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New insights in the regulation of the shikimate pathway after quinate as glyphosate enhancer in *Amaranthus palmeri*

Tesis doctoral
Ainhoa Zulet González
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Tesis Doctoral

New insights in the regulation of the shikimate pathway after quinate as glyphosate enhancer in *Amaranthus palmeri*

Memoria presentada por Dña. Ainhoa Zulet González para optar al grado de Doctor con Mención de Doctor Internacional

Pamplona, Noviembre de 2019

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AUTORIZACIÓN DE LAS DIRECTORAS DE TESIS

Dra. Mercedes Royuela Hernando, Catedrática de Universidad y Dra. Ana Zabalza Aznárez, Profesora Contratado Doctor, profesoras del Área de Fisiología Vegetal del Departamento de Ciencias de la Universidad Pública de Navarra,

HACEN CONSTAR:

Que el trabajo titulado **"New insights in the regulation of the shikimate pathway after quinate as glyphosate enhancer in *Amaranthus palmeri*"** que **presenta Dña. Ainhoa Zulet González** para optar al título de Doctor Internacional por la Universidad Pública de Navarra, ha sido desarrollado bajo su dirección en el grupo de Fisiología Vegetal y Agrobiología en el Institute for Multidisciplinary Research in Applied Biology (IMAB) de la Universidad Pública de Navarra.

Revisado el trabajo, consideran que reúne las condiciones necesarias para su defensa, por lo que

AUTORIZAN:

La presentación de la citada Tesis Doctoral.

En Pamplona- 27 de noviembre de 2019

Fdo. Mercedes Royuela Hernando

Fdo. Ana Zabalza Aznárez

Results obtained in this study have been presented in the following publications and conferences:

Ainhoa Zulet-González, Manuel Fernández-Escalada, Ana Zabalza and Mercedes Royuela. Enhancement of glyphosate efficacy on *Amaranthus palmeri* by exogenous quinate application. Pesticide Biochemistry and Physiology (2019) 158:1-11. DOI:10.1016/j.pestbp.2019.04.004 (Publication)

Ainhoa Zulet-González, María Barco Antoñanzas, Miriam Gil-Monreal, Dierk Scheel, Karin Gorzolka, Ana Zabalza, Mercedes Royuela. Aproximación metabolómica para la mayor eficacia del glifosato tras su combinación con quinato en el control de *Amaranthus palmeri*. Congreso SEMh 2019, Octubre 2019, Vigo (Spain). Oral presentation. Premio PHYTOMA-SEMh 2019.

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Ainhoa Zulet-González, Manuel Fernández-Escalada, Miriam Gil-Monreal, Ana Zabalza, Mercedes Royuela. Efecto del glifosato y diferentes intermediarios metabólicos en la regulación de la ruta del siquimato. Congreso SEMh 2017, Octubre 2017, Pamplona (Spain). Poster presentation.

Ainhoa Zulet-González, Manuel Fernández-Escalada, Miriam Gil-Monreal, Ana Zabalza, Mercedes Royuela. Does the regulation of the shikimate pathway after glyphosate or quinate treatments change in *Amaranthus palmeri* overexpressing EPSPS?. SEFV 2017 Congress, Junio 2017, Barcelona (Spain). Poster presentation.

Ainhoa Zulet-González, Manuel Fernández-Escalada, Ana Zabalza and Mercedes Royuela. Quinate as a potential glyphosate-enhancer in the control of *Amaranthus palmeri*. 7th International Weed Science Congress. ISBN: 978-80-213-2648-4. Junio 2016, Praga (Czech Republic). Poster presentation.

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"Si he llegado a ver más lejos es porque me subí a hombros de gigantes"

Isaac Newton

"Quien tiene un reloj sabe la hora; quien tiene dos, no"

Anónimo

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ABBREVIATIONS

AAA	Aromatic amino acid
AAAs	Aromatic amino acids
ACCase	Acetyl CoA carboxylase
ADH	Arogenate dehydrogenase
ADT	Arogenate dehydratase
AHAIR	Acetohydroxyacid isomer-reductase
AHAS (ALS)	Acetohydroxyacid synthase
ALS	Acetolactate synthase
AMPA	Aminomethylphosphonic acid
ANOVA	Analysis of variance
AS	Anthranilate synthase
BCAA	Branched chain amino acid
BCAAs	Branched chain amino acids
BCIP/NBT	5-bromo-4-chloro'-indolyphosphate/nitro-blue-tetrazolium chloride
BSA	Bovine serum albumin
cDNA	Complementay deoxyribonucleic acid
CM	Chorismate mutase
CPS	Carbamoyl phosphate synthetase
CS	Chorismate synthase
Ct value	Threshold cycle value
CYS	Cysteine
DAHP	3-deoxy-d-arabino-heptulosonate-7-phosphate
DAHPS	3-deoxy-d-arabino-heptulosonate-7-phosphate synthase
DHAD	Dihydroxyacid dehydratase
DHD	3-dehydroquinate dehydratase
DHQ	Dehydroquinate
DHQS	dehydroquinate synthase
DNA	Deoxyribonucleic acid
DQSD	3-dehydroquinate dehydratase/shikimate dehydrogenase
DTT	Dithiothreitol
E4P	Erythrose 4-phosphate
EC_{50}	Half maximal effective concentration
EDTA	Ethylendiaminetetraacetic acid
EOBII	Emission of benzenoids II
EPSP	5-enolpyruvylshikimate 3-phosphate

EPSPS	5-enolpyruvylshikimate 3-phosphate synthase
EPSPS-S3P	5-enolpyruvylshikimate 3-phosphate synthase/shikimate-3-phosphate
FITC	Fluorescein isothiocyanate
GM	Genetically modified
GR	Glyphosate resistant
GRC	Glyphosate resistant crop
GRCs	Glyphosate resistant crops
GS	Glyphosare sensitive
HPP-AT	4-hydroxyphenylpyruvate aminotransferase
HRAC	Herbicide Resistance Action Committee
IGPS	Indole-3-glycerol phosphate synthase
Ile	Isoleucine
Leu	Leucine
M	Molar
MYB	Myeloblastosis family
N	Normal
OD	Optical density
ODO1	Odorant 1
ORCA3	Octadecanoic-derivative responsive Catharanthus AP2-domain
PA-AT	Prephenate aminotransferase
PAI	Phosphoribosylanthranilate isomerase
PAL	Phenylalanine ammonia lyase
PAT	Phosphoribosylanthranilate transferase
PCA	Principal component analysis
PDH	Prephenate dehydrogenase
PDT	Prephenate dehydratase
PEP	Phosphoenolpyruvate
Phe	Phenylalanine
PMSF	Phenylmethylsulfonyl fluoride
PPY-AT	Phenylpyruvate aminotransferase
PVDF	Polyvinylidene difluoride
qPCR	Quantitative real-time PCR
RD	Recommended field dose
RNA	Ribonucleic acid
S3P	Shikimate-3-phosphate
SDH	Shikimate dehydrogenase
SDS	Sodium dodecyl sulfate

SK	Shikimate kinase
TA	BCAA transaminase
TCA	Trichloroacetic acid
TD	Threonine deaminase
Thr	Threonine
Trp	Tryptophan
TSα	Tryptophan synthase α subunit
TSβ	Tryptophan synthase β subunit
TTBS	Twin tris buffer saline
Tyr	Tyrosine
UV	Ultraviolet
Val	Valine

SUMMARY

Glyphosate is the most widely used herbicide worldwide. The site of action of this herbicide is the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in the aromatic amino acid biosynthetic pathway, also known as shikimate pathway. One of the physiological processes triggered by the herbicide is quinate accumulation. Quinate is a secondary metabolite, formed in a lateral branch of the shikimate pathway. The detection of some physiological effects after applying quinate exogenously raised the possibility of using this compound in combination with glyphosate to enhance herbicide efficacy and to reduce the glyphosate doses in the control of *Amaranthus palmeri*. Glyphosate resistant populations have evolved in this species, and one of the most important resistance mechanism is EPSPS gene amplification, which leads to an overexpression of the enzyme target of glyphosate.

The general objective of this work was to gain further insights in the regulation of the shikimate pathway after glyphosate treatment and the use of quinate as an enhancer of this herbicide in the control of glyphosate-sensitive and resistant (due to gene amplification) *A. palmeri* plants.

In this work, it was determined that applying quinate one day after glyphosate (combined treatment), become lethal a sub lethal glyphosate dose in the glyphosate-sensitive population, laying the framework for the application of the both compounds to improve the efficacy of the herbicide and to reduce the doses in the control of the sensitive population. The higher toxicity of the combined treatment was related to an exacerbation of changes in the herbicide physiological markers previously reported (shikimate and free amino acid content).

The pattern of the shikimate pathway after the combined treatment was approached at metabolic and transcriptional level, trying to explain the increase in the efficacy detected of the combined treatment in this population. The results obtained suggest that the enhancement of the toxicity observed after the combined treatment would be related mainly at metabolic level, due to the increase in the quinate derivative content, and not to changes at transcriptional level.

A non-targeted metabolic profiling was performed trying to compare the metabolic profile of both populations treated with glyphosate and to unravel if any change in the metabolomic profile of the plants treated with the combined treatment could contribute to the toxicity increase. Among all primary and

secondary metabolites evaluated, the quinate derivatives were the only compounds that were accumulated differently after the combined treatment, evidencing their role in the increased toxicity of the combined treatment.

Finally, the role of aromatic amino acids and the intermediates (shikimate, quinate, chorismate and anthranilate) in the regulation of shikimate pathway was evaluated by leaf disc incubation. It was observed that aromatic amino acids when applied combined with the herbicide abolished the glyphosate effects at transcriptional level but no shikimate accumulation. Also, none of the intermediates evaluated fully mimicked glyphosate effect on shikimate pathway. However, shikimate was the metabolite that induced the relative transcript level of most of the genes of the shikimate pathway, suggesting that the transcription induction detected after glyphosate treatment would be mediated, at least in part, by shikimate accumulation.

In summary, this study describes new insights in the shikimate pathway regulation after glyphosate treatment and evaluates the combined treatment with quinate from a management and physiological points of view, trying to unravel the causes of its increase of the toxicity.

RESUMEN

El glifosato es el herbicida más empleado a nivel mundial. Su mecanismo de acción es la inhibición de la enzima 5-enolpiruvilsiquimato-3-fosfato sintasa (EPSPS), enzima clave dentro de la ruta de biosíntesis de los amino ácidos aromáticos, también conocida como la ruta del siquimato. Uno de los procesos fisiológicos descritos tras la aplicación de este herbicida es la acumulación de quinato, un metabolito secundario, formado a partir de una ramificación lateral de la ruta del siquimato. Tras detectar efectos fisiológicos por la aplicación de quinato exógeno de forma aislada, se planteó la posibilidad de utilización de este compuesto como potenciador del glifosato para reducir las dosis de herbicida en el control de la mala hierba *Amaranthus palmeri.* En esta especie se han desarrollado poblaciones resistentes a glifosato, entre las cuales se encuentra el mecanismo de resistencia de sobreexpresión del gen EPSPS, lo que lleva a una sobreexpresión del enzima diana del herbicida.

El principal objetivo de esta tesis fue el de profundizar en las alteraciones de la ruta del siquimato por el herbicida glifosato y el uso del quinato como potenciador de este herbicida en el control de plantas de *A. palmeri* sensibles y resistentes a glifosato (por amplificación génica).

En este trabajo se determinó que la aplicación de quinato un día después del glifosato (tratamiento combinado) convertía en letal una dosis sub-letal de herbicida en la población sensible. Estos resultados abren la posibilidad de aplicar ambos compuestos para mejorar la eficacia del herbicida y de reducir las dosis a aplicar en el control de poblaciones sensibles. La mayor toxicidad del tratamiento combinado estuvo relacionada con la exacerbación en los marcadores fisiológicos previamente descritos para este herbicida (acumulación del contenido de siquimato y de aminoácidos libres).

Se estudió el comportamiento de la ruta del siquimato tras el tratamiento combinado a nivel metabolómico y transcriptómico, tratando de encontrar respuesta al incremento de eficacia observado en el tratamiento combinado. Los resultados obtenidos parecen indicar que el incremento de la toxicidad del tratamiento combinado estaría relacionado con cambios a nivel metabólico, debido en concreto a un incremento en el contenido de los derivados del quinato, y no debido a cambios de niveles de transcripción.

Se realizó un perfil metabólico no dirigido para comparar el perfil metabólico de ambas poblaciones tratadas con glifosato y para determinar si otros cambios en el perfil metabólico podrían estar contribuyendo al incremento en la toxicidad del

tratamiento combinado. Entre todos los compuestos del metabolismo primario y secundario evaluados, los derivados del quinato fueron los únicos compuestos que se acumularon en este tratamiento de manera diferencial, evidenciando su papel en el incremento de la toxicidad de este tratamiento.

Por último, se evaluó el papel de los amino ácidos aromáticos y de algunos intermediarios metabólicos (siquimato, quinato, corismato y antranilato) en la regulación de la ruta del siquimato mediante incubación de discos de hojas con ellos. Se observó que los aminoácidos aromáticos, al ser aplicados en combinación con el herbicida, neutralizaron los efectos del glifosato a nivel transcipcional pero no la acumulación del siquimato. Ninguno de los intermediarios metabólicos evaluados mimetizó completamente el efecto del glifosato en la ruta del siquimato. Sin embargo, el siquimato fue el metabolito que indujo una sobreexpresión de la mayoría de los genes de la ruta del siquimato, de una manera similar al herbicida. Estos resultados sugieren que la inducción en la transcripción observada tras el tratamiento con glifosato podría estar mediada, al menos en parte, por la acumulación de siquimato.

Resumiendo, esta tesis aporta nuevos aspectos en la regulación de la ruta del siquimato tras la aplicación de glifosato, y aborda el efecto del tratamiento combinado del herbicida con quinato a nivel práctico y fisiológico, tratando de encontrar las causas de ese incremento en la toxicidad.

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GENERAL INTRODUCTION

GI.1. Herbicide resistance

GI.1.1. Weeds and weed control

Traditionally, weeds have been defined as 'plant growing where is not wanted', focusing the objectives of weed management only at eradicating weeds. However, the new strategies in weed management are leading to an integration of weed science and sustainable agriculture, and weeds need to be evaluated not only for their detrimental impacts on crop quality, but as integral components of agroecosystems that interact with it (Roma-Burgos et al. 2019). For that, the traditional definition of weeds is being replaced for 'plants that are especially successful at colonizing disturbed, but potentially productive, sites and at maintaining their abundance under conditions of repeated disturbances' (Mohler 2001). This new concept focuses the weed management strategies at preventing the causes that lead to weed problems and strives at understanding the agroecological functions of weeds while exploring new management options (Roma-Burgos et al. 2019).

The practice of growing crops in monoculture has exerted a considerable selection pressure in the evolution of weeds. Many characteristics have evolved that contribute to weed success. Although no single weed possesses all of these attributes, there are several biological traits associated with "weediness" such as, long term seed survival, rapid growth, high environmental plasticity or genetic diversity (Ziska and Dukes 2011).

To most growers, yield reduction due to weed competition represents a persistent, undesirable, and almost inevitable problem. Weeds increment crop production costs because they compete with crops for the available resources, increase incidence of plant pathogens or insect pests, and interfere with the recollection. However, there is evidence that, in some cases, the increase in the biodiversity caused by weeds leads to an increase in the benefits of the agroecosystem. For example, leguminous weeds can fix atmospheric nitrogen, reducing the needs for fertilizer inputs; acting as cover crops; or increasing pollen and nectar resources enhancing the pollination services (Roma-Burgos et al. 2019).

Given the damage that weeds do to human activities, there are several practices used to restrict the growth and spread of weeds. In general, these practices focus on three main areas: prevention, eradication, and control. Prevention refers to those actions taken to inhibit the introduction and/or establishment of weed

species into new areas. Eradication is defined as a set of measures taken to completely remove a weed species from an area, which is rarely achieved (Ziska and Dukes 2011).

