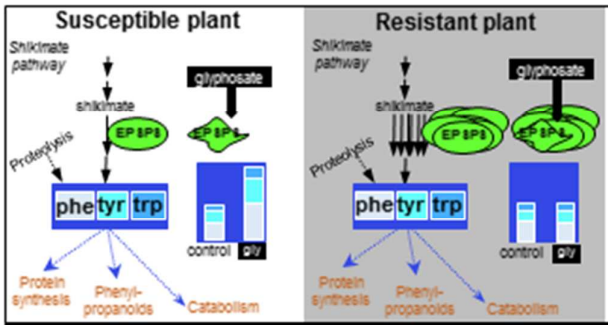
Figure 6. Effect of glyphosate on total soluble sugar (fructose, glucose and sucrose) and starch contents in leaves and roots of glyphosate-susceptible (NC-S) and -resistant (NC-R) *Amaranthus palmeri* populations (3 days after application). Means ± SE (n=6-9). The symbol * indicates significant differences between control plants (without herbicide) of each population. Different capital letters for the NC-R population and different lowercase letters for the NC-S population indicate significant differences between treatments (p value≤0.05).

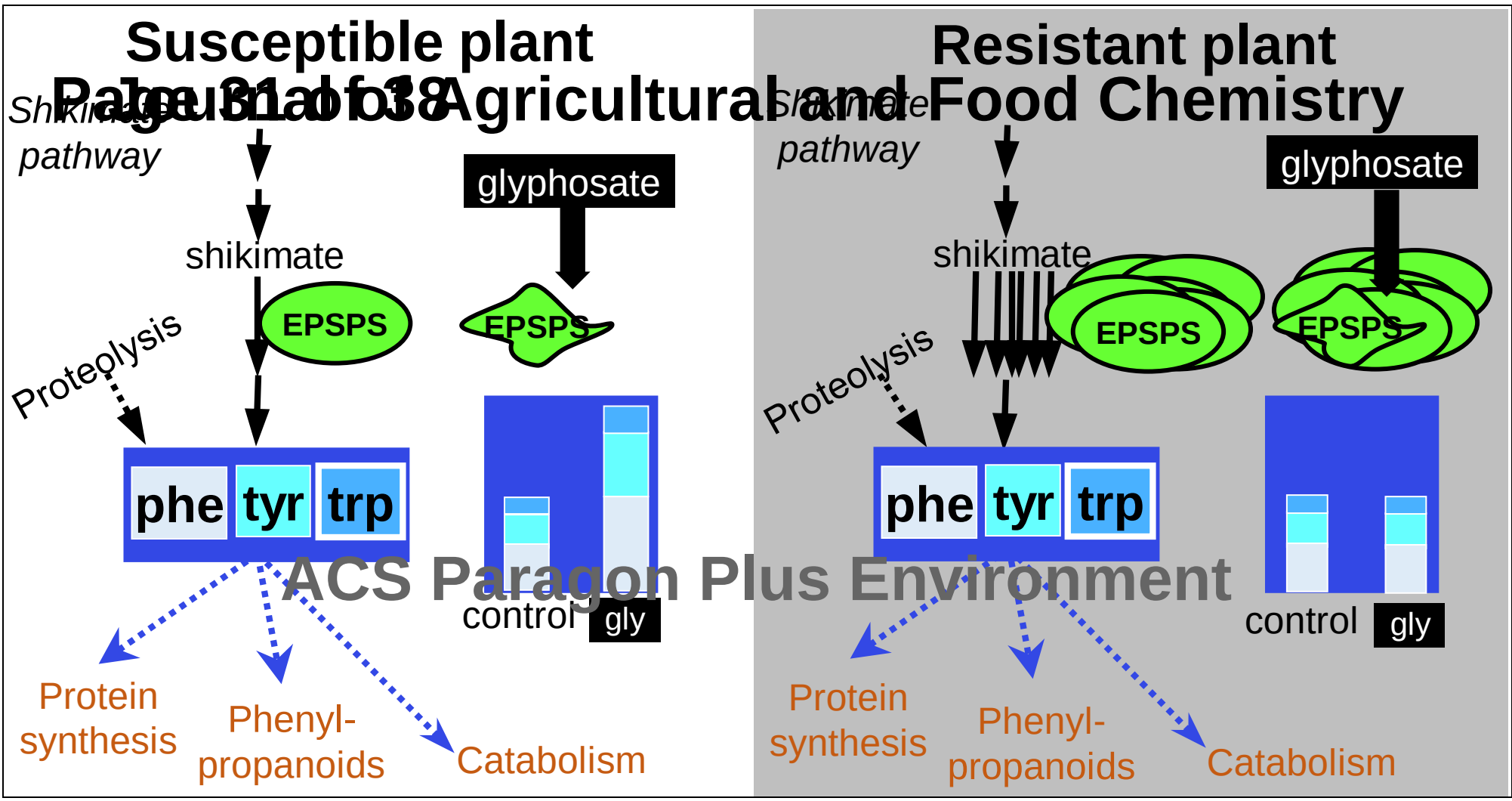
Figure 7. Effect of glyphosate on pyruvate decarboxylase and alcohol dehydrogenase enzymatic activities in roots of glyphosate-susceptible (NC-S) and -resistant (NC-R) *Amaranthus palmeri* populations (3 days after application). Means ± SE (n=6-9). The symbol * highlights significant differences between control plants (without herbicide) of each population. Different capital letters for the NC-R population and different lowercase letters for the NC-S population indicate significant differences between treatments (p value≤0.05).

