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Enhancement of glyphosate efficacy on *Amaranthus palmeri* by exogenous quinate application

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ABSTRACT.

Glyphosate is a widely used herbicide targeting the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) in the aromatic amino acid biosynthesis pathway (shikimate pathway) and provoking accumulation of quinate, a secondary metabolite synthesized through a side branch of this pathway. The objective of this work was to evaluate whether the efficacy of glyphosate activity in *Amaranthus palmeri* is enhanced by quinate application one day after herbicide treatment. To this end, one glyphosate-sensitive and one glyphosate-resistant (due to EPSPS gene amplification) population of *A. palmeri* were used. The 3-day time course study of the quinate treatment alone showed quinate, Tyr and Phe accumulation in both populations. When the herbicide was applied alone at 0.25× the recommended dose, no phytotoxicity or glyphosate effects were detected in the sensitive population 3 days after treatment, but the combined treatment with quinate was lethal, and markers of herbicide activity at the amino acid level could be detected. In the resistant population, an important metabolic perturbation in the flux of the shikimate pathway was detected in the combined treatment. These results raise the possibility of the joint application of quinate and glyphosate to enhance glyphosate efficacy while lowering doses in the sensitive population.

1. Introduction

Glyphosate is a broad-spectrum herbicide whose mode of action is the inhibition of EPSPS in the shikimate pathway, which is responsible for the synthesis of the aromatic amino acids (AAA) phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr) (Duke & Powles, 2008; Amrhein et al., 1980). The shikimate pathway is a key metabolic pathway in plants that links carbon and nitrogen metabolism. This pathway leads to aromatic amino acid synthesis and serves as a major sink for intermediate metabolites from the central carbon metabolism pathways. Any perturbations in the shikimate pathway (e.g., caused by glyphosate treatment) would lead to a disruption in aromatic amino acid synthesis and alter the carbon and nitrogen metabolism (Orcaray et al., 2012; Zulet et al., 2013a; Zulet et al., 2015), affecting many plant physiological processes that could be associated with glyphosate toxicity, such as mineral nutrition, photosynthesis or plant and oxidative status (Gomes et al., 2014), although the precise mechanisms by which plants die after glyphosate treatment remain unclear. Indeed, glyphosate is a specific inhibitor of the EPSPS enzyme, but the effects of this inhibition on the shikimate pathway itself and the regulation of the AAA pathway remain poorly understood in plants (Maeda & Dudareva, 2012). Glyphosate was described to induce the first enzyme of the pathway, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS) (Pinto et al., 1988), and the expression of EPSPS (Baerson et al., 2002; Mao et al., 2016; Fernández-Escalada et al., 2017). Recently, glyphosate has been shown to induce a general accumulation of the transcripts corresponding to genes of the AAA pathway leading to the synthesis of chorismate (Fernández-Escalada et al., 2017). Another well-known effect of glyphosate on the shikimate pathway is the accumulation of the compounds upstream of the EPSPS, as occurs for shikimate, protocatechuate and quinate (Lydon & Duke, 1988; Becerril et al., 1989; Orcaray et al., 2010).

Quinate is formed in a branch pathway of the shikimate pathway, and it is considered a reserve compound of the pathway (Boudet, 2012). Quinate accumulation in glyphosate-treated plants raised the question of the role of quinate accumulation in the toxicity of the herbicide. If it

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were possible to mimic the effect of glyphosate by applying quinate, exogenous application of this compound could potentially be used as a commercial herbicide treatment. Therefore, residual and foliar exogenous quinate were tested on pea plants, and although both treatments affected plant growth, only the residual application was lethal (Zulet et al., 2013b). More recently, the patterns of the shikimate pathway and phenylpropanoids after glyphosate or quinate feeding have been compared (Zabalza et al., 2017). Although both compounds alter the shikimate pathway (glyphosate blocks EPSPS and quinate is an alternative source of carbon for the shikimate pathway), no common effects were found, suggesting that the role of quinate in the toxicity of glyphosate is not mediated by a common alteration of the regulation of the shikimate pathway (Zabalza et al., 2017).

The intensive and continuous use of glyphosate has led to the emergence of glyphosate-resistant (GR) weed populations (Powles, 2008). One of the most damaging GR weed species is *Amaranthus palmeri* S. Wats (Culpepper et al., 2006; Powles & Yu, 2010), and the most important target site mechanism of glyphosate resistance is conferred by gene amplification of EPSPS, which leads to a massive production of the enzyme EPSPS (Gaines et al., 2010). Combined application of more than one herbicide can be a useful tool to increase the diversity of weed species to be controlled. Crops that are altered to exhibit resistance to herbicides with two different mechanisms of action provide new opportunities for the control of herbicide-resistant weeds. The control of GR *A. palmeri* in glyphosate, 2,4-D, and glufosinate-tolerant cotton crops has been achieved after mixing the three herbicides (Merchant et al., 2014). Nevertheless, this management practice continues to mix synthetic herbicides, which, in the end, will increase selection pressure for the evolution of other resistant weeds (Powles & Yu, 2010).

Natural-product-based compounds, such as quinate, are considered safer than synthetic herbicides because of their often relatively shorter persistence in the environment than that of synthetic herbicides (Dayan et al., 2012), which is desirable from an environmental point of view. Enhancement of the efficacy of glyphosate by combined application with biological compounds, such as quinate, would be a new opportunity for glyphosate-resistant weed control, such as *A. palmeri*. As quinate and glyphosate affect the shikimate pathway differently, it can be hypothesized that they may interact in the pathway by enhancing the toxicity process (Kirkwood, 1991). In this context, it makes sense to study the joint effect of both compounds by applying quinate after glyphosate. The supply of exogenous quinate to the previously inhibited pathway at the level of EPSPS might result in exacerbated glyphosate toxicity by increasing the accumulation of the compounds upstream of the EPSPS.

If the efficacy of glyphosate were increased with quinate application, it would be possible to control *A. palmeri* with lower herbicide rates. In addition to this potential practical application, the present study provides an opportunity to obtain new insights into how the AAA pathway is regulated and how glyphosate and/or quinate may affect this regulation. The gene amplification resistance mechanism found in *A. palmeri* offers us the opportunity to study the effect of EPSPS overexpression due to extra EPSPS gene copies on the regulation of the shikimate pathway. Understanding the precise metabolism of resistant and sensitive populations might enable the adoption of alternative herbicides for weed management practices.

The present work aims to study whether the toxicity and physiological effects of glyphosate are affected by its combined application with quinate and whether the effects are different in GR plants. Furthermore, this study explores the impact of EPSPS overexpression on the regulation of the AAA biosynthetic pathway after glyphosate and/or quinate treatments. To this end, the response of glyphosate-sensitive (GS) and GR populations of *A. palmeri* was evaluated at the level of parameters of the AAA pathway and free amino acid content.