Control is the more achievable outcome and refers to those methodologies that can reduce weed impacts to manageable levels. There are several methods to control weed growth, and can be classified as non-chemical or chemical methods.

In the group of non-chemical methods, cultural, mechanical and biological measures are included. Cultural control recognizes those practices to good land and water management, such as crop rotation, false seedbed or cover crops. Mechanical control reflects some of the oldest known means to eliminate weed populations, including hand-pulling and hoening but also refer to flooding, mulching the soil, burning and machine tillage. Biological control is a strategy to employ a specific organism that once introduced will produce a measure of weed control (Ziska and Dukes 2011). Non-chemical weed management increases its efficacy by combining multiple methods (Délye et al. 2013).

Chemical control of weeds through the selective application of herbicides is the most utilized practice (Edwards and Hannah 2014). A great advantage of chemical control over mechanical weed control is the ease of application, which often saves on the cost of labour. That is one of the reasons why, despite of all those non-chemical methods, chemical control is the most extensively used and the most important tool to control weeds. Nowadays chemical weed control has expanded to probably every crop situation in the world (Cobb and Reade 2010).

Integrated control in weed management merges the use of non-chemical and chemical methods, and is the sustainable management needed for evolving from a weed management focused on the eradication of weed species to one that considers the role of the weed community in the agroecosystem. Integrated weed management systems also embrace environmental and financial factors, and need to be effective enough for long-term maintenance of natural resources and agricultural productivity but also to have minimal adverse environmental impact combined with adequate economic returns to the farmer (Cobb and Reade 2010).

GI.1.2. Herbicides

Herbicides are chemical products that limit the growth of plants and are by far the most effective weed control developed (Délye et al. 2013). Herbicides are

phytotoxic, usually organic compounds divided into chemical families which include multiple compounds. Herbicides can be either selective or nonselective, and either be applied to emerged plants (postemergence) or directly to the soil (preplant or preemergence). Application of the herbicide may be as a spray in water or oil, or as dry granules and sometimes surfactants are added to increase the absorption of the active ingredient by the plant.

The mechanism of action, or site of action, of an herbicide is the target site or biochemical process that is specifically inhibited or blocked by the herbicide compound molecule. Herbicides inhibit specific molecular target sites belonging critical plant biochemical and/or physiological processes. Most herbicide target sites are enzymes involved in primary metabolic pathways or proteins carrying out essential physiological functions (Dayan et al. 2010).

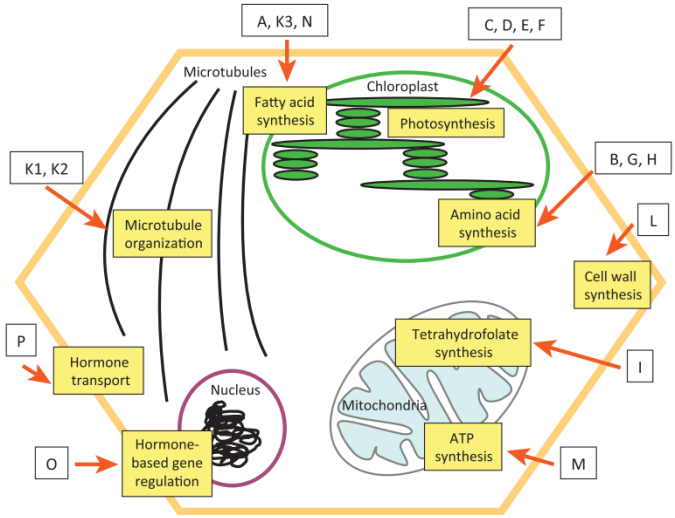

Figure GI.1. Cellular targets of herbicide action and herbicide classification by site of action according to the Herbicide Resistance Action Committee (HRAC). Taken from Délye *et al*., 2013.

The Herbicide Resistance Action Committee (HRAC) has classified herbicides according to their site of action (Figure GI.1 and Table GI.1). This classification system names the groups of herbicides with different letters of the alphabet each one according to the cellular target. In this study, the herbicide of family G (glyphosate) was used, which affects the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), enzyme of the aromatic amino acid (AAA) biosynthetic pathway, located in the plastids.

Table GI.1. The groups of herbicides classified by site of action according to the Herbicide Resistance Action Committee (HRAC). Modified from Délye et al. 2013.

GROUP	Herbicide site of action
A	Inhibition of acetyl-CoA carboxylase (ACCase)
B	Inhibition of acetohydroxyacid synthase (AHAS, ALS)
C	Inhibition of photosystem II protein D1
D	Diversion of the electrons transferred by the photosystem I ferredoxin
E	Inhibition of protoporphyrinogen oxidase
F	Inhibition of phytoene desaturase or 4-hydroxyphenylpyruvate dioxygenase
G	Inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)
H	Inhibition of glutamine synthase
I	Inhibition of dihydropteroate synthase
K1, K2	Enhancement of tubulin depolymerization
K3	Inhibition of fatty acid synthase
L	Inhibition of cellulose-synthase
M	Uncoupling of oxidative phosphorylation
N	Inhibition of fatty acid elongase
O	Simulation of transport inhibitor response protein 1
P	Inhibition of auxin transport

Once the herbicide reaches the target site, many physiological effects are triggered before plant death occurs and this process is known as mode of action. Although the mechanism of action has been well established for most of the commercialized herbicides, the contribution of the physiological effects derived of it is not completely understood; the exact reason by which plant dies after herbicide application is not completely known.

In general, three reasons can be proposed that lead to plant death after herbicide application. Firstly, due to a lack of the products of the pathway where the target enzyme is located, as a consequence of the metabolic pathway inhibition. Secondly, due to an accumulation of the substrates or the metabolites upstream the inhibited enzyme. Finally, due to side reactions elicited after the target inhibition (Siehl 1997). Knowledge of the mode of action, the physiological effects involved in plant death after herbicides application, could provide a better understanding of the plant metabolism and could be useful in the design of new herbicides and improve the use of herbicides.

GI.1.3. Resistance and mechanisms of resistance

Herbicide resistance is the inherited ability of an individual plant to survive an herbicide application that would kill a normal population of the same species. Herbicide-resistant biotypes of weeds are a predictable result of natural selection, of naturally mutations and evolutionary processes (Roma-Burgos et al. 2019), the result of the adaptive evolution of weed populations to the intense selection pressure by herbicide treatment (Neve et al. 2009).

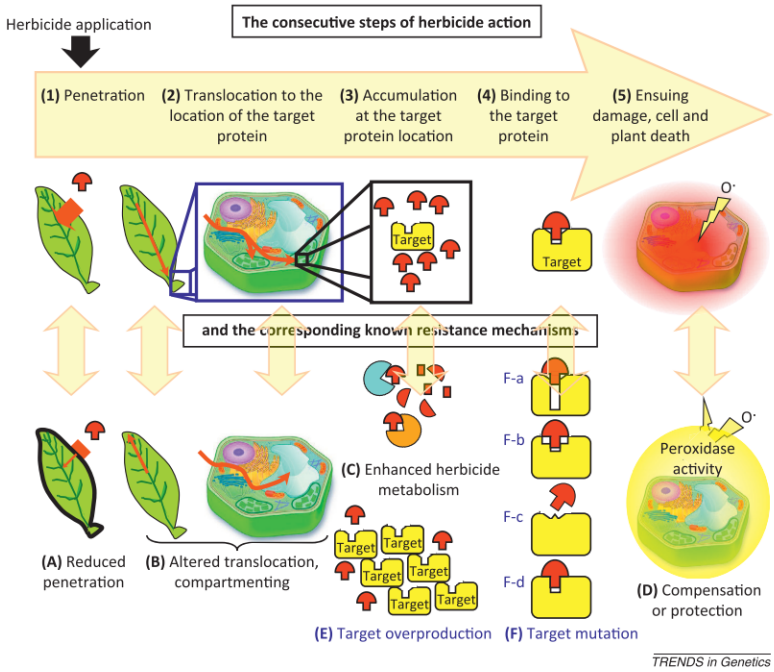

Figure GI.2. Resume of the consecutive steps of herbicide action as a guide (top) and Non-Target site and Target site mechanisms of resistance (bottom). Numbers 1 to 5 indicate the steps of herbicide action. Capital letters in black (A, B, C and D) indicate Non-target site mechanism of resistance and capital letters in blue (E and F) indicate Target site mechanisms of resistance. (Délye et al. 2013).

Figure GI.2 shows a simplification of the mechanisms of resistance and its relation with the steps of the herbicide action. As indicated in the arrow located in the top of the figure, the herbicide action occurs as consecutive steps: after herbicide application, herbicide penetrates into the plant, is translocated to the location of the target protein, accumulated at this location and then binds to the target enzyme. Herbicide then disrupts biosynthetic pathways or vital structures, and causes plant death.

Resistance can be classified into two groups depending on the strategy used by the plant to survive the herbicide treatment: non-target site and target site mechanisms (Powles and Preston 1995; Délye et al. 2013). The former involves mechanisms that minimize the amount of active herbicide reaching the target site. The latter exists when herbicide reach the target site at a lethal dose but there are changes at the target site that limit herbicide impact (Powles and Yu 2010).

G.I.1.3.1. Non-target site mechanisms

Such mechanisms act minimizing the amount of herbicide reaching the target site. There are different mechanisms that confer this type of resistance (Figure GI.2 A-D), such as a decrease of the herbicide penetration into the plant, limitation of herbicide translocation, sequestration of the herbicide into vacuoles, increase of the herbicide detoxification pathways (Powles and Yu 2010) and protection against herbicide effects (Délye et al. 2013). Non-target based resistance is an unspecific resistance mechanism and can confer cross-resistance to herbicides of different sites of action and very little is known about the genetic determinants that cause this type of resistance (Délye 2012).

A decrease of the herbicide penetration (Figure GI.2.A) has been reported in several weed biotypes resistant in all most used herbicides (Délye 2012). Differences in the cuticle properties of resistant plants cause a penetration reduction, but this type of resistance mechanism does not confer high levels of resistance (Délye 2012).

Reduction of herbicide translocation (Figure GI.2.B) involves a limitation on the herbicide movement across the plant and/or the compartmentation of the herbicide in a specific part of the cell, such as vacuoles (Figure GI.2.D) (Délye 2012).

If the herbicide reaches the target site, the active herbicide molecules can be degraded by an enhanced metabolism (Figure GI.2.C) before reaching the target site. Herbicide degradation is the most studied non-target mechanism and it is a multistep process involving the coordinated action of several types of enzymes. For example, it has been described that in the degradation process of aryloxyphenoxypropionate, an acetyl CoA carboxylase (ACCase) inhibitor, the herbicide molecule is first transformed into a more hydrophilic metabolite. Then, it is conjugated to a plant acceptor molecule (usually sugars) and finally the metabolite is transported to a vacuole or the cell walls where further degradation

may occur (Délye 2012). Several enzyme families have been identified in herbicide-degrading processes, such as cytochromes P450 and glutathione-S-transferases families.

Also, protection against herbicide effect can provide the plant resistance. In some cases, herbicides can damage cell components by reactive oxygen species after binding the target enzyme. For example, some resistant plants protects the plant against the collateral herbicide damage in ACCase inhibitors (Figure GI.2.D) by increasing the expression of peroxidases that protect the cells against oxidative stress, and giving the resistant plant time to degrade the herbicide (Délye 2012).

G.I.1.3.2. Target site mechanisms

Target site resistance can be produced by two different mechanisms. Firstly, due to mutations on the gene coding for the target protein that provokes changes on its amino acid sequence. Structural mutations of the target enzyme can modify its structure and properties and will prevent herbicide binding to the enzyme (Figure GI.2.F). Mutations can result in different levels of reduction of herbicide sensitivity (Figure GI.2.F a-d) at the protein level (Délye et al. 2013).

Secondly, due to overproduction of the target enzyme (Figure GI.2.E), by an overexpression due to gene amplification –higher gene copy number- of the gene coding for the target protein or by an enhanced expression of the gene of the target enzyme. Several cases of resistance due to target overproduction have been described in different species to herbicide of type G, glyphosate, since Gaines et al., (2010) identified a glyphosate resistant *Amaranthus palmeri* biotype by EPSPS gene overexpression by gene amplification.

G.I.1.4. Resistance evolution

Long-term and repeated application of the same herbicide, or herbicides with the same site of action, accelerates the selection of resistant biotypes to that family of herbicides (Powles and Yu 2010; Neve et al. 2014). Figure GI.3. shows the evolution of number of species that have developed at least one biotype resistant to the different families of herbicides according to HRAC classification (Heap, 2019). Resistance to herbicides belonging to B group was confirmed few years after herbicide appearance in the market. Afterwards, the number of resistant biotypes to this group has highly increased and nowadays this group of herbicides is the one that presents the largest number of resistant populations. After glyphosate was released in the market in 1974, more than 20 years passed until

the first report of glyphosate resistance. The evolution of resistant populations to G group has increased in the following years after the introduction of the glyphosate-resistant crops (GRCs) in 1996. Several factors have to be taken into account regarding to herbicide-resistance development. The first factor is related to the genetic variability of the species, because it seems that resistance evolution is relatively slow with high ploidy level or in cross-pollinated species (Roma-Burgos et al. 2019), making resistance selection more likely in some species than in others.

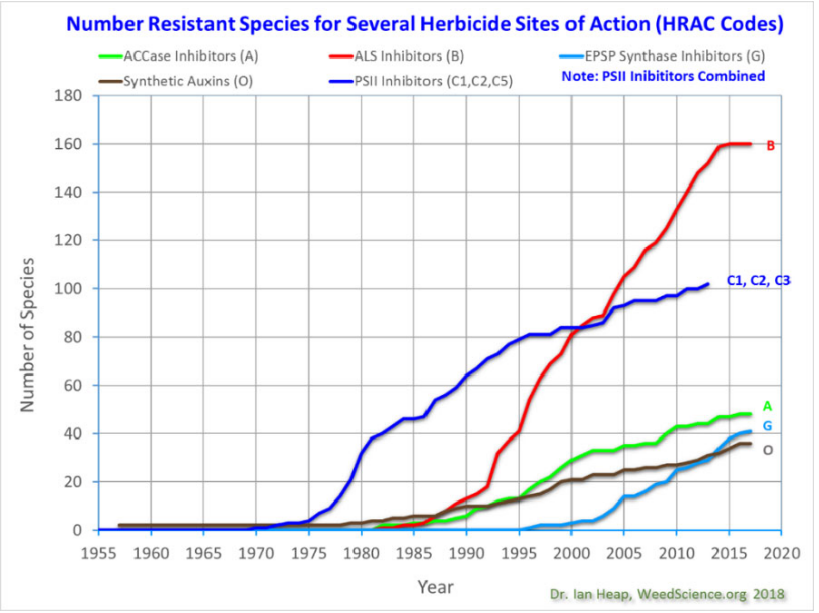

Figure GI.3. The evolution of the increase in the number of resistant biotypes to different herbicide groups classified by their site of action according to HRAC classification (Heap, 2019).

Other factor in the selection of resistant individuals is the nature of the target site. AHAS (acetohydroxy acid synthase) enzyme keeps its function even when mutations are located in the herbicide binding. Indeed, the use of herbicides AHAS inhibitors (Group B) has developed the fastest evolution of resistant genotypes documented (Figure GI.3). For example, on the same population would take fewer generations on the selection of AHAS inhibitor-resistant individuals than other targets. In contrast, EPSPS (5-enolpyruvylshikimate 3-phosphate synthase) enzyme, inhibited by herbicides from Group G, is highly conserved, and the enzyme loses its function after simple mutations in the protein-coding regions (Roma-Burgos et al. 2019). This makes this kind of target-site resistance to glyphosate not very frequent, or not giving a very high resistance level.