Supplementary Information Figure 1. Effect of glyphosate on free amino acid content in leaves of glyphosate-resistant (NC-R) and -susceptible (NC-S) *Amaranthus palmeri* populations (3 days after application). When not indicated Y-axis units are μmol g^{-1} fresh weight. Means ± SE (n=6-9). The symbol * highlights significant differences between control plants (without herbicide) of each population. Different capital letters for the NC-R population and different lowercase letters for the NC-S population indicate significant differences between treatments (p value≤0.05).

Supplementary Information Figure 2. Effect of glyphosate on free amino acid content in roots of glyphosate-resistant (NC-R) and -susceptible (NC-S) *Amaranthus palmeri* populations (3 days after application). When not indicate Y-axis units are μmol g^{-1} fresh weight. Means ± SE (n=6-9). The symbol * highlights significant differences between control plants (without herbicide) of each population. Different capital letters for the NC-R population and different lowercase letters for the NC-S population indicate significant differences between treatments (p value≤0.05).

TABLE OF CONTENTS





ACS Paragon Plus Environment

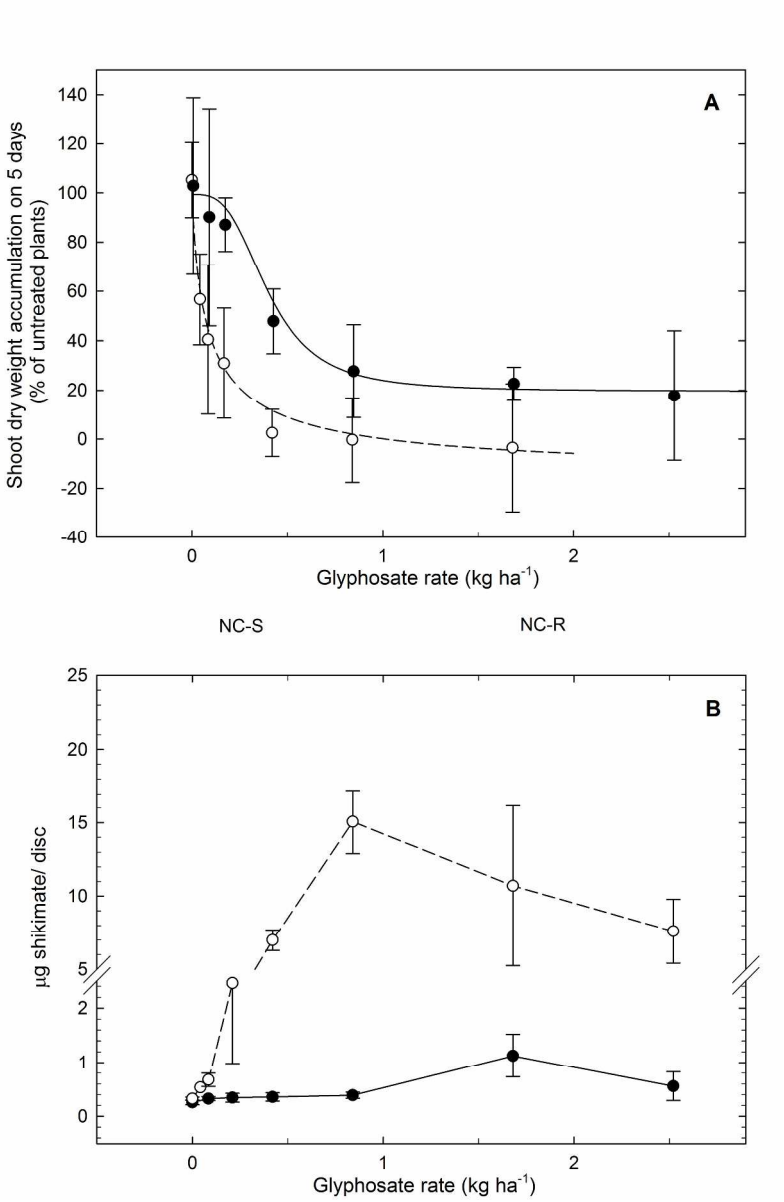


Figure 1.
A. Dose-response of the shoot dry weight accumulation in glyphosate-susceptible (NC-S) and –resistant (NC-R) Amaranthus palmeri plants over the 5 days following glyphosate application, as a percentage of the untreated plants. Log-logistic dose-response curves.
B. Shikimate content in the leaves of glyphosate-susceptible (NC-S) and -resistant (NC-R) Amaranthus palmeri plants 5 days after spraying with glyphosate. Means ± SE (n=4-7).

230x354mm (300 x 300 DPI)