2. Materials and methods

2.1. Plant material and treatment application

A. palmeri GS and GR biotypes were originally collected from North Carolina (USA) (Chandi et al., 2012; Fernández-Escalada et al., 2016). The resistance mechanism of the GR biotype has been described to be EPSPS gene amplification (Chandi et al., 2012), with 47.5 more gene copies in GR than in GS plants (Fernández-Escalada et al., 2016).

Seeds were surface sterilized and germinated as described previously (Fernández-Escalada et al., 2016). The seeds were then transferred to 2.7 L tanks in a phytotron and grown in aerated hydroponic culture under controlled conditions. At three weeks of age, the plants of each population were divided into four groups: one was used as a control, another was used to assess the effect of quinate, another was used to assess the effect of the herbicide, and another was used to assess the effect of quinate applied after glyphosate. Throughout the course of the experiment, the plants remained in the vegetative phenological stage.

A different concentration of glyphosate to combine with quinate was chosen for each population. Nonlethal, low concentrations were used so that the potential enhancement of toxicity when applied in combination with quinate could be detected. Glyphosate was applied (commercial formula, Glyfos, 360 g a.e. L^{-1}, isopropylamine salt, Bayer Garden, Valencia, Spain) at 0.25 and 1 times the recommended field dose (0.84 kg ha^{-1}, (Culpepper et al., 2006)) in the GS population and at 0.5 and 3 times the recommended field dose in the GR population. Quinate was applied at the leaves at a concentration of 400 mM in 5.4% of the adjuvant sodium lauryl sulfate (Zulet et al., 2013b) (commercial formula Biopower 27.65% (p/v) (Bayer Crop Science, Madrid, Spain). Plants of the combined treatment were sprayed with 0.25 times or 0.5 times the recommended field dose (GS and GR populations, respectively) and sprayed with quinate 1 day later. Control plants were sprayed with adjuvant only.

Treatments were performed using an aerograph (Junior Start model; Definik; Sagola, Vitoria-Gasteiz, Spain). Leaf samples were taken 3 days after glyphosate treatment applied alone. In the case of the combined treatment, plants were harvested 3 and 2 days after glyphosate and quinate treatments, respectively. Moreover, in the control and quinate treatments, the study included harvest at days 0, 1 and 3. At harvest, samples were obtained and immediately frozen in liquid nitrogen and stored at −80 °C for analytical determinations. GS and GR plants treated with 1 and 3 times the recommended field dose were only used for visual assessment of lethality.

2.2. Analytical determinations

2.2.1. EPSPS and DAHPS immunoblotting

Proteins were separated by 12.5% SDS-PAGE, and immunoblots were produced according to standard techniques. EPSPS and DAHPS immunoblotting was performed as described previously (Fernández-Escalada et al., 2017).

2.2.2. Enzymatic activities

Protein extraction for chorismate mutase (CM) and anthranilate synthase (AS) and activity assays was developed as described previously (Fernández-Escalada et al., 2017) using leaves of treated plants.

2.2.3. Shikimate determination

The determination of the content of shikimate in leaf disks of treated plants was determined spectrophotometrically as described previously (Fernández-Escalada et al., 2016).

2.2.4. Quinate

The extraction of quinate from leaves of treated plants was performed in trichloroacetic acid (TCA) as described previously (Orcaray

et al., 2010). Quinate levels were analyzed by ion chromatography in a 940 Professional IC Vario 2 instrument (Metrohm AG; Herisau: Suiza) equipped with a Metrosep A Supp16 150/4.0 (Metrohm AG; Herisau: Suiza) column at 45 °C. The solvents were ultrapure water (solvent A) and 60 mM NaOH (solvent B) at a flow rate of 1 mL min^{-1}. The gradient was as follows: 90% in A and 10% in B from 0 to 10 min; linear transition from 90 to 0% in A and from 10 to 100% in B from 10 to 18 min; 100% in B from 18 to 26 min; linear transition from 0 to 90% in A and from 100 to 10% in B from 26 to 28 min; and 90% in A and 10% in B from 28 to 40 min. Detection was performed by conductivity.

2.2.5. Free amino acid determination

The extraction of amino acids from leaves was performed in HCl. After protein precipitation, amino acid concentrations were measured in the supernatant using capillary electrophoresis equipped with a laser-induced fluorescence detector, as previously described (Orcaray et al., 2010).

2.3. Statistical analysis

The mean was used as a measure of central tendency and the SE as a measure of dispersion. Each mean value was calculated using samples collected from different individual plants as biological replicates ($n = 4$).

In the experiment investigating the time-course of quinate (Fig. 1), untreated plants and plants treated with quinate of each genotype on a given day were compared by Student's *t*-test for the means of independent samples. In all cases, statistical analyses were conducted at a significance level of 5%. In the study of glyphosate and/or quinate after 3 days of treatment, two different comparisons were made. First, experimental data (quinate, shikimate, amino acids and enzymes) of the untreated plants of both populations were compared by Student's t-test, showing no significant differences. Second, for each population multivariate one-way analysis of variance (MANOVA) was conducted to test the effect of glyphosate and/or quinate on parameters (metabolite content and enzymes) measured on single plants. If multivariate tests were significant at $p \leq .05$, one way ANOVA were performed individually on each parameter. Where appropriate, means were compared using Tukey's HSD test ($p \leq .05$). Significant differences in each parameter within each population are highlighted in the figures (Fig. 3-7) using different letters. Statistical analyses were performed using SPSS Statistics 25.0 (IBM, Corp., Armonk, NY, United States).

3. Results

3.1. Time course of quinate treatment

The application of quinate to leaves dramatically increased the concentration of quinate in the leaves of both populations (GS and GR), confirming that the compound was absorbed (Fig. 1. A). Quinate accumulation was maintained at similar levels during the time of study in GS and GR plants.

In nontreated plants, a similar AAA content in both populations was detected. While the total AAA content was not significantly modified by quinate in either of the populations (Fig. 1. B), a different pattern was found for each amino acid. In both populations, Tyr and Phe contents were significantly increased during the first 24–48 h of quinate treatment, while Trp content was not modified (Fig. 1.B, C, D).

3.2. Visual symptoms and lethality

Preliminary studies were performed to determine the glyphosate doses to be applied to obtain a very low effect that could facilitate the detection of the enhancement of glyphosate efficacy in the combined treatment with quinate. Glyphosate was applied at one-quarter or half of the recommended field dose for *A. palmeri* to GS and GR populations, respectively, and they were compared to the doses that were shown to be lethal in GS in 5–6 days (recommended field dose) and to severely affect plant growth in GR (3 times recommended field dose) (Supplementary Figs. 1 and 2).

After the dose determination, additional preliminary studies were performed to select the order of the combined treatment. To this end, quinate was evaluated 1 day before, 1 day after and simultaneously with glyphosate application (data not shown). The greatest visual effects were found when quinate was applied 24 h later than glyphosate; therefore, this is the order that was selected to perform the full physiological approach.