And finally, weed management is also an important factor in herbicide resistance. For example, the introduction of herbicide resistant crops has increased the development of weed resistant biotypes due to the application of a single herbicide continuously, and such increasing the selection pressure.

G.I.1.5. Challenges in weed management

The effectiveness of the weed management is facing a critical scenario. Decades of chemical weed management have led to an increase in the number of herbicide resistant populations, with a decrease in the number of available herbicide sites of action (Westwood et al. 2018). At the same time, the world population is increasing with the concomitant increase of food demand. The current crop production levels are not adequate to feed the 9 billion projected population by the year 2050 (Figure GI.4.A). The climate change, the loss of water resources and reduction in arable land are some of the problems that will make more difficult to reach this crop production levels. Weeds are also a persistent problem that increases the crop production costs and the increase of the number of herbicide-resistant biotypes is threatening the productivity rates (Figure GI.4.B) (Westwood et al. 2018). This problem will be turning worse in the following years, because the number of herbicide resistant biotypes is projected to continue to increase drastically, but the number of herbicides sites of action, which stopped in 1995, not.

Over the last years, the concern about environmentally sustainable use of the pesticides has increased and the number of authorized herbicides has decreased. Many herbicide active ingredients had been severely restricted or had been completely disappeared from the market, particularly in Europe, due to safety concerns. For example, the commercialization of pesticides was regulated in Europe by the EC N°1107/2009 and the sustainable use of pesticides by the Directive 2009/128/EC. Since that moment, products that contain risk substances for human health (such as carcinogens, mutagens, toxic for reproduction or endocrine disrupters) or for the environment (such as persistent or bioaccumulative compounds) had not being registered, or had lost their registration. As a result, the herbicide market worldwide is dominated by just six different types of mechanisms of action, leaded by family G, in which glyphosate is included (Figure GI.5) (Peters and Strek 2018).

Due to several reasons, no new sites of action have been introduced since the end of the 1980s. One of the main reasons is the intensifying environmental regulations that led to a substantial increase in the cost to develop and register

new herbicides. Combined with the introduction of GRCs and their easy management, the increasing cost of herbicide development and registration greatly reduced the potential economic benefit of bringing a new herbicide to market, ceasing the introduction of new herbicide sites of action (Roma-Burgos et al. 2019).

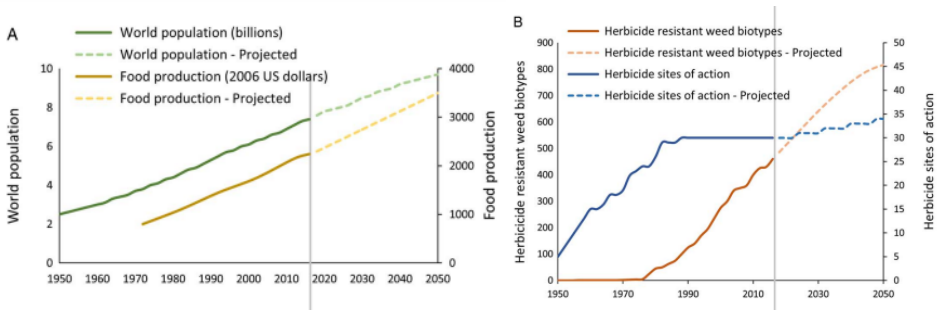

Figure GI.4. The growing world human population and the agricultural production needed to meet growing demand based on World Bank estimation data (A). Rise in herbicide-resistant weed biotypes and herbicide sites of action discovered and their future projections (B).

Therefore, the increase in the selection pressure based on applying repeatedly herbicides with the same site of action is developing the selection of herbicide-resistant biotypes to that family of herbicides. Weed resistance is nowadays a rapidly growing problem with 251 different species resistant to 23 of 26 known herbicide sites of action (Peters and Strek 2018) as has been covered in section G.I.1.4.

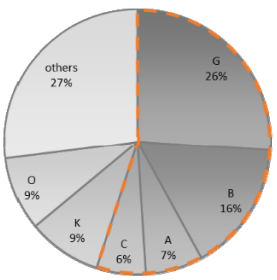

Figure GI.5. Most important herbicidal modes of action in percentages of global market value for 2015, shown for sites of action with HRAC classification. Those with significant resistance issues are enclosed in a dotted line. Modified from Peters and Strek 2018.

On the way of trying to find effective solutions to herbicide-resistant weeds, some alternative strategies can be affordable (Figure GI.6). However, not all the alternatives guarantee a long-term solution. Solutions in which a greater use of herbicide would be contemplated, the selection pressure would increase and

therefore resistant biotypes too. For example, biotechnology companies are currently promoting, as a solution to glyphosate resistant (GR) weed problems, a second-generation of genetically modified crops resistant to additional herbicide sites of action. As explained before, this approach will create new resistant weed challenges, will increase risks to environmental quality and will lead to a decline in the science and practice of integrated weed management. However, in solutions involving integrated weed management the selection pressure would be reduced, alleviating the severity of the problem resistant weeds. Integrated weed management requires the application of different techniques (herbicides combined with other non-chemical control practices) in order to find an effective and stable solution in the long term to reduce the evolution of herbicide-resistant populations.

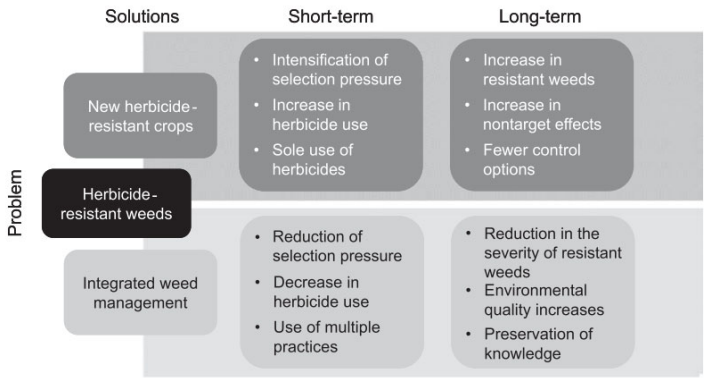

Figure GI.6. A conceptual model of the alternative solutions and their potential consequences presently available for addressing glyphosate-resistant weed problems. Taken by Mortensen et al. 2012.

In this context, it has been described the importance of incorporating an integrated weed management perspective in the design of best management practices to prevent weed spread and to manage herbicide resistance, including the biological and ecological characteristics of agricultural weeds at their agricultural context. (Roma-Burgos et al. 2019). Instead of targeting in the eradication of one specific weed species or intensification of the selection pressure by leading to continue using herbicide application as the main option, integrated management would develop a program considering ecological, environmental, and social issues, which could be maintained through multiple years.

G.I.2. Aromatic amino acid biosynthetic pathway or shikimate pathway

G.I.2.1. The pathway and its regulation

L-tyrosine (Tyr), L-phenylalanine (Phe) and L-tryptophan (Trp) are aromatic amino acids (AAAs) synthetized in the shikimate pathway. They are not only components of proteins, but also precursors of secondary metabolites that play important roles in plant growth, development, reproduction, defense and environmental responses (Tzin and Galili 2010a; Maeda and Dudareva 2012; Tohge et al. 2013a, b). The shikimate pathway is present in plants, bacteria and fungi, but this pathway has been lost in animal lineages, making the AAAs essential components of animal diet. The absence of the AAAs pathway in animals also makes the enzymes of this pathway suitable targets for herbicides (Baylis 2000), such as glyphosate, which site of action is the inhibition of EPSPS, enzyme of this pathway.

Approximately 20 % of the total carbon fixed by the plant can flux to the AAAs biosynthetic pathway (Haslam 1993). The pathway, also known as shikimate pathway, is located in the plastids and can be subdivided in two steps: (a) the pre-chorismate pathway, where carbon from primary metabolism derived glycolysis and the pentose phosphate pathway is used to form chorismate (Maeda and Dudareva 2012). And (b) the post-chorismate pathway, which via two different routes can lead from chorismate to the synthesis of Phe and Tyr, or Trp (Maeda and Dudareva 2012; Tohge et al. 2013b).

The synthesis of chorismate in the shikimate pathway is performed by seven enzymatic reactions that act sequentially: D-arabino-heptulosonate 7-phosphate synthase (DAHPS), dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase (DHD), shikimate dehydrogenase (SDH), shikimate kinase (SK), EPSPS and chorismate synthase (CS). The enzymes DHD and SDH form a bifunctional dimer (DQSD) in plants (Maeda and Dudareva 2012) and in this work the complex was treated as one enzymatic complex and will be named as DQSD.

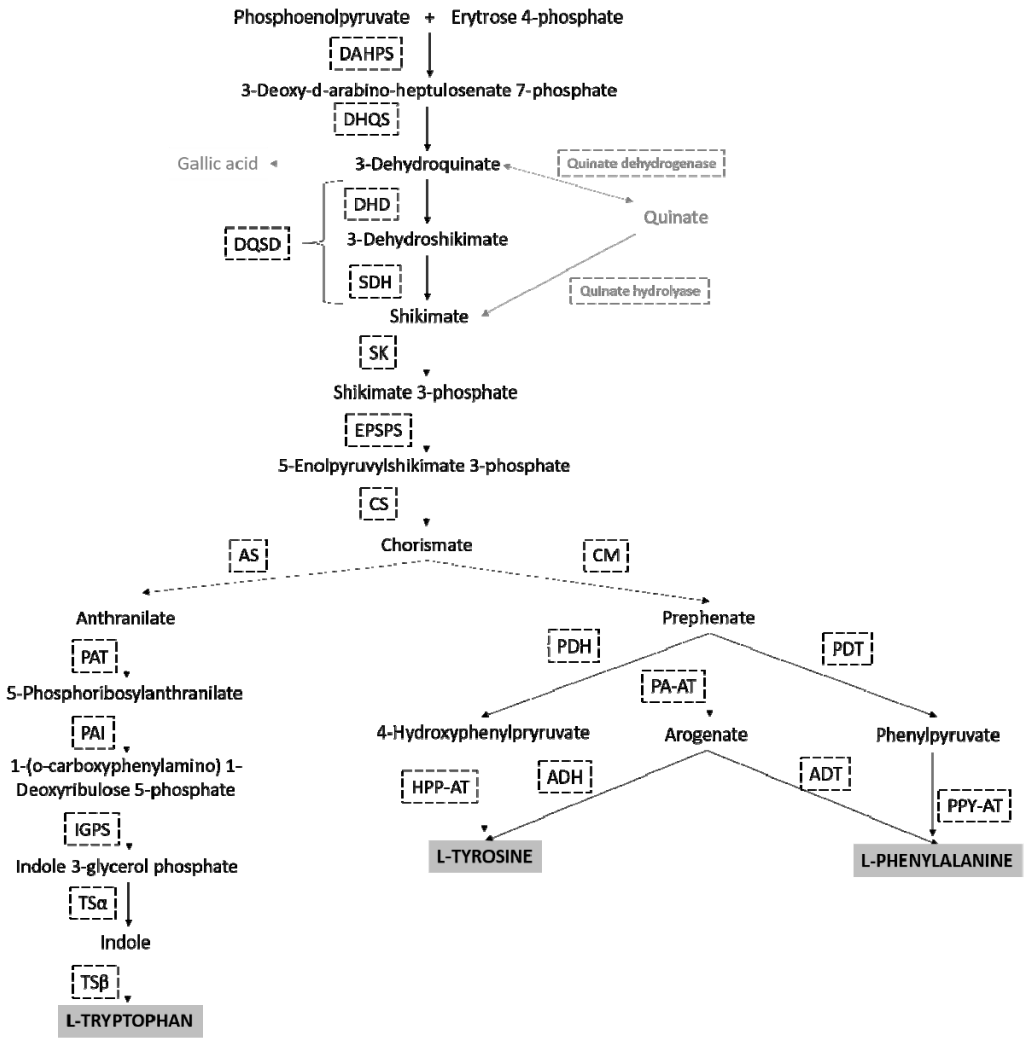

Figure GI.7. Aromatic amino acids biosynthetic pathway in plants. The enzymes belonging the pre-chorismate pathway: D-arabino-heptulosonate 7-phosphate synthase (DAHPS), dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase (DHD), shikimate dehydrogenase (SDH), the bifunctional DHD-SDH dimer (DQSD), shikimate kinase (SK), 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) and chorismate synthase (CS). The enzymes belonging the post-chorismate pathway, towards the tryptophan synthesis: Anthranilate synthase (AS), phosphoribosylanthranilate transferase (PAT), phosphoribosylanthranilate isomerase (PAI), indole-3-glycerol phosphate synthase (IGPS), tryptophan synthase α subunit (TSα), tryptophan synthase β subunit (TSβ). The enzymes belonging the post-chorismate pathway towards the tyrosine and phenylalanine synthesis: chorismate mutase (CM), prephenate dehydrogenase (PDH), 4-hydroxyphenylpyruvate aminotransferase (HPP-AT), prephenate aminotransferase (PA-AT), arogenate dehydrogenase (ADH), arogenate dehydratase (ADT), prephenate dehydratase (PDT), phenylpyruvate aminotransferase (PPY-AT). Secondary metabolites are represented in gray and final products AAA are represented in bold capital letters and gray squared.

Chorismate can be used as substrate by anthranilate synthase (AS) or chorismate mutase (CM) at the first step of the post-chorismate pathway (Figure GI.7). The synthesis of Trp converts chorismate to Trp via six enzymatic reactions, and its first step is mediated by AS enzyme. The other branch, leading to Tyr or Phe biosynthesis, is mediated in its first step by CM enzyme for prephenate biosynthesis. After prephenate, the synthesis of Tyr or Phe may occurs via two alternative pathways, the arogenate pathway or the phenylpyruvate/4-hydroxyphenylpyruvate pathway (Figure GI.4).

Many factors strongly regulate the key pathway of the synthesis of AAAs (Tzin and Galili 2010b; Tohge et al. 2013b; Galili et al. 2016), and the carbon flux trough the pathway is regulated at transcriptional, post-transcriptional and post-translational level (Bentley and Haslam 1990; Maeda and Dudareva 2012).

All enzymes belonging to the shikimate pathway have been characterized, and their corresponding genes have been identified. Although genetic studies have been performed about this pathway in plants, its regulation has not been completely clarified. Nevertheless, some studies have described in plants possible transcriptional and post-translational regulations.

G.I.2.1.1. Transcriptional regulation of the shikimate pathway

In plants the expression of genes encoding enzymes in the AAAs and their pathways downstream seems to be closely regulated, often by the same transcription factors (Maeda and Dudareva 2012).

In *Arabidopsis* it was described that some MYB transcription factors activated the genes encoding DAHPS and AS (Bender and Fink 1998; Gigolashvili et al. 2007). Three transcription factors have been described in *Petunia hybrida*: EPF1, ODORANT1 (ODO1) and EMISSION OF BENZENOIDS II (EOBII), the two last belonging to MYB transcription factor family (Takatsuji et al. 1992; Spitzer-Rimon et al. 2010). EPF1 directly binds to the EPSPS promoter and controls its spatial and developmental expression (Takatsuji et al. 1992). ODO1 and EOBII transcription factors regulate CM and phenylalanine ammonia lyase (PAL) expression and EOBII can bind ODO1 (Verdonk 2005; Spitzer-Rimon et al. 2010). ODO1 transcription factor affects also DAHPS and EPSPS expression (Verdonk 2005). AS expression is also regulated by ORCA3 transcription factor (Van Der Fits and Memelink 2000).

Besides transcription factors, other mechanisms have been proposed. For example, it seems that also reduced levels of AAAs would act as signal to induce the expression of the shikimate pathway genes to increase the flux through the pathway, including the DAHPS enzyme (Maeda and Dudareva 2012).

Interestingly, a perturbation on the pathway such as EPSPS inhibition caused by glyphosate treatment has been described to provoke an increase of the transcript abundance of the genes of the shikimate pathway (Fernández-Escalada et al. 2017).