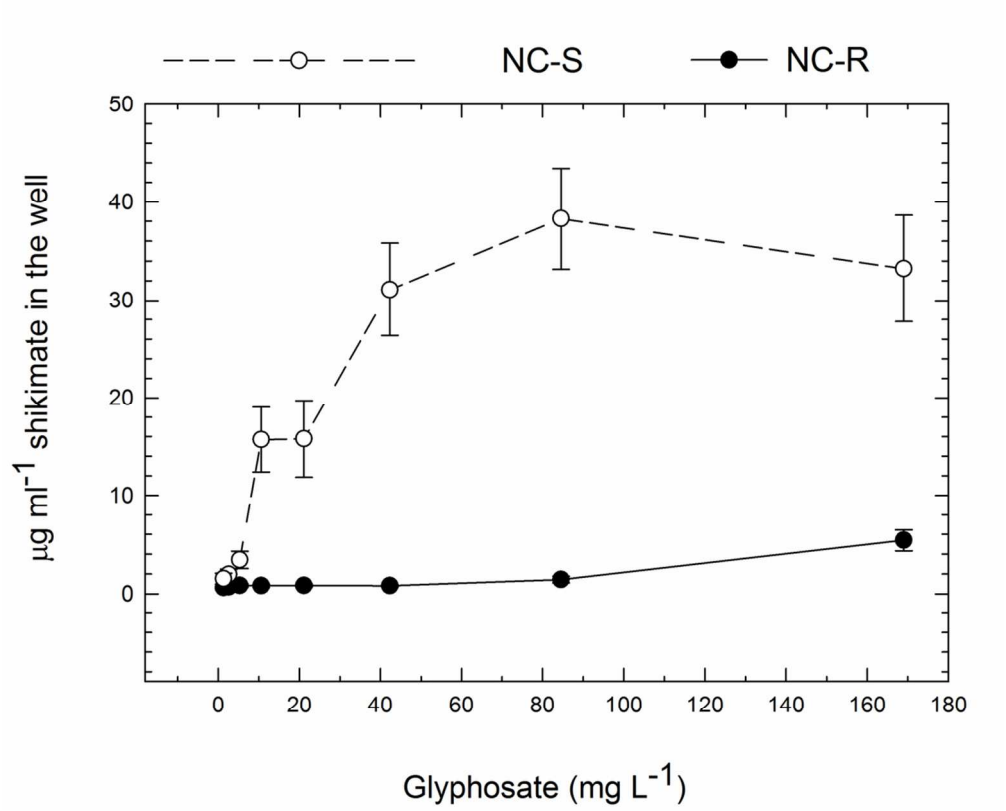


Figure 2. Effect of glyphosate concentration on shikimate levels in excised leaf discs from glyphosate-susceptible ((NC-S) and –resistant (NC-R) Amaranthus palmeri biotypes. Means ± SE (n=16).
101x82mm (300 x 300 DPI)

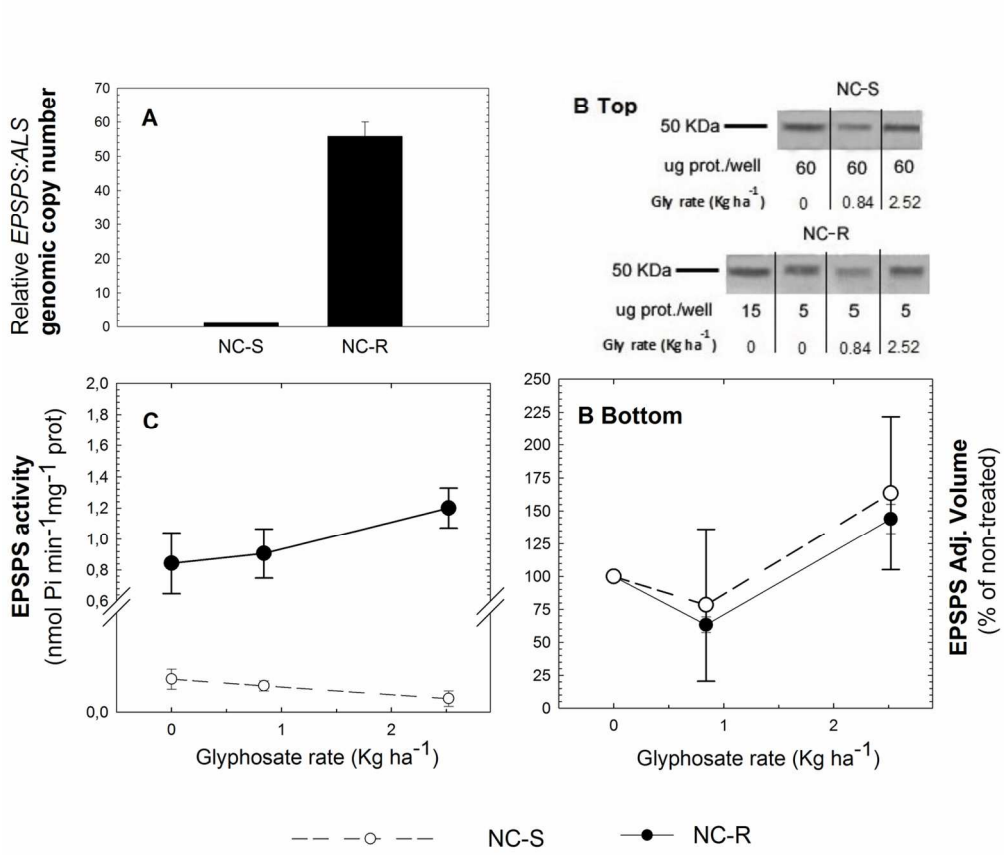


Figure 3.
A: Amaranthus palmeri genomic copy number of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) relative to acetolactate synthase (ALS) in glyphosate-susceptible (NC-S) and -resistant (NC-R) biotypes. Mean ± SE (n=9).
B: EPSPS protein levels in the leaves of glyphosate-susceptible (NC-S) and -resistant (NC-R) Amaranthus palmeri populations untreated (0) or treated with 0.84 or 2.52 kg ha-1 glyphosate, 3 days after application. Top: A representative sample blot is shown, and μg protein loaded/well is indicated. Bottom: Normalized EPSPS quantity. Mean ± SE (n=3).
C: Effect of glyphosate on EPSPS activity in leaves of glyphosate-susceptible (NC-S) and -resistant (NC-R) Amaranthus palmeri populations (3 days after application). Means ± SE (n=4).