The visual status of the plants was monitored for 10 days (Supplementary Figs. 1 and 2). No visual symptoms were detected after any of the treatments in either of the populations at harvest: 2 and 3 days after quinate and glyphosate treatments, respectively.

GS plants treated with 0.25 times the recommended field dose of glyphosate alone were affected and showed growth arrest, but they did not die during the course of the experiment (10 days) (Supplementary Fig. 1). When this dose of glyphosate was combined with quinate, it was more effective and caused plant death in approximately 7–10 days (Supplementary Fig. 1). Indeed, the visual effects of the combination of the low dose of glyphosate with quinate closely resembled the effects induced by the high dose of glyphosate after 7 days (Fig. 2A).

No treatment was lethal in the case of the GR population. Nevertheless, as shown in Fig. 2B, 10 days after treatment, GR plants treated with the combined treatment were more affected than were plants treated with glyphosate alone and resembled the plants treated with the highest dose of glyphosate.

3.3. Effect of quinate and/or glyphosate on each population

For each population, MANOVA analysis identified a significant ($p \leq .05$) effect of treatments on measured variables. When analyzed individually by ANOVA in the GS population, DAHPS content, EPSPS content, CM activity, Trp and amide amino acids content were non-significant different parameters (Figs., 4, 5 and 7). More parameters were detected as non significant different in the GR population and the corresponding figures do not contain letters (Figs. 3-7).

3.4. Pattern of the AAA biosynthetic pathway

Quinate and glyphosate were applied alone or in combination, and their effects on growth, the AAA pathway and the amino acid pattern were measured for both populations. In the combined treatment, quinate was applied 1 day after glyphosate; thus, at harvest, plants of the combined treatment had been treated with quinate for only 2 days. Quinate and glyphosate applied alone were sampled after 2 and 3 days after spraying, respectively, in concordance with the elapsed time of the combined treatment.

The effect of individual and combined treatments on the pattern of the biosynthetic pathway of AAA was evaluated by determining the content of the metabolites quinate and shikimate and certain important enzymes of the pathway. All measured parameters of the AAA pathway were similar between the untreated plants of both populations.

Quinate did not accumulate in either of the two populations when glyphosate was applied alone, most likely due to the low doses applied (Fig. 3A). The quinate content after the combined treatment differed depending on the population: while in GS plants, quinate accumulation was similar after quinate alone or after quinate and glyphosate, in GR plants, quinate accumulation due to exogenous quinate was abolished if glyphosate was applied before.

Shikimate content was very low in the untreated plants of both populations (approximately 0.8 μg $disc^{-1}$). Glyphosate treatment (0.25 times the recommended field dose) provoked a substantial shikimate accumulation (40-fold that in the untreated plants) in the GS population, while 0.5 times the recommended field dose of glyphosate induced

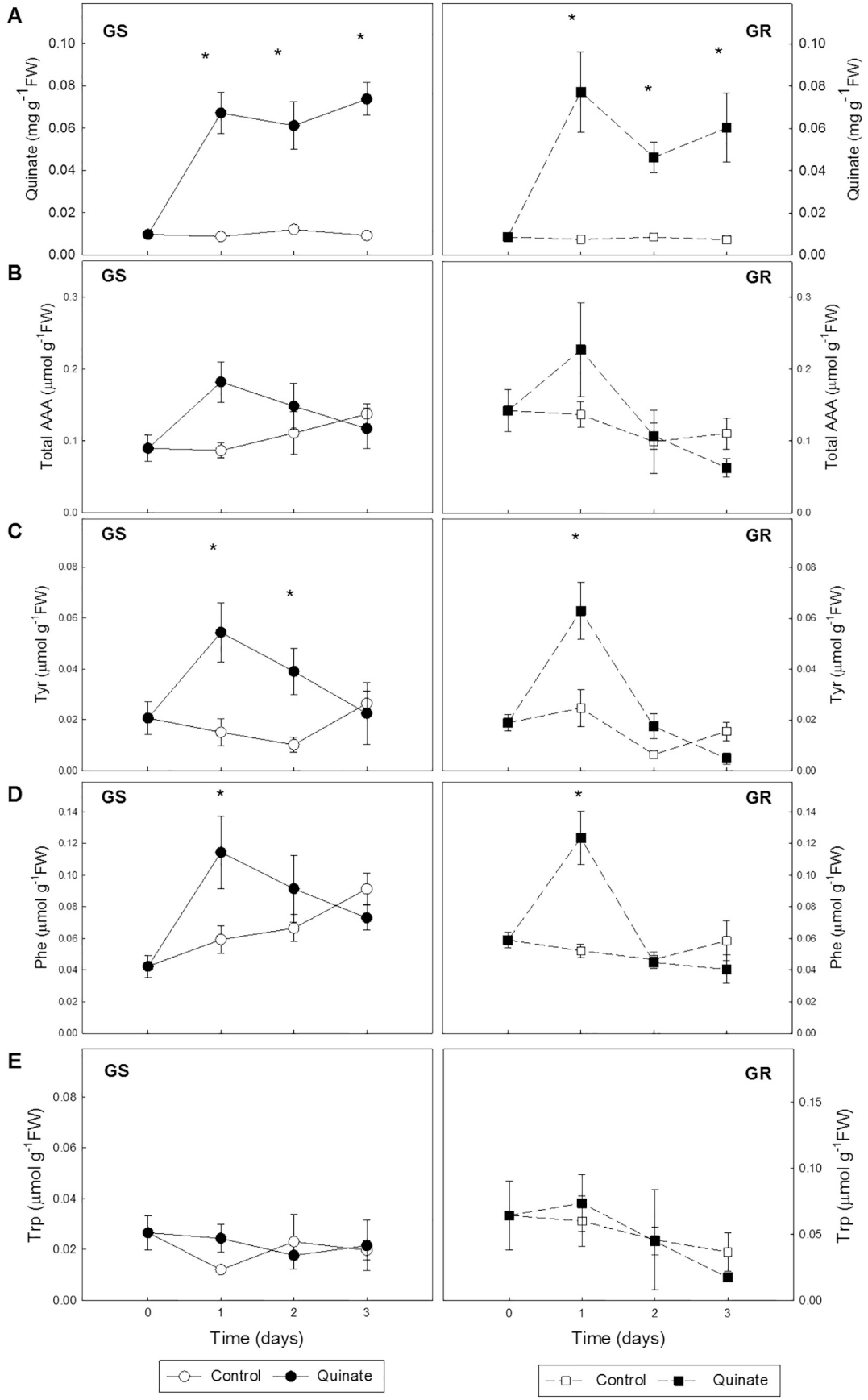

Fig. 1. Quinate and aromatic amino acid contents during four harvest times (0, 1, 2 and 3 days after treatment). Quinate (A), total aromatic amino acid content (B), tyrosine (Tyr; C), phenylalanine (Phe; D) and tryptophan (Trp; E) were measured in glyphosate-sensitive (circles, left; GS) and glyphosate-resistant (squares, right; GR) populations untreated (represented in white) or treated with quinate (represented in bold) (Mean ± SE; $n = 4$). The symbol * indicates significant differences between treatment and control for each harvest time in each population (p value ≤ .05).