G.I.2.1.2. Post-transcriptional / post-translational regulation of the shikimate pathway

Regulation at the entrance of the pathway

In plants, how the carbon flux into the shikimate pathway is specifically regulated (Figure GI.8) is ambiguous (Maeda and Dudareva 2012). The entrance of carbon through the pathway is mediated by DAHPS enzyme. It catalyzes the first reaction of the pathway from phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to 3-deoxy-D-arabino-heptulosonate 7-phosphate. In microbes, the regulation of the expression of this enzyme is very well known. It has been described that the expression of DAHPS enzyme is regulated in response to cellular levels of AAAs. However, there is limited information about its regulation in plants. DAHPS activity seems to not be inhibited by AAAs with few exceptions *in vitro*: Trp and Tyr produced a DAHPS inhibition in maize shoots and a DAHPS activity activation by Trp in carrot and potato (Graziana and Boudet 1980; Suzich et al. 1985). Phe-sensitive DAHPS has not been identified in plants (Maeda and Dudareva 2012). Inhibition of the DAHPS activity *in vitro* was observed with arogenate, suggesting that this intermediate may exert short-term feedback control of flux into the pathway (Rubin and Jensen 1985; Siehl 1997). The *in vivo* role of arogenate regulation remains to be investigated.

Interestingly, EPSPS inhibition due to glyphosate treatment induced DAHPS protein level and activity (Pinto et al. 1988) and the protein level of this enzyme also increased in a dose-dependent way after different glyphosate doses applied (Fernández-Escalada et al. 2017)

Regulation at the branch point (AS/CM)

The bifurcation of the pathway towards Trp and Phe/Tyr pathways is controlled by AS and CM enzymes. Both enzymes use chorismate as a substrate (Maeda and

Dudareva 2012). AS and CM are feedback inhibited by the AAA of their corresponding pathways: Trp inhibits AS activity and Phe and Tyr inhibits CM activity (Romero et al. 1995; Bohlmann et al. 1996). Trp also activates CM activity to redirect flux from Trp to Phe/Tyr biosynthesis (Kuroki and Conn 1988; Benesova and Bode 1992; Lopez-Nieves et al. 2017). Tyr activates ADT to redirect the flux from Tyr to Phe biosynthesis (Siehl and Conn 1988) to ensure that the major carbon flux is directed toward Phe biosynthesis in plants.

In many plants, CM activity exists in two isoenzyme forms, CM1 and CM2 (Maeda and Dudareva 2012), which are regulated quite differently:

CM1 is located in the plastid and its regulatory behavior is consistent with a role as a committing enzyme in an amino acid biosynthetic pathway. CM1 is feedback-inhibited by each of the end of the products, Phe and Tyr, and is activated by Trp, the product of the other branch of the pathway (Tzin and Galili 2010b; Buchanan et al. 2015). This mechanism regulates flux into the two competing pathways by increasing synthesis of Phe and Tyr when Trp is plentiful, and suppressing synthesis of Phe and Tyr when the supply of these amino acids is adequate (Benesova and Bode 1992; Eberhard et al. 1996). The *Arabidopsis* genome contains an additional gene encoding another isoenzyme: CM3 (Kuroki and Conn 1988). Like CM1, CM3 contains a putative plastid transit peptide and is subject to allosteric regulation.

On the other hand, the isoform CM2 is located in the cytosol, and lacks a putative plastid transit peptide. The activity of CM2 is usually insensitive to allosteric regulation by AAAs (Benesova and Bode 1992; Eberhard et al. 1996). This isoform has roughly a 10-fold higher affinity toward chorismate than CM1. This is consistent with a presumably lower chorismate concentration in the cytosol. Because the substrate for CM is produced in the plastid, the function of this isoenzyme remains unknown, but it seems that could play a critical role in interactions between the plant with nematodes and fungi (Buchanan et al. 2015).

Arogenate dehydrogenase (ADH) catalyzes the synthesis to Tyr from arogenate in one of the possible synthesis branches of this amino acid (Figure GI.7). Two ADH isoforms have been described in *Arabidopsis,* ADH1 and ADH2, whose individual functions remain to be determined. Two isoforms have also been described in *Beta vulgaris,* encoded by two ADH genes (called *BvADH*α and *BvADH*β) that are differently regulated: *BvADH*β is strongly inhibited by Tyr, and *BvADH*α exhibited relaxed sensitivity to Tyr (Lopez-Nieves et al. 2017).

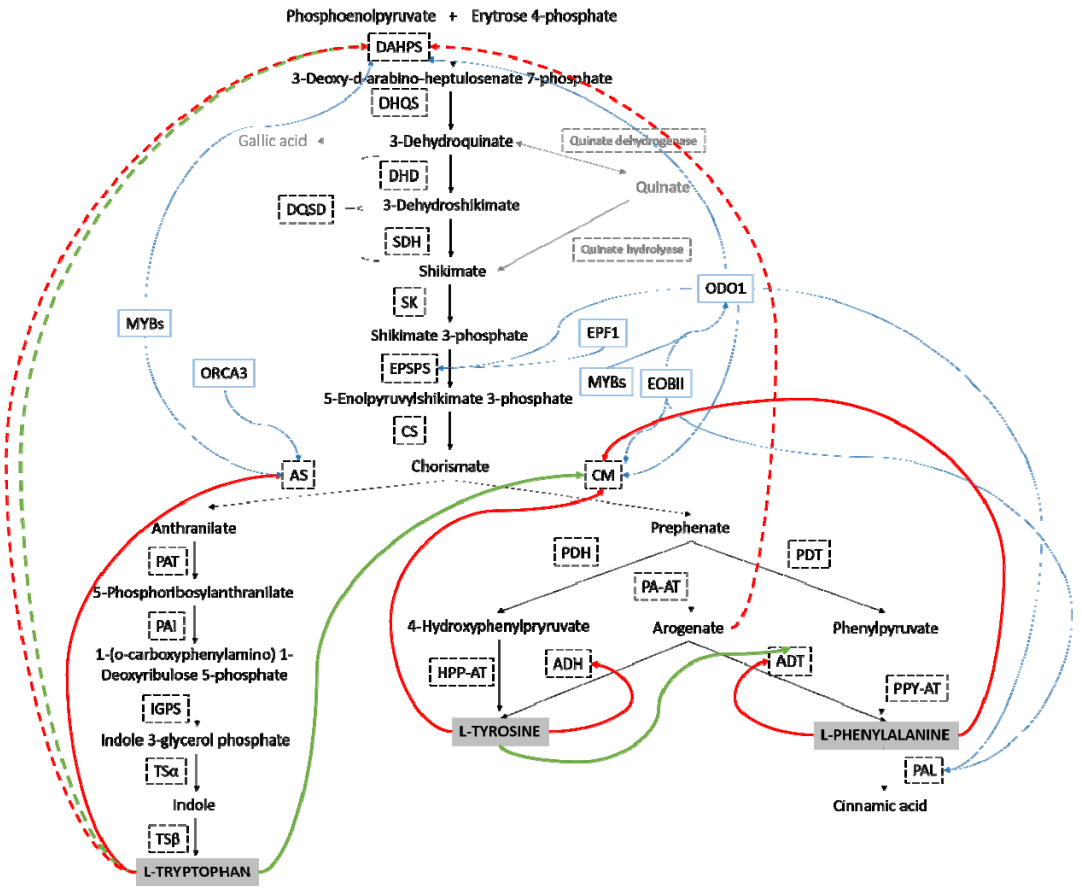

Figure GI.8. Regulation of the aromatic amino acids biosynthetic pathway in plants. Transcriptional regulation, transcription factors squared in blue and blue arrows for representing their effect. ODORANT1 (ODO1); Post-translational regulation, being red for inhibition loop, green for enhanced loop. Discontinued loop for *in vitro* results. The enzymes belonging the pre-chorismate pathway: D-arabino-heptulosonate 7-phosphate synthase (DAHPS), dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase (DHD), shikimate dehydrogenase (SDH), the bifunctional DHD-SDH dimer (DQSD), shikimate kinase (SK), 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) and chorismate synthase (CS). The enzymes belonging the post-chorismate pathway, towards the tryptophan synthesis: Anthranilate synthase (AS), phosphoribosylanthranilate transferase (PAT), phosphoribosylanthranilate isomerase (PAI), indole-3-glycerol phosphate synthase (IGPS), tryptophan synthase α subunit (TSα), tryptophan synthase β subunit (TSβ). The enzymes belonging the post-chorismate pathway towards the tyrosine and phenylalanine synthesis: chorismate mutase (CM), prephenate dehydrogenase (PDH), 4-hydroxyphenylpyruvate aminotransferase (HPP-AT), prephenate aminotransferase (PA-AT), arogenate dehydrogenase (ADH), arogenate dehydratase (ADT), prephenate dehydratase (PDT), phenylpyruvate aminotransferase (PPY-AT), phenylalanine ammonia lyase (PAL). Secondary metabolites are represented in gray and final products AAA are represented in bold capital letters and gray squared.

G.I.2.2. Cross-regulation with branched chain amino acid biosynthetic pathway

Branched chain amino acid (BCAA) biosynthetic pathway (Figure GI.9) can be found in microorganisms and plants, and leucine (Leu), valine (Val) and isoleucine (Ile) are essential amino acids for animals. In plants, the BCAA biosynthetic pathway is located in plastids (Wittenbach and Abell 1999). This pathway is formed by two different branches, where Ile is synthesized in one branch, and Leu and Val through the other branch. Both branches are parallel and the synthesis of the three amino acids share the 4 enzymes (Galili et al. 2016). Three more enzymatic reactions are needed for the synthesis of Leu and one previous for Ile synthesis (Figure GI.9).

The first step of the common pathway is catalyzed by the acetohydroxy acid synthase (AHAS) enzyme, also known as acetolactate synthase (ALS). It catalyzes the condensation of two molecules of pyruvate to 2-acetolactate in one branch, or one molecule of pyruvate and one molecule of 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate in the other branch (Binder et al. 2007). 2-ketobutyrate is synthetized from threonine mediated by threonine deaminase (TD). Once 2-acetolactate or 2-aceto-2-hydroxy-butyrate are formed, acetohydroxyacid isomer-reductase (AHAIR) catalyzes the reductive isomerization to 2,3-dihydroxy-3-isovalerate or to 2,3-dihydroxy-3-methylvalerate, respectively (Durner et al. 1993). Dihydroxyacid dehydratase (DHAD) catalyses the dehydration of these acids to the 2-oxo acids: 3-methyl-2-oxobutanoate or 3-methyl-2-oxopentanoate. The first one serve as a substrate for the biosynthesis of Val and Leu, and the second one is the substrate for the biosynthesis of Ile (Binder 2010). The last step in the biosynthesis is the action of BCAA transaminase (TA), which catalyzes the transamination of the respective 2-oxo acids into their corresponding amino acids: 4-methyl-2-oxopentanoate to Leu, 3-methyl-2-oxobutanoate to Val, and 3-methyl-2-oxopentanoate to Ile (Singh 1999).

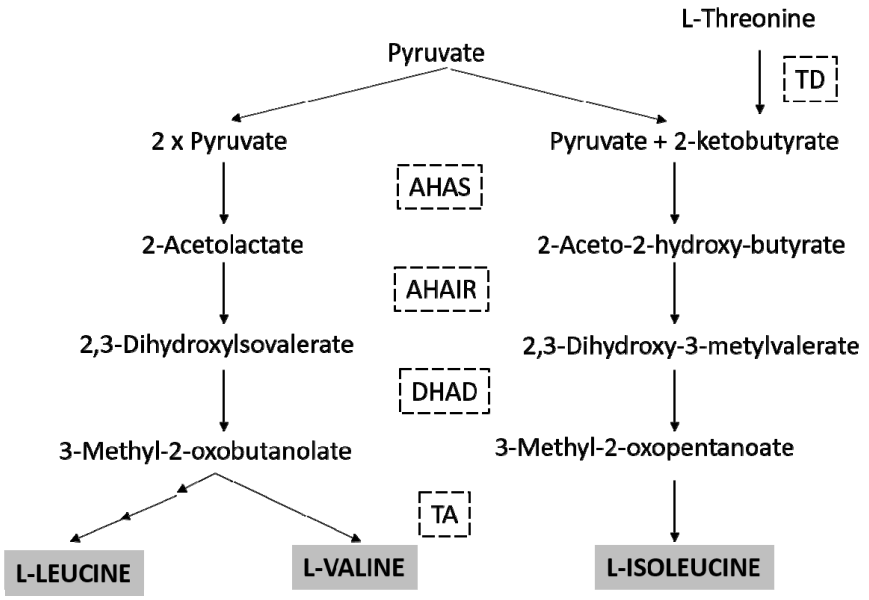

Figure GI.9. Branched chain amino acids biosynthetic pathway in plants with main enzymes. Threonine deaminase (TD), acetohydroxy acid synthase (AHAS), acetohydroxyacid isomer-reductase (AHAIR), dihydroxyacid dehydratase (DHAD) and BCAA transaminase (TA). Final products BCAA are represented in bold capital letters and gray squared.

The hypothesis that amino acid biosynthetic pathways are close cross-regulated has been previously proposed and different interactions between pathways have been described (Guyer et al. 1995; Mohapatra et al. 2010; Pratelli and Pilot 2014). In contrast, it has also been proposed that the apparent cross-regulation of the pathways is the consequence of a stress response caused by alterations in the activity of specific amino acid pathways that modify normal amino acid levels that were caused by amino acid perturbations (Hey et al. 2010).

Several studies suggest a cross relationship between AAA and BCAA biosynthetic pathways. The contents of many minor amino acids vary in concert with different amino acid biosynthetic families, and the closest correlation in these variations occurs between AAA and BCAA (Noctor et al. 2002; Orcaray et al. 2010). For example, treatment in *Lemna minor* with an aminotransferase inhibitor showed the concomitant accumulation of BCAAs and two AAAs (Tyr and Phe) (Brunk and Rhodes 1988). Interactions between Leu synthesis and levels of Tyr and Phe were also observed (Wittembach et al. 1994).

G.I.3. *Glyphosate*

G.I.3.1. Overview

Glyphosate has been by far the most widely used herbicide in the world for about two decades (Duke et al. 2018) and it is a broad-spectrum herbicide that has changed modern agriculture. Glyphosate is the common name of the molecule N-(phosphonomethyl) glycine (Figure GI.10) and it was tested as a herbicide for the first time in 1970 (Franz et al. 1997). This molecule is active as a salt with various cations and the isopropylamine salt was first commercialized in 1974 as a post-emergence, non-selective herbicide called Roundup® (Duke and Powles 2008).

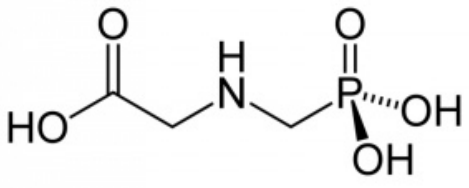

Figure GI.10. Structure of glyphosate: N-(phosphonomethyl) glycine.

Glyphosate penetrates rapidly through leaf cuticle and is diffused across it. After that, the herbicide is translocated from the leaves via phloem to actively growing tissues or organs (Duke and Powles 2008; Vila-Aiub et al. 2012), such as apical meristems, young roots and leaves, and storage organs although acropetal glyphosate movement through apoplast has also been reported (Dewey and Appleby 1983).