128x110mm (300 x 300 DPI)

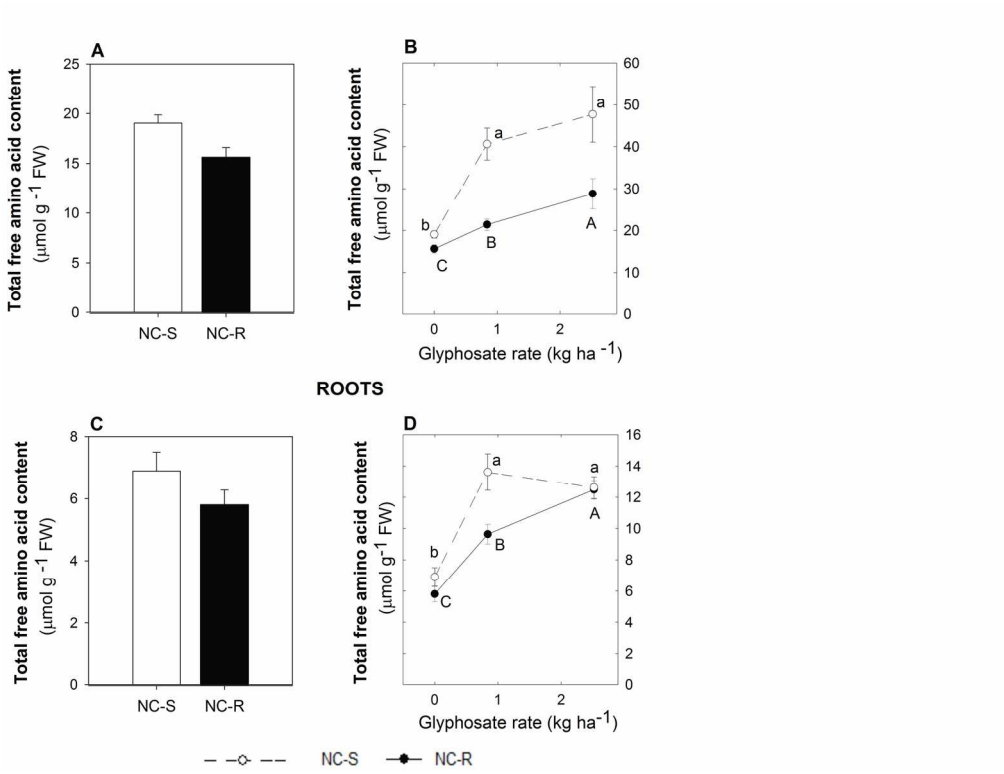


Figure 4.
Total amino acid content in leaves (A) and roots (C) of untreated plants of glyphosate- susceptible (NC-S) and -resistant (NC-R) Amaranthus palmeri populations. The effect of glyphosate on the total amino acid content in leaves (B) and roots (D) 3 days after treatment. Means ± SE (n=6-9). The symbol * indicates significant differences between control plants (without herbicide) of each population. Different capital letters in the NC-R population and different lowercase letters in the NC-S population indicate significant differences between treatments (p value<0.05).

170x130mm (300 x 300 DPI)