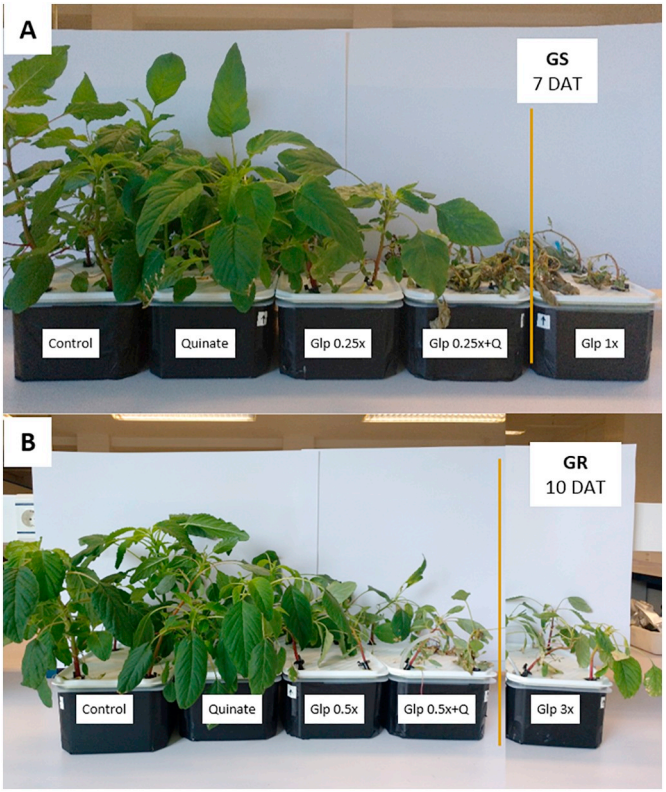

Fig. 2. Visual appearance of the glyphosate-sensitive (A) and glyphosate-resistant (B) *Amaranthus palmeri* plants 7 and 10 days after treatment (DAT) respectively. Plants were untreated (Control) or treated with quinate, glyphosate (Glp), or quinate with glyphosate (GLP + Q). Glyphosate was applied at 0.25 or 1 times the recommended field dose on the GS population and 0.5 or 3 times on the GR populations.

a very low shikimate accumulation (approximately 1.8-fold). The higher shikimate accumulation in GS than in GR after glyphosate treatment (even though the dose was higher in GR) confirms the inhibition of EPSPS by glyphosate in GS and resistance in GR. Quinate treatment alone did not affect shikimate content in either population. In the GS population, the combined treatment provoked a similar shikimate accumulation to that induced by the herbicide treatment alone. This lack of enhancement of shikimate accumulation in the combined treatment compared to that in the glyphosate treatment cannot be related to reaching the maximum shikimate accumulation only with glyphosate because shikimate has been reported to increase proportionally to glyphosate from 0.25 up to 1 times the recommended dose in this population (Fernández-Escalada et al., 2016). Interestingly, in GR plants, the combined treatment induced the highest shikimate accumulation (approximately 3.3-fold that in the untreated plants) (Fig. 3B).

DAHPS and EPSPS are key enzymes in the AAA biosynthetic pathway, the former as the enzyme regulating the carbon entrance into the shikimate pathway and the latter as the target site of glyphosate. The abundance of both proteins was tested by immunoblotting, and no changes were detected in either of the enzymes in the GS population or in the DAHPS of the GR population (Fig. 4A, B). In contrast, EPSPS abundance was significantly decreased after quinate alone and after the combined treatment in the resistant population.

After chorismate, CM and AS are key enzymes regulating the carbon flux through the Tyr/Phe branch or the Trp branch. While CM or AS enzymatic activities (Fig. 4C, D) were not affected by any of the treatments in the GR population, in the GS population, AS activity was increased after the combined treatment (Fig. 4D).

3.5. Amino acid profile of treated plants

The effect of treatments on the free amino acid content of both populations was monitored (Figs. 5, 6, 7). Treatment with glyphosate also resulted in amino acid accumulation; thus, this parameter can also be used as a physiological marker of herbicidal activity. AAAs whose biosynthesis is inhibited by glyphosate were evaluated (Fig. 5).

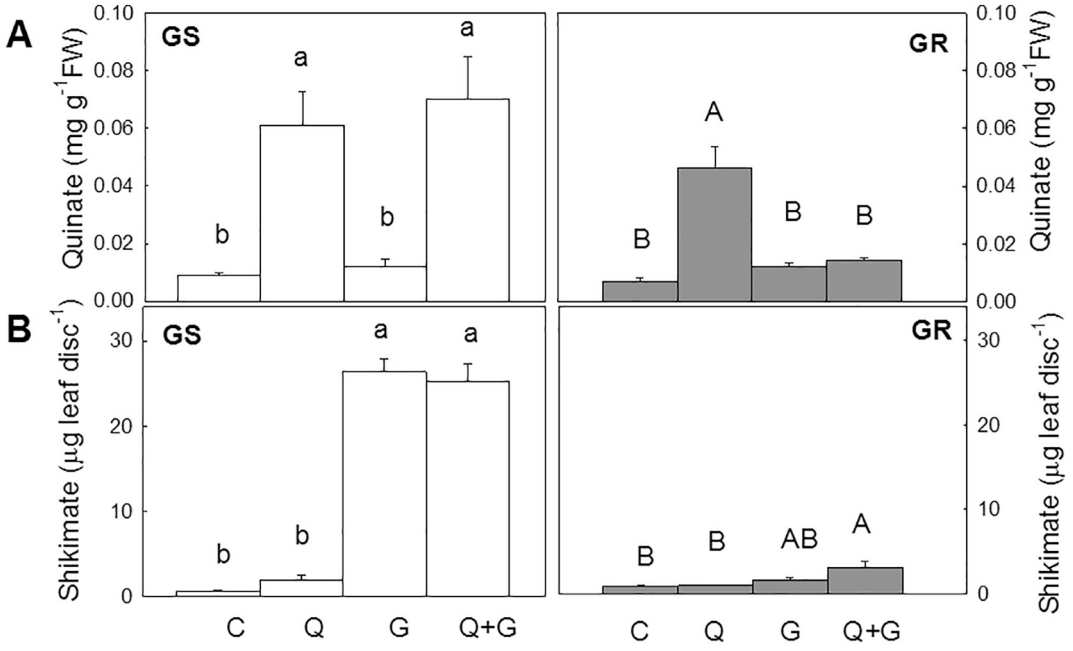

Fig. 3. Quinate (A) and shikimate (B) content. Glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations were untreated (Control, C) or sampled 2 days after quinate (Q), 3 days after glyphosate (G) or 3 days after glyphosate and 2 days after quinate (Q + G). (Mean ± SE; n = 4). Different capital letters in the GR population and different lowercase letters in the GS population indicate significant differences between treatments (p value ≤.05, Tukey).