Glyphosate belongs to the G family of herbicides, so, as described for this family, its mechanism of action is the inhibition of the EPSPS. This enzyme catalyzes the penultimate step of the pre-chorismate part of the shikimate pathway, the AAA biosynthetic pathway (See section GI 1.2 and figure GI.7), a pathway found in microorganisms, fungi and plants (Dill 2005). EPSPS activity is absolutely required for the survival of the plants (Funke et al. 2006) and catalyzes in the plastids the reaction from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to 5-enolpyruvilshikimate-3-phosphate (EPSP). EPSPS functions as following: it firstly binds to S3P and the resulting complex (EPSPS-S3P) changes its conformation (Figure GI.11) and binds to PEP as the second substrate. The glyphosate is a competitive inhibitor of PEP, so it binds to the EPSPS-S3P complex once it is formed. The resulting complex glyphosate-EPSPS-S3P is very stable, with a very

slow reversal rate, blocking its enzymatic activity (Duke 1990; Schönbrunn et al. 2001; Dill 2005) and preventing the binding with PEP. Glyphosate is the only compound found to be an excellent EPSPS inhibitor, and no alternative chemicals targeting this enzyme have been commercialized (Duke 1990; Duke and Powles 2008).

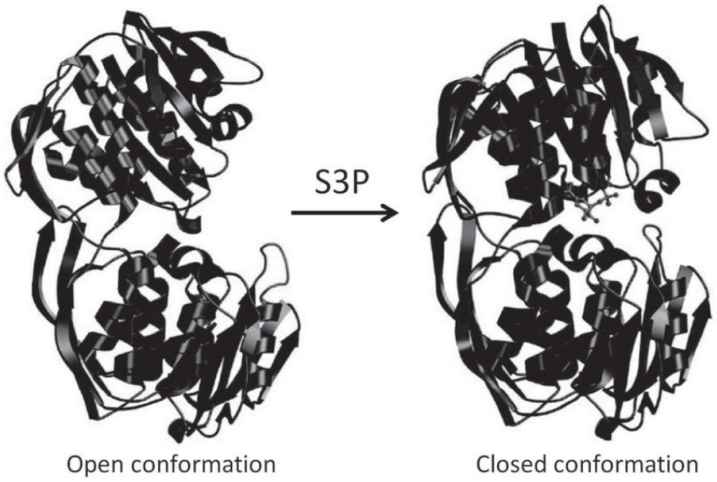

Figure GI.11. Induced fit model of EPSPS enzyme showing open (left) and closed (right) conformation before and after shikimate 3-phosphate (S3P) binding. (Taken from Dev et al. 2012).

At the beginning, when glyphosate was commercialized in 1974, it was used as total herbicide in non-crop situations and in pre- and post-cropping on fields. However, the introduction of the first GRC in 1996 started a weed management revolution and its use has increased to dominate the herbicide market (Duke 2018a). GRCs are the most successful transgenic crops and are a major reason for the heavy use of glyphosate (Duke 2018b). Although glyphosate continues to be used in non-crop situations and in pre- and post-cropping of fields, the use of glyphosate as a selective post-emergence herbicide on GRCs has become the most important use of this herbicide (Heap and Duke 2018) in places were the GRCs are authorized (Duke 2018a).

Most GRCs carry the EPSPS gene of *Agrobacterium* sp. strain CP4 (CP4-EPSPS) a glyphosate-insensitive form of this enzyme (Funke et al. 2006) while all plants and most bacteria have glyphosate-sensitive EPSPS. Once the CP4-EPSPS gene plus a promoter is placed into the genome of the future GRC, it is expressed and high levels of glyphosate resistance are conferred (Duke and Powles 2008). The result is an ability to bypass the endogenous EPSPS system with the CP4-EPSPS insertion

that allows the shikimate pathway to function normally and plants to survive glyphosate treatment (Figure GI.12) (Dill 2005).

Despite of the fact that the CP4-EPSPS gene has been used as the transgene for most GRCs, other possibilities have been developed in GRCs designing. In addition to the CP4 gene, a gene from *Ochrobactrum anthropi* encoding glyphosate oxidoreductase (GOX) was introduced to contribute to resistance in canola by glyphosate metabolization. For maize, the EPSPS has been altered by site-directed mutagenesis of a non-sensitive maize gene to provide a form of GR EPSPS that is used in some GR maize varieties. Genes that encode other forms of GR- EPSPS and glyphosate detoxification enzymes was proposed for GR crops (Duke and Powles 2008).

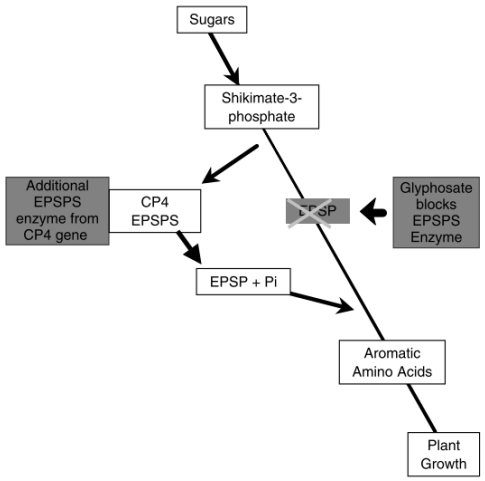

Figure GI.12. Strategy for the development of glyphosate-resistant crops by CP4-EPSPS gene. Taken form Dill, 2005.

The success of GRCs drastically devalued the use and prize of other herbicides, reducing the research on finding new herbicides (Vila-Aiub et al. 2012; Duke 2018a), and the introduction of new herbicide modes of action stopped (Duke 2012). Weed resistance to existing sites of action begun to increase logarithmically. The overuse of glyphosate in GRC, with constant and widespread use of this herbicide, has produced a massive selection pressure and has developed the evolution of glyphosate resistant weed populations (Duke 2018a). The effect of using high or low glyphosate rates on the evolution of herbicide resistance has been analyzed, and the most effective way for avoiding glyphosate

resistance would be to maximize the percent killed by using the highest recommended rate of glyphosate (Heap and Duke 2018).

G.I.3.2. Mode of action

The precise mechanism by which glyphosate-treated plants die remains unclear. According to Siehl (1997), plant death after herbicide application could be related to the accumulation of substrates upstream the enzyme inhibited, a lack of the products of the pathway, or due to a deregulation of the pathway leading to side reactions.

Accumulation of substrates and intermediates: The inhibition of the EPSPS by the herbicide provokes an accumulation on the metabolites upstream the enzyme on the pathway. Shikimate is so highly accumulated after glyphosate treatment that its content is commonly used as an indicator of glyphosate sensitivity (Lydon and Duke 1988; Becerril et al. 1989; Hernandez et al. 1999; Orcaray et al. 2010, 2012; Fernández-Escalada et al. 2016). Although the substrate of the EPSPS is the S3P, and it is also accumulated after glyphosate treatment (Siehl 1997), the accumulation is observed in a lower scale than the accumulation of shikimate. However, although shikimate accumulation has been proposed to have toxic effects in plants (de María et al. 2006), no direct toxic effects of shikimate have been reported. Gallic acid and protocatechuic acid, derivates of the 3-dehydroshikimate, are also accumulated in glyphosate-treated plants (Lydon and Duke 1988; Becerril et al. 1989; Hernandez et al. 1999; de María et al. 2006; Zabalza et al. 2017).

Quinate accumulation has also been reported after glyphosate application (Orcaray et al. 2010). This compound is a secondary metabolite formed on a secondary branch of the shikimate pathway. It will be covered in the section GI.4.2.

Lack of end products: Glyphosate provokes a blockage of the pathway, and the lack of the end products of the pathway is another possible cause of plant death (Siehl 1997). The inhibition of the pathway would provoke a decrease in the AAA levels, not enough to maintain essential protein synthesis (Duke and Powles 2008). In fact, it is generally assumed that, as glyphosate inhibits AAA biosynthetic pathway, it is the lack of the AAAs which causes the death of the treated plants. However, an increase in the AAA content is observed after glyphosate treatment (Zulet et al. 2013a, 2015; Fernández-Escalada et al. 2016; Zabalza et al. 2017). This accumulation would be related to an increase in the

protein turnover, with an increased total free amino acid pool, including AAAs, and a decrease in the soluble protein levels (Zabalza et al. 2013; Zulet et al. 2015).

Phenylpropanoids and other secondary metabolites synthesis could also be affected after glyphosate application. These are important metabolites for plant regulation (Tzin and Galili 2010a; Maeda and Dudareva 2012; Tohge et al. 2013a) and they will be introduced in the section GI.4.1.

Side effects due to blockage of the pathway: Lethality could also be related to the deregulation of the pathway caused by the EPSPS inhibition. It has been described an increase of the transcripts of the genes from the shikimate pathway after glyphosate treatment (Fernández-Escalada et al. 2017).

Glyphosate has been shown to affect other several plant physiological processes, beyond the pathway which is specifically inhibited, which could also be linked to glyphosate-herbicidal effects. Some studies have shown the glyphosate effects being strictly due to EPSPS inhibition (Gomes et al. 2014). Alterations in carbon metabolism in plants has been described after glyphosate treatment. Carbohydrate accumulation was observed in both leaves and roots of glyphosate-treated plants (Orcaray et al. 2012; Armendáriz et al. 2016). As growth was stopped, carbohydrate accumulation in roots was attributed to a lack of utilization of available sugars, which also caused soluble carbohydrate accumulation in leaves (Orcaray et al. 2012), mediated by the inhibition of translocation due to the lack of sink strength. Ethanol fermentation and the activity of alternative oxidase were induced in glyphosate treated roots (Zulet et al. 2015; Armendáriz et al. 2016).

The disruption on the shikimate pathway by glyphosate could have potential adverse effects on other cellular processes, resulting in secondary effects such as creation of reactive radicals (Maroli et al. 2015) and oxidative stress (Ahsan et al. 2008; Gomes et al. 2014). As a metal chelator, glyphosate could deprive plants of important nutrients, which have major roles as enzymatic co-factors and biomolecular constituents (Gomes et al. 2014).

G.I.3.3. Common physiological effects of AHAS-inhibitors and glyphosate

Glyphosate and AHAS-inhibitors herbicides directly target amino acid biosynthetic pathways. Glyphosate specifically inhibits the AAA biosynthetic pathway; and AHAS-inhibitors the branched chain amino acid (BCAA) biosynthetic pathway.

AHAS-inhibitors herbicides are classified in the B group of the HRAC classification (Figure GI.1). The commercialized herbicides of this group belong to five chemical classes: sulfonylureas, imidazolinones, pyrimidinyl (thio) benzoates, triazolpyrimidines and sulfonylamino-carbonyl-triazolinones. The herbicides belonging this group are highly selective, and effective at a low application rates; they are usually applied in post-emergence to control a wide spectrum of grasses and broadleaf weeds in different crops. It is also important to have into account its high persistence on the soil when it comes not only to weed management but also to herbicide contamination.

Although glyphosate and AHAS inhibitors have different enzymatic targets, several common physiological effects have been reported after both type of herbicides. It was observed an accumulation of free amino acid content and a decrease in total soluble protein content, which has been related to an increase of proteolytic activity (Orcaray et al. 2010; Zulet et al. 2013a, 2015; Maroli et al. 2015; Fernández-Escalada et al. 2016; Zabalza et al. 2017). Both types of herbicides also provoked an increase in quinate content (Orcaray et al. 2010) and carbohydrate accumulation (Orcaray et al. 2012; Zulet et al. 2015; Maroli et al. 2015). These common physiological effects suggest that both types of herbicides kill plants by similar mechanisms and a close relation between both pathways. It has been recently proposed that the shared responses stimulated by AHAS and EPSPS inhibitors could be part of a general plant stress response (Dyer 2018). According to this model, sub lethal herbicide exposure activates a common signal transduction cascade, which leads to translation or modification of stress-related proteins that play a role in defense, repair reactive oxygen species management and xenobiotic inactivation (Dyer 2018).

G.I.3.4. Resistance to glyphosate

The widespread use of GRCs has led to large increases in glyphosate use. The extremely high selection pressure due to the overuse of glyphosate has developed resistance to this herbicide (Figure GI.13) in populations of a growing number of weed species (Powles 2008; Vila-Aiub et al. 2012). Glyphosate needs to be used in a more integrated weed management, including herbicide mixtures, rotations, with mechanical and cultural strategies (Heap and Duke 2018) because glyphosate resistance is a serious problem in modern agriculture and resistance management practices are becoming more common. But integrated practices are not applied in the absence of (GR) weeds (Duke 2018a).

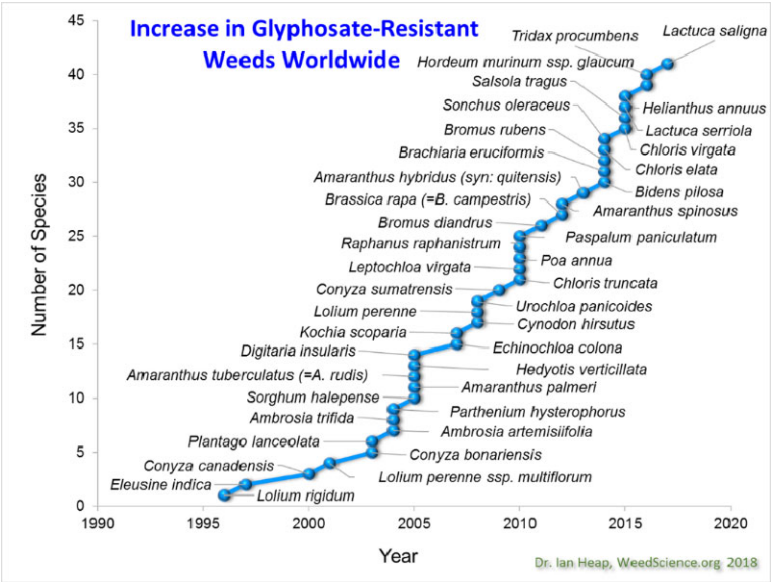

Figure GI.13. Evolution of the number of species that have developed at least one glyphosate-resistant population (Heap, 2019).

Several types of glyphosate resistance have been identified, related to the target site or not:

G.I.3.4.1. Non-target site resistance to glyphosate

Non-target site resistance mechanisms usually confer low levels of resistance over a range of 3-12-fold. In that cases, although the EPSPS enzyme is still sensitive to glyphosate, weeds have developed some mechanisms that reduce the amount of glyphosate reaching the EPSPS enzyme (Heap and Duke 2018). The mechanisms developed that confer resistance could be related with a decrease in the absorption or translocation of the herbicide, sequestration or metabolization of the herbicide molecule.

A decrease in the absorption or translocation of the herbicide does not let the herbicide to reach the site of action at an enough concentration to kill the plant. This mechanism is the main non-target site resistance mechanism of glyphosate described (Powles and Yu 2010) and has been found in some biotypes of *Sorghum halepense* (Vila-Aiub et al. 2012), *Lolium multiflorum* (Michitte et al. 2007) and others (De Carvalho et al. 2012; Alcántara-de la Cruz et al. 2016; Brunharo et al. 2016).

Secondly, sequestration of the herbicide into the vacuoles also keeps the herbicide out of the plastids, where the EPSPS is located. This mechanism has been found in *Conyza canadensis* and *Lolium* spp. (Ge et al. 2012; Sammons and Gaines 2014).

Finally, if the resistance mechanism is metabolization, glyphosate is metabolized to glyoxylate and aminomethylphosphonic acid (AMPA), a very weakly phytotoxic compound (Duke 2011). However, the metabolism of glyphosate in plants at least is rare and is only documented in a few cases. Although examination of glyphosate metabolism in multiple species found no relationship between conversion to AMPA and glyphosate resistance level (Sammons and Gaines 2014), a recent study has reported direct experimental evidence of an aldo-keto reductase that metabolizes glyphosate and thereby confers glyphosate resistance in *Echinocloa colona* (Pan et al. 2019).