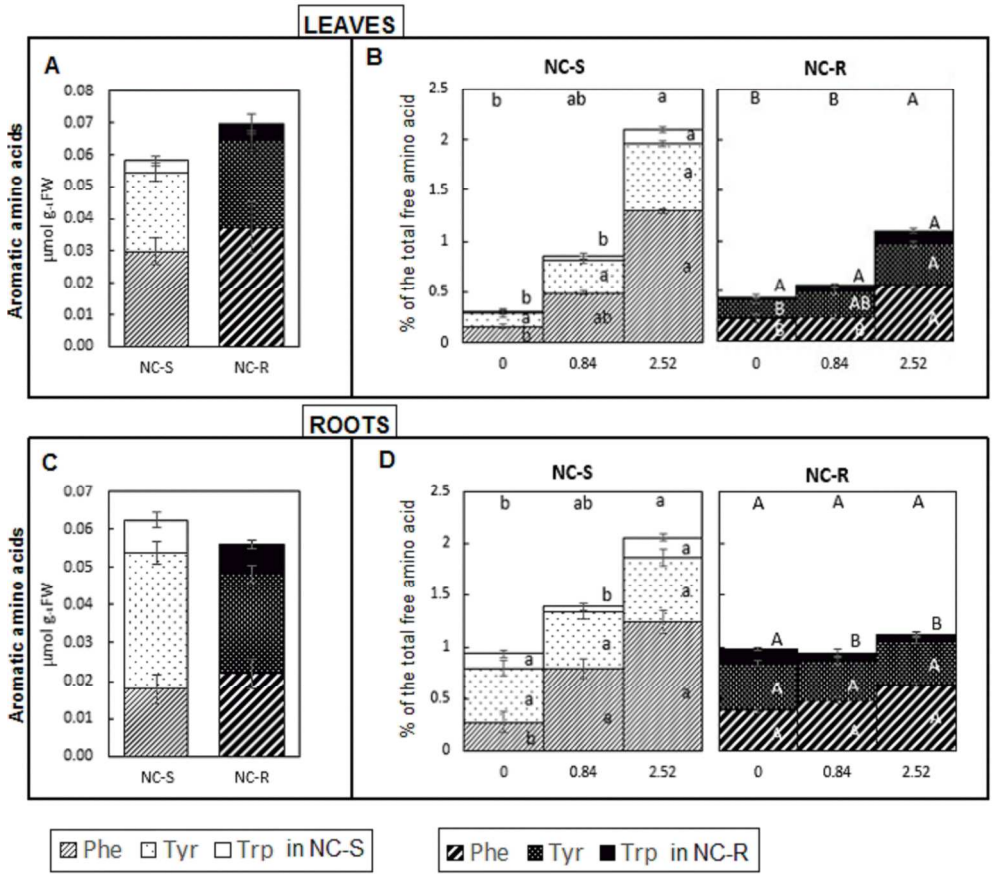


Figure 5
Aromatic amino acid (Phe, Tyr and Trp) content in leaves (A) and roots (C) of untreated plants of glyphosate-susceptible (NC-S) and -resistant (NC-R) Amaranthus palmeri populations. Effect of glyphosate on aromatic amino acid (Phe, Tyr, Trp) content with respect to the total free amino acids (percent of the total free amino acids) in leaves (B) and roots (D) 3 days after treatment. Means ± SE (n=6-9). Different capital letters for the NC-R population and different lowercase letters for the NC-S population indicate significant differences between treatments (p value<0.05). The letters embedded in the columns indicate differences for each individual amino acid, and letters above the columns indicate differences between the sums of aromatic amino acids.