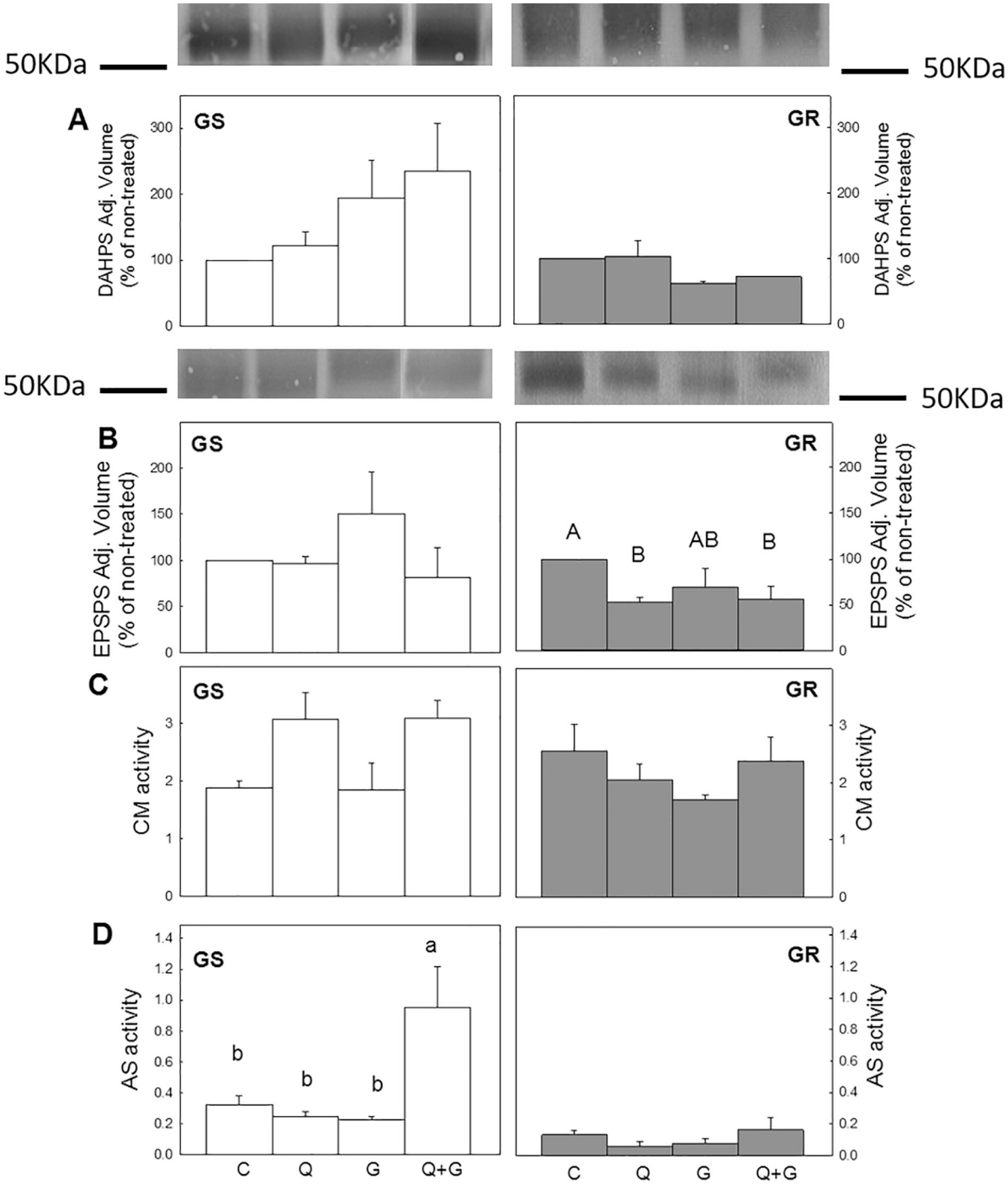

Fig. 4. Enzymes of the aromatic amino acid biosynthetic pathway. (A) Normalization of the quantity of 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (DAHPS) (Mean ± SE; $n = 3$). Top: Representative immunoblots for DAHPS are plotted, and lanes contained 40 μg of protein. (B) Normalization of the quantity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Mean ± SE; n = 3). Top: Representative immunoblots for EPSPS are plotted. Lanes contained 80 μg protein for GS or 15 μg protein for GR of total soluble proteins. (C) Chorismate mutase (CM) enzymatic activity (Mean ± SE; n = 4). (D) Anthranilate synthase (AS) enzymatic activity (Mean ± SE; n = 4). Glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations were untreated (Control, C), sampled 2 days after quinate (Q), 3 days after glyphosate (G) or 3 days after glyphosate and 2 days after quinate (Q + G). Figures without letters denote no significant differences between treatments. Different capital letters in the GR population and different lowercase letters in the GS population indicate significant differences between treatments (p value ≤.05, Tukey).

Moreover, three other groups previously reported to be affected by glyphosate were studied: branched-chain (valine, (Val); leucine (Leu) and isoleucine (Ile)) (Fig. 6); amide (glutamine (Gln) and asparagine (Asn)); and acidic (aspartate (Asp) and glutamate (Glu) (Fig. 7B, C) amino acids. Untreated plants of both populations showed similar content of these amino acids (no significant differences).

Different patterns of AAA content after treatments were detected in each population (Fig. 5). In the GS population, quinate alone did not change the total AAA content. The total AAA content increased significantly only after combined treatment, which was related mainly to the specific accumulation of Phe. Tyr was accumulated after glyphosate, whereas Trp remained unaffected. Contrary to these changes