G.I.3.4.2. Target-site resistance to glyphosate

Target site glyphosate resistance can be provoked by mutation or amplification of the target EPSPS. The former, glyphosate resistance due to EPSPS mutation, is not very frequent. The main reason for the lack of this type of glyphosate resistance is that EPSPS enzyme, and specifically its binding domain, is highly conservated. In addition, most single point mutations in the EPSPS enzyme result in a non-functioning or an inefficient fitness-compromised enzyme (Heap and Duke 2018; Roma-Burgos et al. 2019). Given that inhibition of EPSPS by glyphosate is competitive in relation to PEP, mutations that give structural changes in the EPSPS active site preventing efficient binding in both glyphosate and PEP will endow glyphosate resistance but EPSPS enzyme will reduce its activity (Vila-Aiub et al. 2019). However, substitutions of proline in position 106 change the properties of the enzyme. Pro-106 substitutions cause a narrowing of the EPSPS-S3P complex for the glyphosate or PEP site cavity, which preserves EPSPS functionality but does not allow the glyphosate to bind the complex (Healy-Fried et al. 2007). Substitutions in Pro-106 by serine, alanine, threonine or leucine have been reported to confer glyphosate resistance in *Eleusine indica, Lolium rigidum, Lolium multiflorum, Amaranthus tuberculatus* and *Echinochloa colona* (Sammons and Gaines 2014). Substitution of threonine in position 102 by serine also confers glyphosate resistance to a *Tridax procumbens* population (Li et al. 2018). However, these single EPSPS gene mutations generally confer low levels of resistance while preserving EPSPS catalytic efficiency. Several double EPSPS gene mutations have been described to confer a higher level of resistance (Thr-102-Ile+Pro-106-Ser, TIPS, or Thr-102-Ile+Pro-106-Thr, TIPT) (Murphy and Tranel

2019). It has been recently described a GR population of *Amaranthus hybridus* which have evolved a triple mutation, with substitutions in the positions Thr-102, Thr-103 and Pro-106 with a high resistance level. Although the consequences of the novel substitution at position 103 on the EPSPS kinetic and regulatory properties have not yet been elucidated, its localization within the conserved region suggests that it could be contributing to the extremely high levels of GR observed in that species (Perotti et al. 2019).

On the other hand, glyphosate resistance can also be evolved by other mechanism: the amplification of the EPSPS. The resistance mechanism conferred by amplification of the EPSPS has been described as EPSPS gene duplication. Gene duplication is the correct term to define the heritable replication of a DNA segment, resulting in one or more additional gene copies within the genome of an organism. Although gene amplification is defined as the non-heritable replication of a DNA segment, it is commonly used synonymously with gene duplication (Sammons and Gaines 2014). In this work, the term used to define this concept will be gene amplification.

An increase in the EPSPS gene copy number due to gene amplification increases the production of the EPSPS enzyme and consequently increases the amount of glyphosate needed to kill the plant (Heap and Duke 2018). EPSPS gene amplification is correlated with an increase in the EPSPS transcript level and activity (Gaines et al., 2011), and correlates with the enzyme amount and glyphosate resistance (Powles and Yu 2010). While EPSPS enzyme activity from resistant and susceptible plants is equally inhibited by glyphosate (Gaines et al. 2010), the amplification of the EPSPS gene produced an abundant supply of EPSPS able to act as a molecular sponge to absorb glyphosate, enabling uninhibited EPSPS to continue functioning following glyphosate treatment, conferring high levels of resistance (Powles and Yu 2010).

This resistance mechanism was first detected in a population of *Amaranthus palmeri from Georgia* (Gaines et al. 2010). Since the first report in 2010, glyphosate-resistant biotypes due to EPSPS overexpression by EPSPS gene amplification have been reported in in other weed species, including *Amaranthus tuberculatus* (Lorentz et al. 2014), *Amaranthus spinosus* (Nandula et al. 2014), *Bromus diandrus* (Malone et al. 2016)*, Chloris truncata* (Ngo et al. 2018)*, Eleusine indica* (Chen et al. 2015a)*, Lolium perenne* (Salas et al. 2012), *Lolium multiflorum* (Fernández-Moreno et al. 2017) and *Kochia scoparia* (Wiersma et al. 2015). This suggests that genetic variation for this glyphosate 'molecular sponge' mechanism

is more frequent among plant species than originally anticipated (Vila-Aiub et al. 2019).

Fitness cost is considered as any 'fitness penalty' or 'resistance cost' incurred by resistant plants in the absence of the herbicide to which resistance had evolved (Cousens and Fournier-Level 2018), and integrates all of the genetic, biochemical and physiological changes that the resistant plants suffer that can affect its survival and/or reproduction rates (Vila-Aiub et al. 2019). In the case of *Amaranthus palmeri* GR populations, despite of the massive EPSPS enzyme production is surprising that no fitness cost has been associated with the increase of EPSPS gene expression (Giacomini et al. 2014; Vila-Aiub et al. 2014; Heap and Duke 2018).

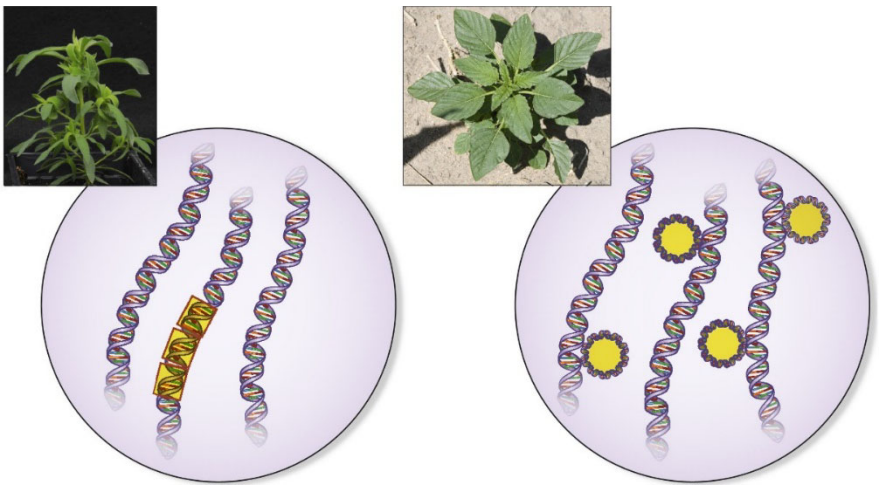

Figure GI.14. EPSPS gene duplication in *Kochia scoparia* occurs as a 45–70 kbp tandem duplication at a single locus, with predictable inheritance and potential for changes in copy number in progeny as a result of unequal recombination; the duplication may have been triggered by insertion of a mobile genetic element next to EPSPS. (c) EPSPS gene duplication in *Amaranthus palmeri* occurs as a 300 kbp extrachromosomal circular DNA carrying a single copy of EPSPS. Taken by Gaines et al., (2019).

Recently, access to genomic resources, combined with cytogenetics, has provided critical evidence for our understanding of the molecular mechanisms of gene duplication, that are different depending on the species. In *A. palmeri*, it was firstly proposed that EPSPS gene amplification occurred via transposon activity, in which DNA-mediated transposon activity and/or unequal recombination between different genomic regions would result in replication of the EPSPS gene (Gaines et al. 2013). More recently, it has been proposed that amplified EPSPS gene copies of GR *A. palmeri* are due to extrachromosal circular DNA transferred to the

following generation by tethering to mitotic and meiotic chromosomes (Koo et al. 2018) (Figure GI.14). In the case of *Kochia scoparia*, it is proposed that an additional sequence insertion containing mobile genetic elements generated a site of unequal recombination, leading to the generation of extra EPSPS copies. In other species, less is known about the molecular mechanisms (Gaines et al. 2019).

G.I.3.5. Glyphosate resistance in *Amaranthus palmeri*

Amaranthus palmeri is a C4 weed with a rapid growth rate and tall stature that makes this species extremely competitive with crops (Culpepper et al. 2006). It is a dioecious annual species, that can grow up to 2 m with high reproduction capacity, genetic variability and stress tolerance (Chaudhari et al. 2017; Palma-Bautista et al. 2019). It produces unbranched terminal seedheads that can reach up to 0.5 m (Culpepper et al. 2006). Compared with other *Amaranthus* species, this weed has the greatest values for volume, dry weight and leaf area (Horak and Loughin 2000).

Amaranthus palmeri is one of the most problematic weeds in corn, cotton and soybean in USA (Culpepper et al. 2006) and is probably the worst GR weed worldwide (Palma-Bautista et al. 2019). A GR population was firstly described in Georgia, USA in 2006 (Culpepper et al. 2006). Since 2006, 42 populations of *Amaranthus palmeri* have developed glyphosate resistance (Heap, 2019). The first mechanism of resistance described in this species was gene amplification of the EPSPS gene due to an increase in the EPSPS gene copy number (Figure GI.15) (Gaines et al. 2010). The number of copies of the EPSPS gene reported in several GR population ranges from 5 to more than 160 (Gaines et al. 2010). The population used in this study was described as having 47.5-fold more EPSPS gene copy number than the glyphosate sensitive population (Fernández-Escalada et al. 2016).

In the last years, *A. palmeri* multiple resistant populations have been described having simultaneous resistance to glyphosate and at least other herbicide with different site of action, increasing the troublesome on the control of this species (Heap, 2019; Küpper et al., 2017). The vast majority of the multiple resistant populations described in this species are resistant to the two groups of herbicides G and B, although two different multiple resistant populations, resistant to G and C1 or to G and E herbicide group respectively have been described. In Arkansas, the first population which evolved multiple resistance to 5 different herbicide

sites of action (B, E, G, K1 and K3 groups) was described in 2016, in cotton and soybean crops (Heap, 2019).

The first case of glyphosate resistance in *A. palmeri* involving exclusively NTSR mechanisms has been recently described (Palma-Bautista et al. 2019), in which was observed a low absorption and impaired translocation of glyphosate as the main resistance mechanisms.

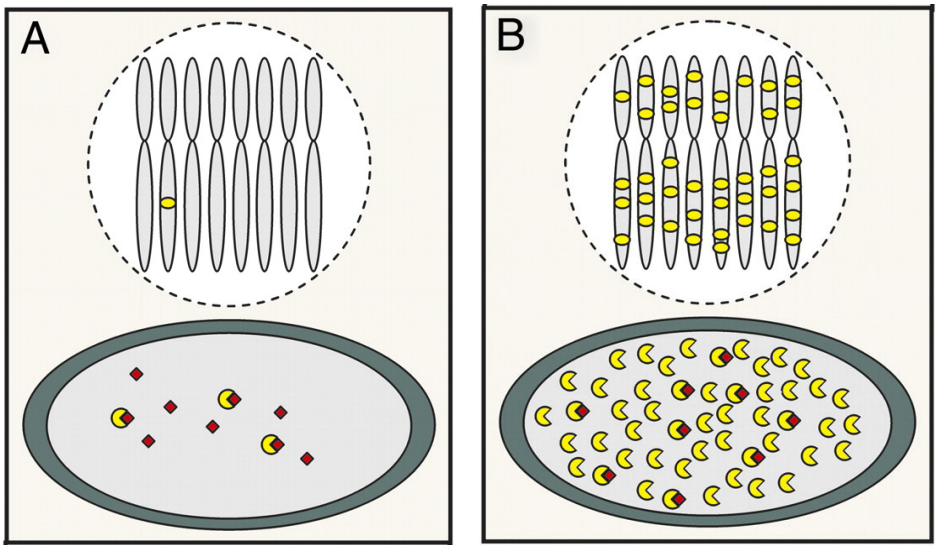

Figure GI.15. Glyphosate resistance resulting from gene amplification. In a sensitive individual (A), the normal number of EPSPS gene copies (yellow dots on chromosomes) produce EPSPS (yellow) in chloroplasts that is targeted and overwhelmed by the normal usage rate of glyphosate (red), and the plant dies. In a resistant individual (B) with amplified EPSPS gene copies present on multiple chromosomes, there is increased EPSPS, and the normal glyphosate rate cannot inhibit all of the available EPSPS. Taken by Powles, 2010.

G.I.4. *Metabolites derived from the AAA biosynthetic pathway*

G.I.4.1. Phenylpropanoids

Phenolic compounds can account for as much as 35% of the plant biomass (Franz et al. 1997), with different functions, particularly protecting against various abiotic and biotic stresses and different interactions (Maeda and Dudareva 2012). Phenolics are synthesized by several different routes so, from the metabolic point of view, is a heterogeneous group (Taiz and Zeiger 2010). Two basic pathway are involved in their synthesis: the shikimate pathway and the malonic acid pathway. The malonic acid pathway is few significant in higher plants, so the most abundant classes of phenolic secondary compounds in plants are derived from shikimate pathway (Figure GI.16) (Taiz and Zeiger 2010).

Thus, the AAA (Tyr, Phe and Trp) are not only components for protein synthesis, but also precursors for a wide range of secondary metabolites that are important for plant growth and regulation (Tzin and Galili 2010a; Maeda and Dudareva 2012; Tohge et al. 2013b, a). Trp is a precursor of alkaloids, phytoalexins, and indole glucosinolates, as well as auxin, a plant hormone. Tyr is a precursor of isoquinoline alkaloids, pigment betalains, and quinones. Phe serves as a precursor for a large number of secondary metabolites, such as phenylpropanoids, flavonoids, lignin and anthocyanins (Tzin and Galili 2010a; Maeda and Dudareva 2012; Castrillón-Arbeláez and Délano Frier 2016).

Phenylpropanoids are derived from Phe via PAL to produce cinnamic acid (Duke et al. 1979; Tzin and Galili 2010a; Mobin et al. 2015), whose encoding genes are generally highly regulated by different stresses. Some of the phenylpropanoids detected in this study, represented in Figure GI.15., are gallic acid, caffeic acid, ferulic acid, vanillic acid and salicylic acid.

Glyphosate treatment provokes a disruption on the shikimate pathway, by blocking the aromatic amino acid biosynthetic pathway at the level of the EPSPS enzyme. Phenylpropanoids metabolism, and Tyr- and Trp-derived compounds, are affected after the inhibition of the EPSPS by the herbicide. Glyphosate treatment causes an accumulation of the phenylpropanoids in plants, specially a rapid accumulation of gallic acid and protocatechuic acid (Lydon and Duke 1988; Becerril et al. 1989; Hernandez et al. 1999; de María et al. 2006). An accumulation of caffeic acid after glyphosate treatment was also observed in *Echinacea purpurea* roots (Mobin et al. 2015).

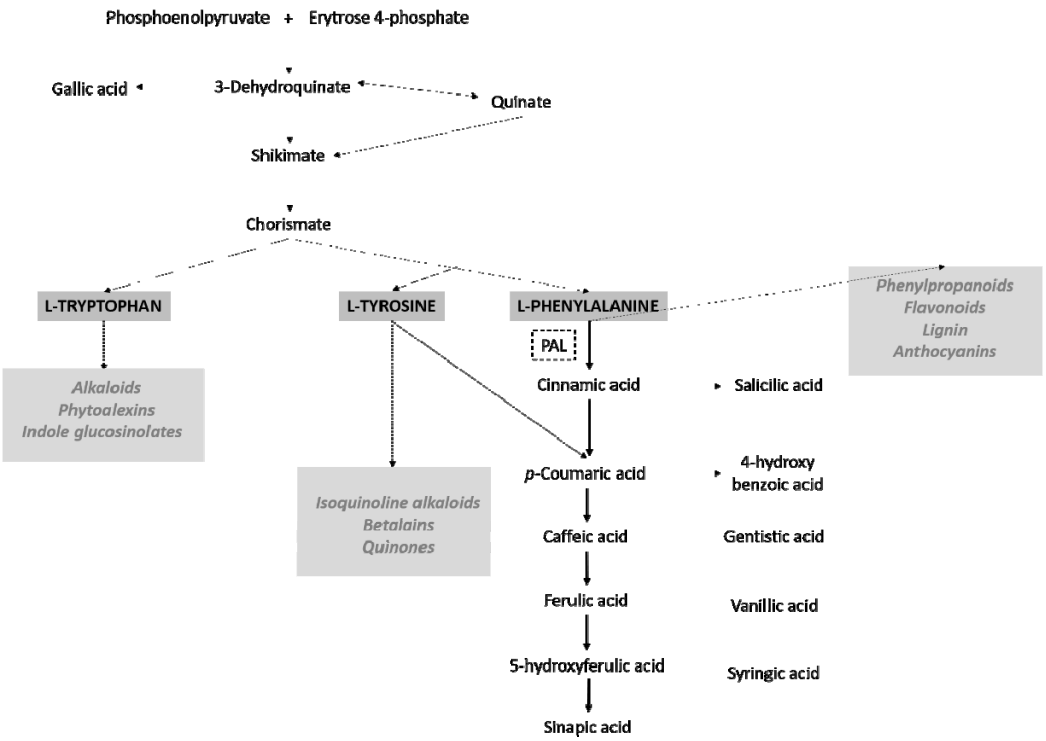

Figure GI.16. Simplification of the secondary metabolites biosynthesis derived from the shikimate pathway. Phenylalanine ammonia lyase (PAL). AAA are represented in bold capital letters and gray squared. Secondary metabolites group derived from each AAA are represented in italic letters and gray squared. One-step reactions are represented by arrows with continuous line. Reactions containing more than one step are represented by arrows with discontinuous line.