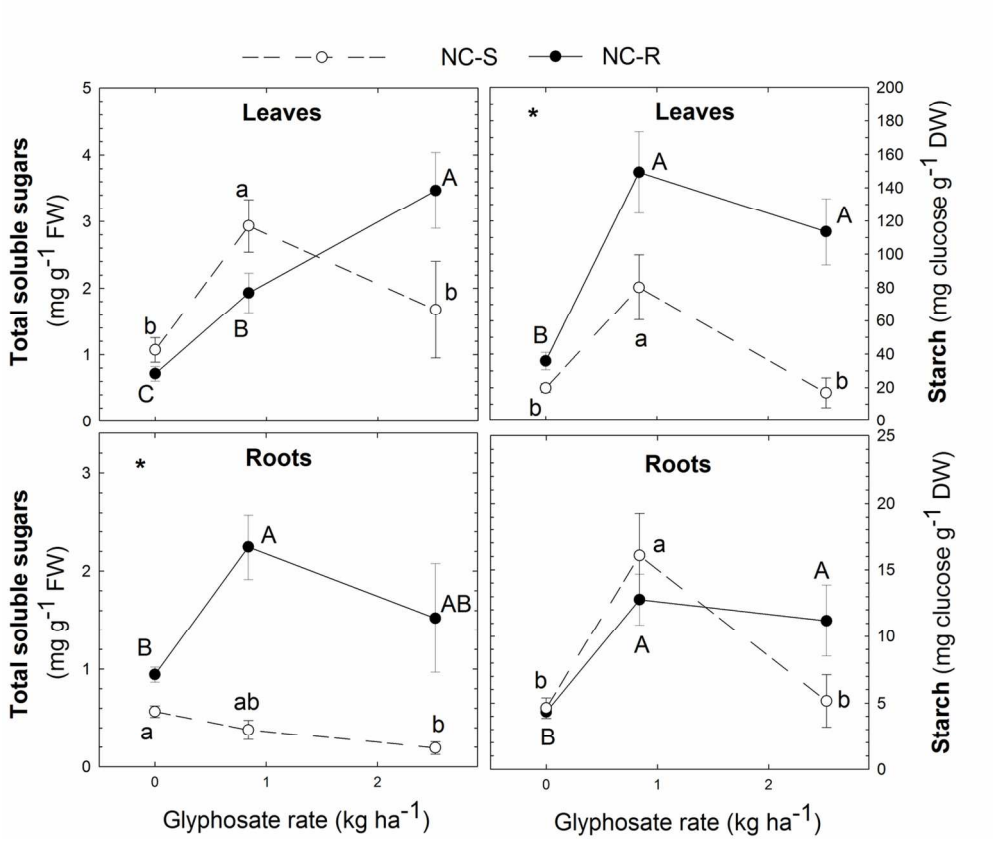


Figure 6. Effect of glyphosate on total soluble sugar (fructose, glucose and sucrose) and starch contents in leaves and roots of glyphosate-susceptible (NC-S) and -resistant (NC-R) Amaranthus palmeri populations (3 days after application). Means ± SE (n=6-9). The symbol * indicates significant differences between control plants (without herbicide) of each population. Different capital letters for the NC-R population and different lowercase letters for the NC-S population indicate significant differences between treatments (p value<0.05).
125x105mm (300 x 300 DPI)

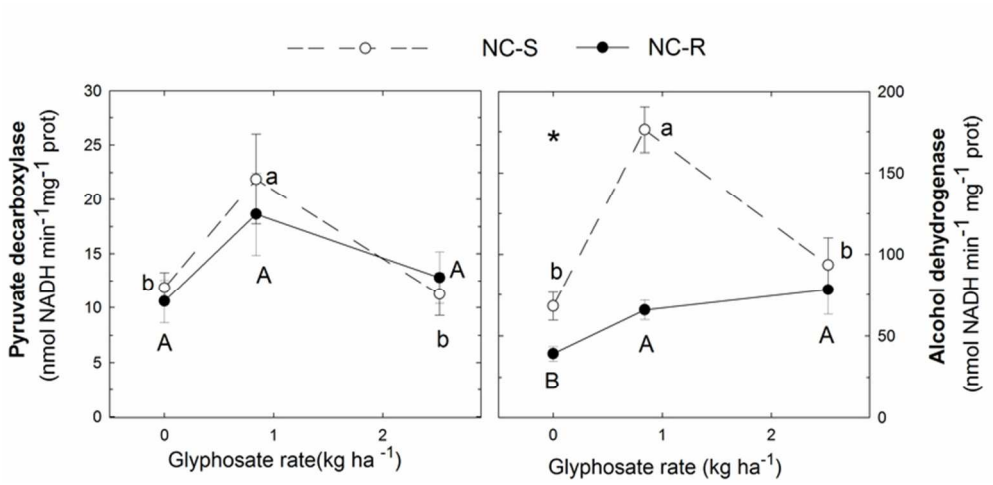


Figure 7. Effect of glyphosate on pyruvate decarboxylase and alcohol dehydrogenase enzymatic activities in roots of glyphosate-susceptible (NC-S) and -resistant (NC-R) Amaranthus palmeri populations (3 days after application). Means ± SE (n=6-9). The symbol * highlights significant differences between control plants (without herbicide) of each population. Different capital letters for the NC-R population and different lowercase letters for the NC-S population indicate significant differences between treatments (p value<0.05).
72x34mm (300 x 300 DPI)