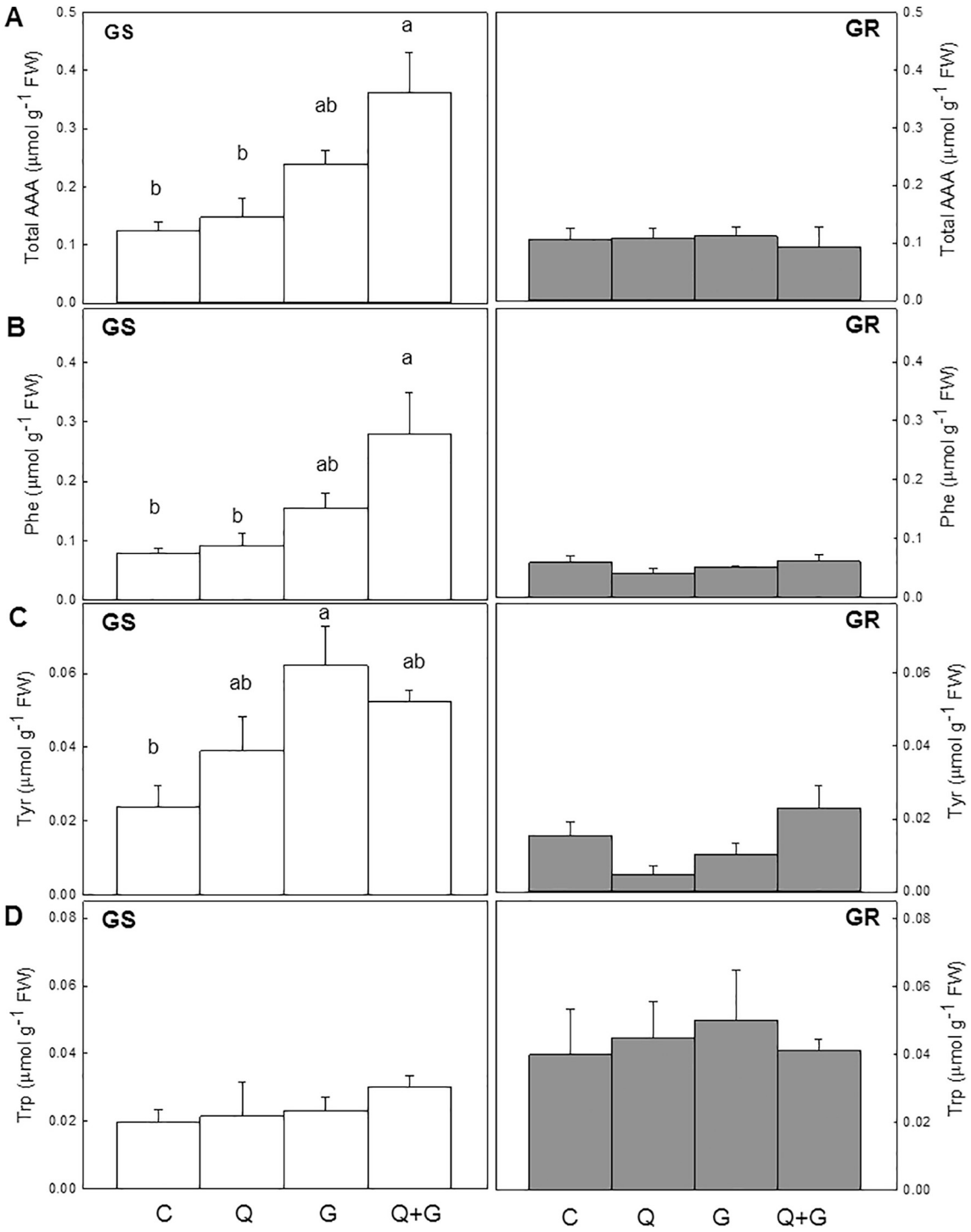

Fig. 5. Aromatic amino acid profile. Total aromatic amino acid (A), phenylalanine (Phe; B), tyrosine (Tyr; C), and tryptophan (Trp; D) contents were measured in leaves of plants of glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations. Plants were untreated (Control, C), sampled 2 days after quinate (Q), 3 days after glyphosate (G) or 3 days after glyphosate and 2 days after quinate (Q + G). (Mean ± SE; n = 4). Figures without letters denote no significant differences between treatments. Different capital letters in the GR population and different lowercase letters in the GS population indicate significant differences between treatments (p value ≤.05, Tukey).

detected in the GS population, in the GR population, the AAAs were not affected by any of the treatments.

Next, branched-chain, total, acidic and amide amino acids were measured (Figs. 6 and 7). The total free amino acid content was measured in both populations (Fig. 7A). In GS plants, only the combined treatment induced a significant increase in the total amino acid content, while in the GR plants, the free amino acid pool was increased in the presence of glyphosate alone. In the GS population, total branched-chain and amide amino acid contents were significantly increased only after the combined treatment. In the GR population, glyphosate alone induced valine accumulation (Fig. 6A) and glyphosate alone or combined provoked accumulation of acid amino acids (Fig. 7C).