Several *Amaranthus* species are abundant sources of secondary metabolites, mostly phenylpropanoids, many of which may confer benefits associated with their properties (Castrillón-Arbeláez and Délano Frier 2016). The vast majority of plants synthetize their red pigments as anthocyanins, which are Phe-derived metabolites. Interestingly, red pigments of plants belonging to the order Caryophyllales, in which *Amaranthus* species are included, are not anthocyanins but betalains, a class of Tyr-derived pigments exclusive of this order (Lopez-Nieves et al. 2017).

G.I.4.2. Quinate

Quinic acid (1,3,4,5-tetrahydroxyciclohexancarboxylic acid) (Figure GI.17) is widely distributed and an abundant metabolite in higher plants and its concentration can reach up to 8 % of the dry mass in the leaves (Boudet 1973,

2012). At physiological pH, quinic acid is found in its ionic form, quinate. Quinate is a secondary metabolite synthetized in plants from a lateral branch of the shikimate pathway (Figure GI.7).

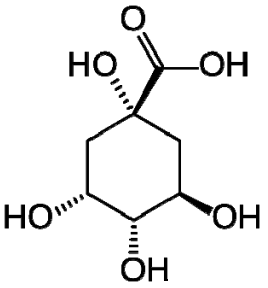

Figure GI.17. Structure of quinate: 1,3,4,5-tetrahydroxyciclohexancarboxylic acid

Quinate is synthetized from the metabolite 3-dehydroquinate through the reversible Quinate dehydrogenase enzyme (Figure GI.17). This process would produce quinate when the carbon flux through the pathway is high (Boudet 2012) and quinate has been described as a reserve compound in plants. It seems that quinate would be first accumulated in the vacuole as a result of active photosynthetic activity in the leaves and then used as carbon sources for the synthesis of different phenolic compounds and lignins (Osipov and Aleksandrova 1982; Schmid and Amrhein 1999; Boudet 2012). It has been observed a differential accumulation in plants in an annual cycle, with a peak in spring during the period of intense growth, and a decrease in summer (Boudet 2012).

Quinate can be incorporated afterwards in the pathway via two different pathways (Figure GI.18): via the reversible quinate dehydrogenase to 3-dehydroquinate; and to shikimate catalyzed from the quinate hydrolyase enzyme (Ossipov et al. 2000). It has been revealed that exogenous quinate application is incorporated into the pathway to shikimate, Phe and Tyr (Weinstein et al. 1959, 1961; Boudet 2012). Quinate is also precursor for the synthesis of other secondary metabolites, such as coumarines, caffeoyl and feruloyl derivatives (Boudet 2012).

The herbicide glyphosate, inhibiting the EPSPS enzyme from the shikimate pathway, also provokes an increase in quinate content (Ulanov et al. 2009; Orcaray et al. 2010; Zabalza et al. 2017). Quinate accumulation was also detected in plants when AHAS-inhibitors were applied (Orcaray et al. 2010), proposing quinate and its accumulation as a key factor on the toxic response to both

herbicides (Orcaray et al. 2010; Zulet et al. 2013b; Zabalza et al. 2017) and suggesting quinate toxicity.

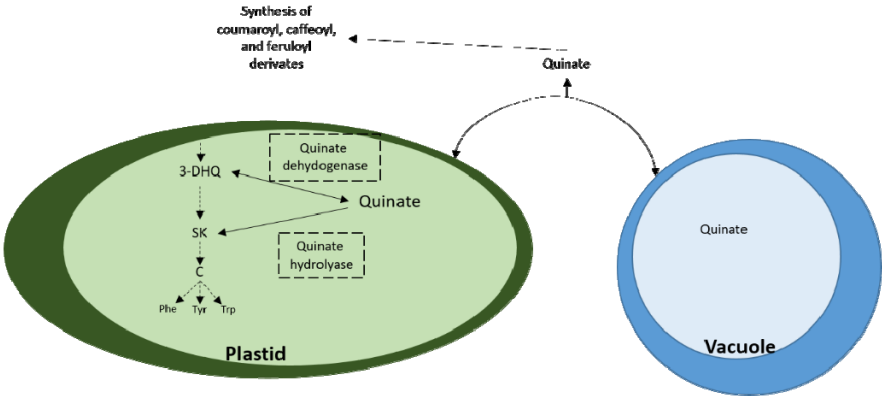

Figure GI.18. Potential routes for quinate synthesis and utilization in plants and subcellular compartments. 3-dehydroquinate (3-DHQ), shikimate (SK), chorismate (C), phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp). Modified from Boudet (2012).

Previous studies were performed trying to mimic herbicide effect with quinate (Zulet et al. 2013b). In those studies, quinate was applied exogenously through the nutrient solution or spraying to the leaves in pea plants. Quinate applied through the nutrient solution was lethal, while the phytotoxic effect after spraying application was only temporal. Exogenous application of quinate affected the carbohydrate content in the leaves and roots in a similar way to the toxic effects of herbicides so it was confirmed that quinate plays an important role in the toxicity induced by glyphosate and other inhibitors of amino acid biosynthesis (Zulet et al. 2013b).

It was hypothesized that quinate may not have a target by itself, but it would mimic the mode of action of glyphosate by entering the shikimate pathway and deregulating different processes related with this pathway. In order to gain new insights in possible similarities of the toxicity of glyphosate and quinate, it was compared the effect of the glyphosate and of the exogenous quinate on several metabolites and enzymes of the shikimate pathway, trying to elucidate similarities on quinate toxicity pattern compared with glyphosate (Zabalza et al. 2017). The results elucidated that each compounds affect differently in the pathway, glyphosate blocking it and quinate fueling it (Zulet et al. 2013b; Zabalza et al. 2017). 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, laying the framework for applying both compounds combined, enhancing glyphosate effect.

GENERAL AIMS

Although the site of action of the herbicide glyphosate is very well known as the inhibition of the EPSPS enzyme in the shikimate pathway, its mode of action is not completely elucidated. That means that the exact reason by which plants die after glyphosate treatment is not known. One of the physiological processes characterized as part of the mode of action of this herbicide is quinate accumulation. Quinate has been applied exogenously and affects differently the shikimate pathway that glyphosate, fueling it.

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 of the glyphosate and it was raised the chance of applying both compound combined.

The comparison of the effects of quinate and/or glyphosate treatments can provide new insights in the regulation of the shikimate pathway. In addition, the gene amplification resistance mechanism found in *Amaranthus palmeri* offers us the opportunity to obtain new insights into how the pathway is regulated after EPSPS overexpression due to extra EPSPS gene copies and how glyphosate and/or quinate may affect this regulation.

The main objective of this work is **to gain further insights on glyphosate effect on the shikimate pathway,** focusing on the physiological response caused by the herbicide alone or in combination with quinate applied exogenously, and whether the effects are similar in glyphosate sensitive and resistant *A. palmeri* plants.

To this end, the responses of two populations of glyphosate-sensitive and resistant populations, due to EPSPS overexpression by EPSPS gene amplification, treated with both compounds were compared at molecular and biochemical level.

This general aim was approached by four specific objectives that are covered in the four individual chapters of this thesis:

1. Elucidate the **possible use of quinate as a glyphosate enhancer**, by evaluating whether the toxicity and the most common stress markers of glyphosate are affected by its combined application.

2. **Unravel the physiological pattern of the shikimate pathway after the combined treatment of quinate and glyphosate.** The physiological study focused on the shikimate pathway and its derivates because is the common pathway affected by quinate and glyphosate.

3. To **characterize the metabolic profiling** of sensitive and resistant populations of *A. palmeri* and to compare the effect of the treatments applied (quinate and/or glyphosate) by metabolomics.

4. To unravel the specific effect of glyphosate **on the regulation of the shikimate pathway, comparing its effect with the effect of quinate and other intermediates of such pathway,** by assessing the expression level of the enzymes and protein content of the pathway.

MATERIAL AND METHODS

M.M.1. *PLANT MATERIAL AND TREATMENT APPLICATION*

M.M.1.1. Plant material

Glyphosate resistant (GR) and sensitive (GS) populations of *Amaranthus palmeri* were used to develop the experiments in all chapters. Seeds provided by Dr. Todd Gaines (Colorado State University, Fort Collins, Colorado) were originally collected from North Carolina (United States) with a described copy number variation between 22 and 63 fold more in GR population respect to GS population (Chandi et al. 2012). Some biotypes from North Carolina have been reported to exhibit levels of resistance ranging from 3- to 22-fold after 21 days (Culpepper et al. 2008). The GR population used in this studied was described before (Fernández-Escalada et al. 2016) as a population with an EPSPS copy number of 47.5-fold more than the GS population. It was also determined that the half maximal effective concentration (EC_{50}) (reduced the shoot dry weight accumulation by 50%) over 5 days was 3.7-fold greater in GR population than in GS population (Fernández-Escalada et al. 2016).

Seeds of *A. palmeri* were surface sterilized according to Labhilili et al. (1995). First, they were placed in a 1 % (w/v) sodium hypochlorite and 0.01 % (w/v) SDS mixture containing solution for 40 min and rinsed several times with deionized water. Then, they were soaked in 0.1 N HCl for 10 min and rinsed again with deionized water several times.

For germination, seeds were placed on Seedholders (Araponics SA, Belgium) (Figure M.M.1 B) filled with 0.65 % (w/v) plant agar and were incubated for 7 days at 4 °C in darkness. Then, they were maintained for 48 h in a 16/8 h light/night cycle photoperiod and 28/18 °C day/night temperature (Figure M.M.1 A, Table M.M.1) before they were transferred to the growth chamber.

Figure M.M.1. *Amaranthus palmeri* plants in Seedholders during the germination period in a small growth chamber (A). Seedholders used to place the seeds on (B).

Seven seedholders with one plant each were then transferred to each 2.7 L hydroponic tanks in a growth chamber (Figure M.M.2) (18/6 h day/night cycle photoperiod; 500 µmol s^{-1} m^{-2} light; 60-70 % relative humidity; and 22/18 °C day/night temperature). The water of the tanks was then replaced with nutrient solution and refreshed every week. The nutrient solution was prepared according to Hoagland & Arnon (1950) and supplemented with 15 mM KNO_3. The final composition of the nutrient solution was as follows: 3 mM $Ca(NO_3)_24H_2O$; 2 mM $MgSO_47H_20$; 1.6 mM KH_2PO_4; 0.1 mM Na_2Fe EDTA; 43.3 µM H_3BO_3; 9.1 µM $MnCl_24H_2O$; 0.3 µM $CuSO_45H_2O$; 0.8 µM $ZnSO_47H_2O$; 0.1 µM $Na_2MoO_42H_2O$; 15 mM KNO_3. Throughout the course of the experiment, the plant remained in the vegetative phenological stage. To prevent roots from hypoxia, aeration was set in the banks and maintained onward. When the treatments were applied, 2.7 L hydroponic tanks were replaced for 4.7 L tanks.

Table M.M.1. *Amaranthus palmeri* germination conditions before being transferred to the growth chamber.

	Period of time (days)	Temperature (°C) (day/night)	Photoperiod (h) (day/night)
Pre-germination	7	4	darkness
Germination	2	28/18	16/8

Figure M.M.2. *Amaranthus palmeri* plants in the 4.7 L hydroponic tanks in the growth chamber before applying treatments.

M.M.1.2. Treatment application for physiological studies

These treatment application were developed in chapters one, two and three. Treatments were applied to 21 day-old plants.

M.M.1.2.1. Glyphosate

Amaranthus palmeri plants were treated with glyphosate (commercial formula, 360 g a.e. L^{-1}, isopropyl amine salt; FORTIN Green, KEY, Lleida, Spain). The doses applied (Table M.M.2) were adapted to each population due to the resistance of the GR, in order to obtain comparable response in both of them. In this study, as the main objective was to determine the effect of the combination with quinate, sub lethal doses of glyphosate were used. Too high doses of glyphosate would have covered up the effect of quinate in the combined treatments, and the results would probably have been similar to those with glyphosate applied alone. On the other hand, a high dose was also desirable in order to compare it with the mixtures. The doses applied in the GS population were 0.25 the recommended

field dose (0.25 RD = 0.21 Kg ha^{-1}) as the sub lethal dose and the recommended field dose (RD = 0.84 kg ha^{-1}) (Culpepper et al. 2008) as the high one; the doses applied in the GR population were 0.5 the recommended field dose (0.5 RD = 0.42 kg ha^{-1}) in chapter one, and the recommended field dose (RD = 0.84 kg ha^{-1}) in chapters two and three as the sub lethal doses, and 3 times that dose (3 RD = 2.52 kg ha^{-1}) as the high one. Doses were changed in after Chapter 1 because doses used in the first chapter were too low to induce any changes in the herbicide physiological markers in GR

Table M.M.2. Overview of the glyphosate treatments applied to two populations of *Amaranthus palmeri* plants, glyphosate sensitive (GS) and glyphosate resistant (GR). RD = Recommended field dose (Culpepper et al. 2008).

	Amaranthus palmeri			
	GS		GR	
Sub lethal dose	0.25 RD	0.21 kg ha^{-1}	0.5 RD RD	0.42 kg ha^{-1} 0.84 kg ha^{-1}
High dose	RD	0.84 kg ha^{-1}	3 RD	g ha^{-1}

M.M.1.2.2. Quinate

Amaranthus palmeri plants were treated with quinate (Quinic acid 98 %, Sigma Aldrich, St.Louis, MO, USA). According to previous studies, the dose applied was 400 mM (Zulet et al. 2013b), as it was the highest soluble dose. Quinate was applied in the adjuvant sodium lauryl sulfate 0.2 % (commercial formula, Biopower 27.65 % (p/v) Bayer CropScience, Madrid, Spain). Control treatment was performed only with the adjuvant in the same concentration.

The adjuvant was selected after testing different adjuvants. Firstly, Tween 20® (Polyoxyethylenesorbitan monolaurate, SygmaAldrich, St.Louis, MO, USA) and DMSO (dimethyl sulfoxide 99.9 %,SygmaAldrich, St.Louis, MO, USA) were tested, but these both adjuvants did not increase quinate solubility. After that, adjuvants frequently used with commercial herbicides were tested: Biopower; Aquilphenol etilenic oxide (commercial formula, Agral, 20 % (p/v), Syngenta, Madrid, Spain); and paraffinic acid 60 %, polietoxilated sorbitol oleate 24 %, polietoxilated tridecyl alcohol 12 % (commercial formula, Canplus, Bayer CropScience, Madrid, Spain). Biopower was chosen due to no provoking phytotoxicity and improving the wettability (Zulet, 2009).

M.M.1.2.3. Glyphosate and Quinate

Amaranthus palmeri plants were also treated with quinate and glyphosate. Preliminary studies were performed to determine the most suitable moment to apply both compounds (Chapter 1). Application of quinate 24 h before, simultaneously and 24 h after were evaluated trying to ascertain which combination was more effective. The results showed that the combination that was the most effective was the application of quinate 24 h after glyphosate, and it was used Chapters 1, 2 and 3 (Figure M.M.3).