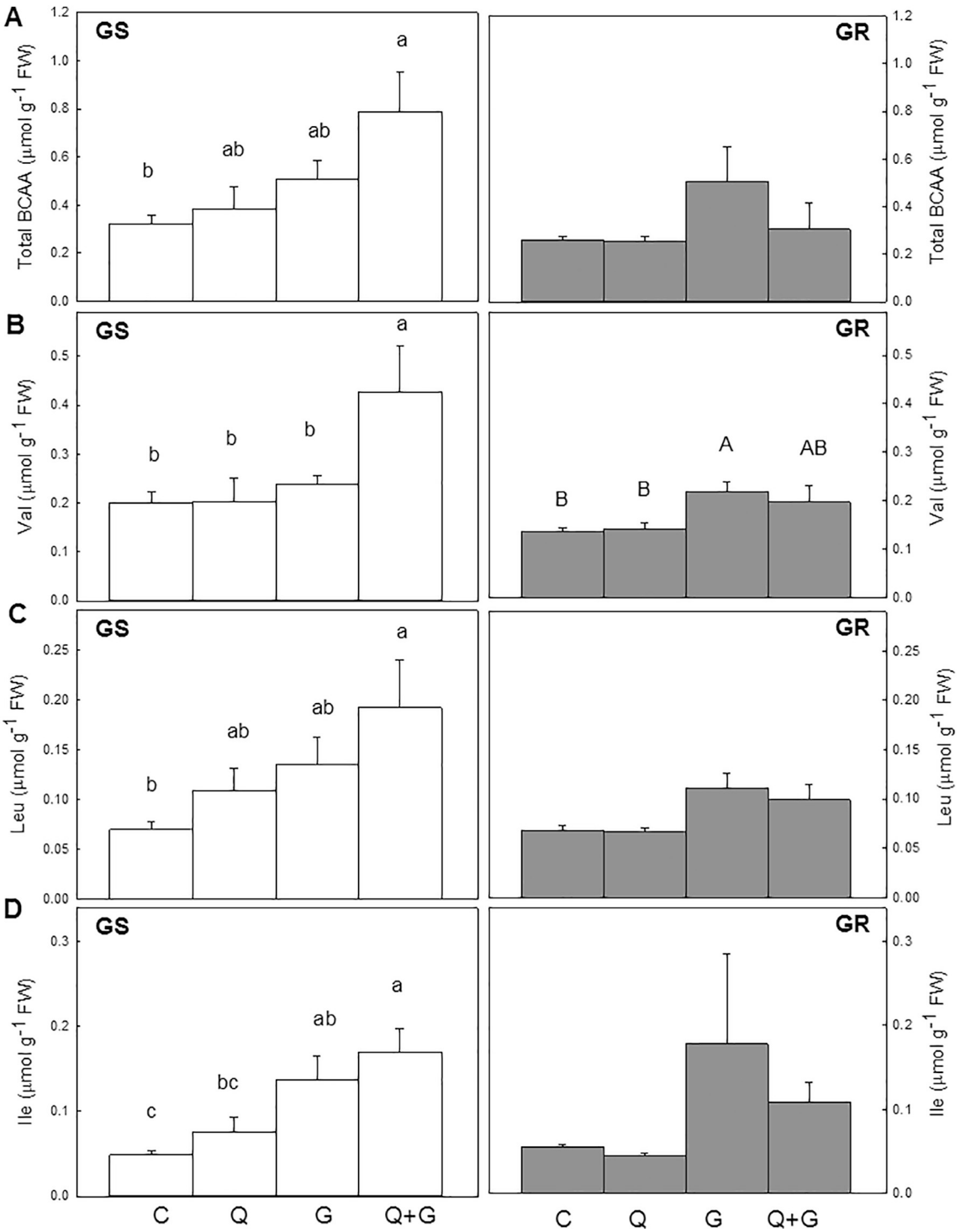

Fig. 6. Branched-chain amino acid profile. Total branched-chain amino acids (A), valine (Val; B), leucine (Leu; C) and isoleucine (Ile; D) contents were measured in leaves of plants of glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations. Plants were untreated (Control, C), sampled 2 days after quinate (Q), 3 days after glyphosate (G) or 3 days after glyphosate and 2 days after quinate (Q + G). (Mean ± SE; n = 4). Figures without letters denote no significant differences between treatments. Different capital letters in the GR population and different lowercase letters in the GS population indicate significant differences between treatments (p value ≤.05, Tukey).

4. Discussion

4.1. Tyr and Phe peak accumulation after quinate is independent of EPSPS overexpression

Quinate is a compound that occurs in relatively high concentrations in herbaceous plants and in the developing tissues of conifers (Yoshida et al., 1975; Osipov & Aleksandrova, 1982). Although its physiological role has not been completely clarified, quinate is thought to be a reserve compound of the shikimate pathway. Indeed, quinate can re-enter the shikimate pathway upon conversion to dehydroquinate by quinate dehydrogenase or to shikimate by quinate hydrolyase (Bentley & Haslam, 1990; Leuschner et al., 1995; Guo et al., 2014).

Exogenous spraying of quinate onto the leaves of *A. palmeri* induced

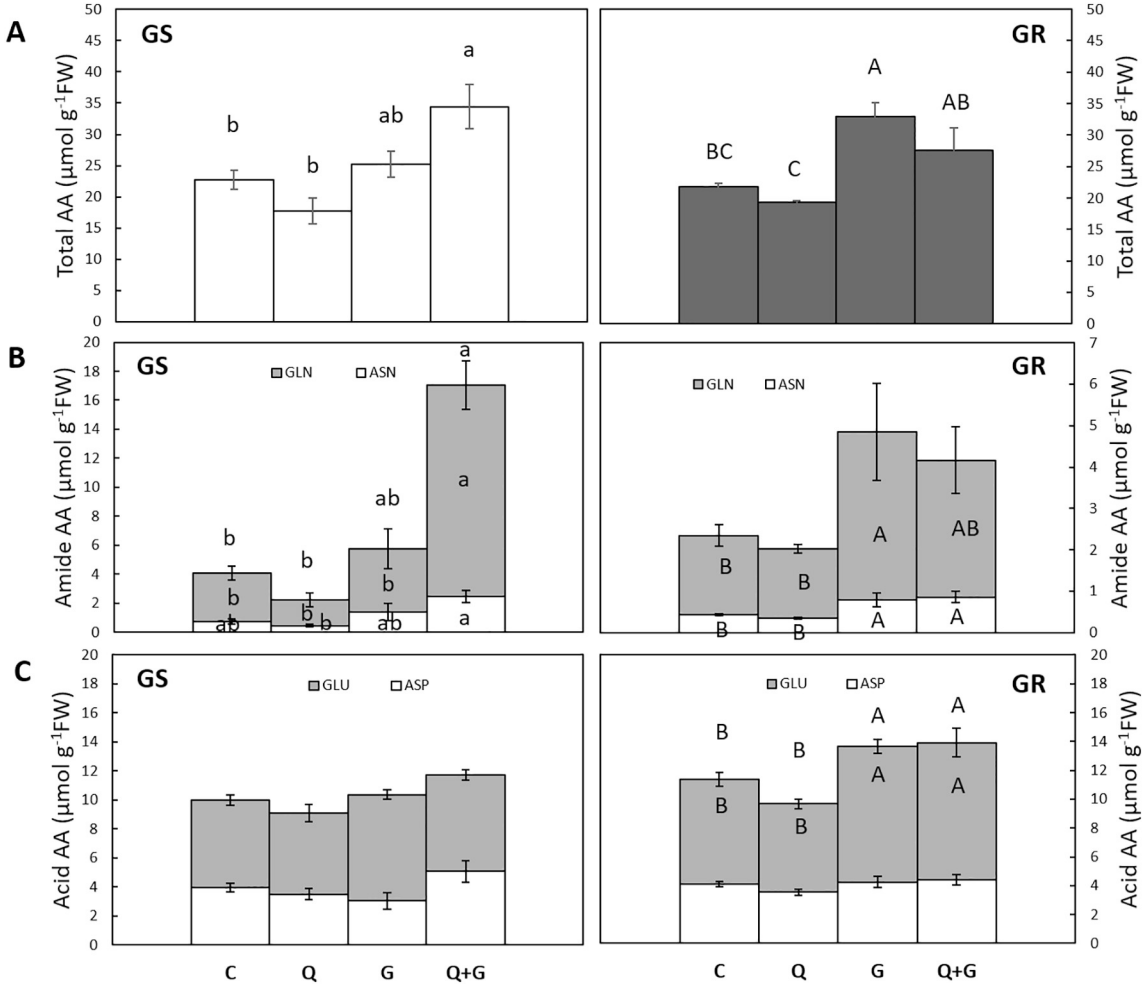

Fig. 7. Total free amino acid (A), amide amino acid (glutamine (Gln) and asparagine (Asn); B) and acid amino acid (aspartate (Asp) and glutamate (Glu); C) contents were measured in leaves of plants of glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations. Plants were untreated (Control, C), sampled 2 days after quinate (Q), 3 days after glyphosate (G) or 3 days after glyphosate and 2 days after quinate (Q + G). (Mean ± SE; $n = 4$. Figures without letters denote no significant differences between treatments. Different capital letters in the GR population and different lowercase letters in the GS population indicate significant differences between treatments (p value ≤.05, Tukey).

quinate accumulation throughout the study period, as has been detected in other organs and species (Orcaray et al., 2010; Zulet et al., 2013b; Zabalza et al., 2017). Quinate was proposed to serve as a carbon source for the biosynthesis of AAAs (Leuschner & Schultz, 1991a; Leuschner & Schultz, 1991b), and the percentage of AAAs in pea leaves increased when quinate was applied to the nutrient solution or sprayed onto the leaves (Zulet et al., 2013b). Leaves of *A. palmeri* sprayed with quinate showed a peak in Phe and Tyr contents 1 day after the application of the treatment (Fig. 1), and Trp was not affected. In contrast to these results, only Trp accumulation was detected in pea roots after 7 or 15 days of quinate supply through the nutrient solution (Zabalza et al., 2017). Such different behavior can probably be explained by the different species, organ, type of application or time of treatment used in the two studies. Indeed, although no Phe or Tyr accumulation was detected in pea roots, accumulation of the secondary metabolites formed from Phe and Tyr (Zabalza et al., 2017) was detected, suggesting a coordinated response of the shikimate pathway.

In the postchorismate pathway, the branch point to the synthesis of Phe and Tyr by CM or to the synthesis of Trp by AS appears to be a checkpoint in the tightly regulated process of synthesis of AAAs (Maeda & Dudareva, 2012). The accumulation of Tyr and Phe occurred during the first 24 h after the treatment and was detected in both populations. This result suggests that, after quinate supply, carbon flux increases in the postchorismate pathway through Tyr and Phe biosynthesis, independent of the EPSPS overexpression by gene amplification of the GR population.

Tyr and Trp are precursors of different types of secondary metabolites. Tyr is a precursor of tocochromanols (vitamin E), plastoquinones, isoquinoline alkaloids, several nonprotein amino acids, and certain phenylpropanoids. Trp is catabolized into many indole-containing secondary metabolites, such as indole-3-acetic acid (Tzin & Galili, 2010). The preferential flux of the quinate to the Tyr/Phe branch of the shikimate pathway might involve a different pattern of secondary metabolism after treatments and between populations. Indeed, previous studies have reported that changes in Phe biosynthesis generate flux changes in various branches of Phe-derived secondary metabolites (Tzin et al., 2009).

4.2. Glyphosate efficacy is enhanced when quinate is applied after glyphosate in the sensitive population

Quinate and glyphosate directly affect the AAA biosynthetic pathway by providing an alternative source of carbon for the shikimate pathway (Leuschner & Schultz, 1991a; Leuschner & Schultz, 1991b) or by blocking the shikimate pathway at the level of EPSPS (Steinrücken & Amrhein, 1980), respectively. No common effects on the regulation of the shikimate pathway were detected between quinate and glyphosate. Quinate is probably incorporated into the main trunk from the branch

pathway and accumulated in Tyr and Phe (Fig. 1) in the final products of the pathway, such as lignin, hydroxybenzoic and hydroxycinnamic acids, concomitant with a decrease in the amount of DAHPS protein (Zabalza et al., 2017). Glyphosate provokes an increase in the DAHPS and EPSPS protein (Fernández-Escalada et al., 2016; Pinto et al., 1988) and accumulation of the metabolites upstream of the enzyme EPSPS, such as shikimate and quinate (Lydon & Duke, 1988; Becerril et al., 1989; Orcaray et al., 2010). In the postchorismate pathway, glyphosate has been reported to induce AS activity (Fernández-Escalada et al., 2017). Nevertheless, only shikimate accumulation after glyphosate treatment was detected in the present study (Fig. 3B). All other previously known effects of glyphosate on the shikimate pathway were not detected in the GS population due to the low doses of glyphosate used and the short time of study.

The toxic effect of glyphosate cannot be considered only in terms of its interaction at the target site because the inhibition of EPSPS results in a metabolic roadblock, with physiological consequences such as an important alteration of the amino acid content, and thus, this parameter can be used as a physiological marker of herbicide activity. The total free amino content pool increase has been widely reported (Zulet et al., 2013a; Zulet et al., 2015; Orcaray et al., 2011; Vivancos et al., 2011; Liu et al., 2015) and has been attributed to increases in protein turnover (Zulet et al., 2013a). With respect to the specific amino acid groups, glyphosate has been reported to induce the accumulation of AAAs (Fernández-Escalada et al., 2017; Orcaray et al., 2010; Fernández-Escalada et al., 2016; Maroli et al., 2018) and amides (Orcaray et al., 2010) and a higher relative increase in branched-chain amino acids (Orcaray et al., 2010; Fernández-Escalada et al., 2016; Maroli et al., 2015). Although the low glyphosate dose applied to the GS population in this study did not provoke these changes in the amino acid profile, the combined treatment was the only treatment that induced significant accumulation of AAAs and branched-chain, amide and total free amino acids, evidencing an increase in the herbicide activity (Figs. 5, 6 and 7).

The most remarkable evidence of the enhanced phytotoxicity of glyphosate by combination with quinate is the lethality provoked in the treated plants, as the combination of one-quarter of the recommended field dose of glyphosate with quinate was as lethal as the recommended field dose (Fig. 2A).

4.3. Metabolic disturbances are enhanced when quinate is applied after the herbicide in the resistant population

The comparison of GS and GR populations offers the opportunity to study the effect of EPSPS overexpression due to extra EPSPS gene copies on the physiological response to the combination of quinate and glyphosate. In the absence of treatments, high EPSPS gene copy number did not affect any of the parameters evaluated in the present study, as has been reported previously for AAA content and expression of AAA and branched-chain amino acid biosynthetic genes ((Fernández-Escalada et al., 2017; Fernández-Escalada et al., 2016). This finding is consistent with previous reports suggesting that the overexpression of EPSPS may have no fitness cost in *A. palmeri* (Giacomini et al., 2014; Vila-Aiub et al., 2014).

Only a few physiological changes were detected in the resistant population after glyphosate treatment, evidencing the resistance of the population and the low dose of glyphosate applied. Changes were related to free amino acid content, including the accumulation of valine (Fig. 6B), the free amino acid pool (Fig. 7A) acid amino acids (Fig. 7C). A decrease in the amount of EPSPS protein was not detected (Fig. 4B), as previously reported for low doses of glyphosate (including the doses used in the present study) (Fernández-Escalada et al., 2016).

Only in the GR population did the combined treatment abolish quinate accumulation (Fig. 3A) and enhance shikimate accumulation (Fig. 3B). Although interpretation based on pool sizes of the pathway intermediates is difficult because pool size does not reflect pool flux, it can be proposed that quinate is incorporated faster if resistant plants have been previously treated with sublethal doses of glyphosate. This effect would explain the attenuation of quinate accumulation and the enhancement of shikimate of the combined treatment compared to individual quinate and glyphosate treatments. Interestingly, this effect was only detected in the GR plants, suggesting that a specific effect of glyphosate on plants overexpressing EPSPS would increase the capacity to assimilate external carbon from quinate.

5. Conclusion

In the GS population, quinate spraying 1 day after glyphosate spray increased the phytotoxicity of the herbicide: one-quarter of the recommended dose of glyphosate was as lethal as the full recommended dose and changes were induced in the amino acid profile. This study lays the framework for the application of the environmentally innocuous organic acid quinate after glyphosate to improve the efficacy of the herbicide and to lower the doses in the control of sensitive *A. palmeri*.

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