To determine that the possible enhancer effect of the quinate in the combined treatment was not due to an increase of the penetration of the glyphosate for the additional surfactant Biopower, an extra treatment of Glyphosate and Biopower was evaluated and an increase of the toxicity was discarded.

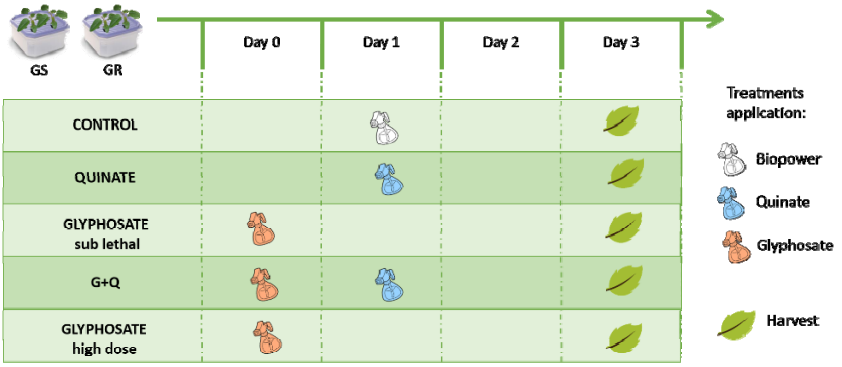

Figure M.M.3. Overview of the treatment application and harvest in *Amaranthus palmeri* plants. Two different doses of glyphosate were applied to each population, a sub lethal one and another one higher. Quinate was applied 24 h after glyphosate application. Plants from all treatments were harvested 3 days after glyphosate application. Control and quinate plants were also harvested 0, 1, 2 and 3 days after their treatment application.

M.M.1.2.4. Application and harvest

Treatments were performed using an aerograph (Junior Start model, Definik, Sagola, Spain) (Figure M.M.4 A) connected to a compressor (Werter One, Breverrato) (Figure M.M.4 B) and applied directly to the plant leaves in a flow cabinet (Table M.M.3). Plant leaves were completely dry after absorbing the treatment before return to the growth chamber.

Figure M.M.4. Detail of the aerograph used for the treatment application (A) and the aerograph connected to a compressor (B).

Table M.M.3. Treatments applied is *Amaranthus palmeri* sensitive (GS) and resistant (GR) plants.

GS		GR	
Control	Biopower	**Control**	Biopower
Quinate	400 mM	**Quinate**	400 mM
Glp 0.25RD	0.21 kg ha^{-1}	**Glp 0.5RD**	0.42 kg ha^{-1}
G+Q	400mM + 0.21 kg ha^{-1}	**Glp RD**	0.84 kg ha^{-1}
Glp RD	0.84 kg ha^{-1}	**G+Q (Chap1)**	0.42 kg ha^{-1} +400mM
		G+Q (Chap2 and 3)	0.84 kg ha^{-1} + 400mM
		Glp 3 RD	2.52 kg ha^{-1}

For the analytical measurements, leaves were harvested 3 days after glyphosate treatment and 2 days after quinate and control treatments and immediately frozen in liquid nitrogen and stored at -80 °C for analytical determinations. In chapter one, it was also performed a time course harvest in control and quinate treatments from day 0 to day 3 after their treatments application. Later, frozen samples were ground to fine powder under liquid nitrogen using a Retsch mixer mill (MM200, Rescht®, Haan, Germany) maintaining separately individual plants as biological replicates.

Additionally, 3 leaf disks were excised from the youngest leaf of each plant to determine shikimate content three days after treatment. Leaf disks were excised using Harris Uni-Core puncher (4 mm-diameter) (Healthcore, Bucks, UK), avoiding the leaf nerves, and then were frozen in liquid nitrogen and stored at -80 °C.

M.M.1.3. Regulation studies with leaf disks incubation

This experiment was developed only in Chapter four. Before performing the incubation, a leaf of each plant of *A. palmeri* GR population was harvested and immediately frozen in liquid nitrogen in order to determine the EPSPS relative genomic copy number of those individuals. After evaluating the results, 30 out of 48 plants evaluated were selected to obtain a homogeneous population with a similar relative EPSPS genomic copy number (between 60 and 100) in order to perform the experiment with them.

When the GS and selected GR plants were 21 days old, leaf disks were excised from the leaves using Harris Uni-Core puncher (4 mm-diameter) (Healthcore, Bucks, UK), avoiding the leaf nerves. From each plant, only two leaves were used: the youngest leaf of the plant was used to determine the shikimate content and other leaf was used to determine transcript levels and enzyme content measurements.

Solutions were prepared freshly and pH were adjusted to 7.0 with NaOH in all the treatments. Glyphosate (Glyphosate, isopropylamine salt, 61 %. Dr. Ehrenstorfer GmbH, Augsburg, Germany) was used in this experiment. The same treatments (Table M.M.4) and doses were applied in both populations.

Table M.M.4. Treatments applied to glyphosate sensitive and glyphosate resistant *Amaranthus palmeri* leaf disks. a.e. acid equivalent.

Identification	Treatment	Dose
C	Control	-
G	Glyphosate	1.75 g a.e.L^{-1}
S	Shikimate	20 mM
Q	Quinate	50 mM
Ch	Chorismate	1 mM
At	Anthranilate	1 mM
AAA	Aromatic amino acids	10 µM (each AAA)
AAA + G	Aromatic amino acids + Glyphosate	10 µM+ 1.75 g a.e.L^{-1}

For the shikimate content determination experiment, one disk was placed in a well of a 96-well microtiter plate. From the same leaf, there were excised as much disks as treatments, in order to use from the same plant one disk for each treatment. Each well contained 100 µL of each treatment (Figure M.M.5. A). Plates were incubated at 24 °C under continuous light for 24 h. After incubation, the plates were placed in a freezer (-20 °C). Only in the shikimate and quinate

treatments, the incubating liquid was replaced by water before freezing to avoid data perturbations in content measurements.

To determine the transcript levels and enzyme content measurements, 45 and 25 disks were placed in a well of a 6-well microtiter plate, respectively (Figure M.M.5. B). In each well, a mixture of disks of different leaves were incubated with the same proportion of each leaf in each treatment. Each well contained 2.5 mL of each treatment. The plates were incubated at 24 °C under continuous light for 24 h. After incubation, the disks were removed from the incubating liquid and immediately frozen in liquid nitrogen and stored at -80 °C.

Figure M.M.5. Leaf disk incubation system. Leaf disks were excised from glyphosate sensitive and glyphosate resistant plants of *Amaranthus palmeri* and incubated for 24 h. One disk per well was incubated for shikimate content determination (A) and 25 or 45 were incubated for enzyme content and transcript levels determination, respectively (B).

M.M.2. *ANALYTICAL DETERMINATIONS*

M.M.2.1. Nucleic acid determinations

Genomic DNA for EPSPS enzyme (Chapter four) and mRNA level for all main enzymes in AAA (Chapters two and four) and BCAA pathway (Chapter four) were determined.

M.M.2.1.1. EPSPS relative genomic copy number

Quantitative real-time PCR (qPCR) were performed to determine the EPSPS genomic copy number relative to carbamoyl phosphate synthetase (CPS) in untreated GR plants. Genomic DNA was extracted from approximately 0.1 g of previously ground *A. palmeri* leaves. The plant material was homogenized in 375 µL of 2×lysis buffer (0.6 M NaCl, 0.1 M Tris-HCl (pH 8.0), 40 mM ethylendiaminetetraacetic acid (EDTA) (pH 8.0), 4 % sarcosyl, and 1 % sodium dodecyl sulfate (SDS)) and 375 µL of 2 M urea. One volume (750 µL) of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the mixture and mixed briefly. The homogenates were centrifuged at 20000 *g* for 10 min at room temperature. To precipitate the DNA, 0.7 volume (525 µL) of cold isopropanol was added to the supernatants, and the tubes were centrifuged at 20000 *g* for 15 min at 4 °C. The DNA pellet was washed twice with 1 mL of 70% ethanol, air-dried, and resuspended in 25 µL of resuspension buffer (10 mM Tris-HCl (pH 8.0), containing 30 µg mL^{-1} RNase A). Samples were briefly incubated at 37 °C for 5 min to degrade contaminating RNAs.

The extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). OD 260 and 280 nm were read for every sample. DNA concentrations were adjusted to 5 ng μL^{-1}. Quantitative real-time PCR was performed as in Gaines et al. (2010) with some modifications. The following primer sets were used: EPSPS forward (5′-atgttggacgctctcagaactcttggt-3′) and EPSPS reverse (5′-tgaatttcctccagcaacggcaa-3′) specified in Gaines et al. (2010); and CPS forward (5′ -attgatgctgccgaggatag-3′) and CPS reverse (5′- gatgcctcccttaggttgttc-3) specified in Ma et al. (2013). To determine the efficiency of the primers, a standard curve using a 1, 1/5, 1/25, 1/125, and 1/500 dilution series of genomic DNA from GR was conducted.

qPCR were performed in an optical 96-well plate using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each reaction was performed using 10 ng of genomic DNA in a total volume of 20 µL

containing 1× SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Shiga, Japan), 300 nM specific forward primer, and 300 nM specific reverse primer. The following thermal profile was used for all PCRs: 50 °C for 2 min, 95 °C for 1 min, and 40 cycles of (95 °C for 30 s and 60 °C for 1 min). To calculate the final Ct values, 48 biological replicates were performed. The average increase in EPSPS copy number relative to CPS and the standard deviation were calculated for each sample. The increase in EPSPS copy number was expressed as $2^{\Delta Ct}$, where ΔCt = (Ct, CPS –Ct, EPSPS) (Livak and Schmittgen 2001).

M.M.2.1.2. Transcript level

All the transcript levels of all the enzymes in AAA (in chapter one, two and four) and BCAA (in chapter four) biosynthetic pathways were determined, as described previously (Fernández-Escalada et al. 2017). As *A. palmeri* was not sequenced, primers were designed using a near sequenced species of Amaranthaceous family and crossed with the one of *Arabidopsis thaliana*. The primers CM1 and CM2, CPS and ADH were specifically designed in this study. For the primers of CM1 and CM2, the sequences alignment of that genes of *A. hypochondriacus* were performed in order to obtain specific primers for each sequence.

cDNA (complementary deoxyribonucleic acid) extraction was performed using Bi-Rrad iScriptTM cDNA Synthesis Kit with 1 µg of total RNA following manufacturer's instructions. Each sample contained 4 µL of 5x iScript reaction mix, 1 µL of iScript reverse transcriptase and 15 µL of nuclease-free water and RNA template, being this concentration calculated to obtain 1 µg of RNA per sample. The reaction protocol was 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C and hold at 4 °C until their storage at -20 °C.

qPCR were performed using a Thermocycler Biorad CFX Connect TM Real-Time System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The reaction kit used for qPCR was PowerUp™ SYBR™ Green Master Mix (AppliedBiosystems, Termo Fisher Scientific Baltics UAB, Lithuania). Each reaction was performed using 1 µL of cDNA template, 5 µL of PowerUp™ SYBR™ Green Master Mix and 4 µL of a solution with forward and reverse primers (1 µmol for primer forward and another 1 µmol for primer reverse) in nuclease-free water. The following thermal profile was used for all PCRs: denaturation at 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 52–61 °C for annealing and extension for 20 s. Optimal annealing temperature for each primer was determined using gradient PCR. All primers and annealing temperatures are listed in the Table M.M.5. EPSPS primer was modified from Gaines et al. (2010). Melting curve analysis was conducted to verify

amplification of single PCR products. Gene expression was monitored in 6 biological replicates in chapter two, and in 4 replicates in chapter four. Relative transcript level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Table M.M.5. Primers of genes from aromatic amino acid pathway enzymes: D-arabinoheptulosonate 7-phosphate synthase (DAHPS), dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase/shikimate dehydrogenase (DQ/SD), shikimate kinase (SK), 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), chorismate synthase (CS), chorismate mutase (CM) and anthranilate synthase (AS); branched-chain amino acid pathway: acetohydroxy acid synthase (AHAS), acetohydroxyacid isomer-reductase (AHAIR), dihydroxyacid dehydratase (DHAD) and BCAA transaminase (TA); and normalization genes selected for this study, β tubulin and carbamoyl phosphate synthetase (CPS). For each primer is shown the annealing temperature.

GENE	FORWARD	REVERSE	ANNEALING TEMP
AAA biosynthetic pathway			
DAHPS	cctcataggatgataagggc	ctttgcatggcagcataacc	55
DHQS	gcattgttggctagggatcc	aacctcggccttgttttcac	61
DQSD	ggtgtactcaagcaaggagc	tgtggactcttactatggcc	57
SK	gattctgaagcacaaagcagc	cagttgttttcccagagccc	55
EPSPS	aatgctaaaggaggccttcc	tcaatctccacgtctccaag	61
CS	cttgatagaaggaggcctgg	gtttctttcctaggagtagtg	61
AS	tttggagggaaggttgtgcg	ctggtgagctttttccatgc	52
CM1-3	gaatccaagcccgcgtataa	cttcaatccaatcgtctcaacaag	59
CM 2	aagggtactgaagctgttcaag	tgtgctaatgaaggcggtaag	59
ADHα	accctcgctcttctctctatc	cggccgtgttggaattagta	52
ADHβ	cgggaatcttcctttcgtctc	aggttgagctgcgtcaatag	59
BCAA biosynthetic pathway			
AHAS	cttcctcgacatgaacaagg	attagtagcacctggacccg	57
AHAIR	atggctcagattgagatcttg	ccacggcttcaatcacactc	52
DHAD	taccatggcatcagctatcg	ggtgttgacgagctgtaagg	55
TA	gtgaagatgatcttcgtcggc	tcacaatcagacttgaaagatg	52
Normalization genes			
βTUBULIN	gatgccaagaacatgatgtg	tccacaaagtaggaagagttc	61
CPS	attgatgctgccgaggatag	gatgcctcccttaggttgttc	61

M.M.2.2. Enzymes (EPSPS and DAHPS) content measurements

In this study, protein content of the enzymes EPSPS and DAHPS (Chapters two and four) were determined. All protein determinations were done in leaf tissues of sensitive and resistant populations.

M.M.2.2.1. Protein extraction

In chapter two protein extraction was performed using 0.1 g of ground leaf tissue in 0.2 mL of extraction buffer (100 mM MOPS, 5 mM EDTA, 1% Triton-X 100, 10% glycerin, 50 mM KCl, 1 mM benzamidine, 100 mM iodoacetamide, 5% polyvinylpyrrolidone (PVP) and 1 mM phenylmethylsulfonyl fluoride (PMSF) pH 7). In chapter four protein extraction was performed using 25 leaf disks and adding 150 µL of extraction buffer and mixing using a a Retsch mixer mill (MM200, Rescht®, Haan, Germany). Then they were centrifuged at 4 °C 18000 *g* for 30 min, and the supernatant was collected for protein content determination.

M.M.2.2.2. Soluble protein content

The soluble protein content was monitored as Bradford (1976) in the crude extracts. Protein aliquots were diluted with deionized water to 1:60, and 60 µL of the dilutions were mixed with 200 µL Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA). The suspensions were incubated at room temperature for 5 min and the optical density (OD) was measured at 595 nm. A curve ranging from 0 µg to 6 µg of bovine serum albumin (BSA) was performed to calibrate the protein determination of each sample. For absorbance measurements a Sinergy™ HT Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA) was used.

M.M.2.2.3. SDS-PAGE transference and immunoblotting

Proteins were separated by 4.6 % (w/v) stacking gel and 12.5 % (w/v) resolving gel SDS-PAGE. The gel was run in a vertical electrophoresis cell (Mini protean III; (Bio-Rad 170, Bio-Rad Laboratories, Inc., Hercules, CA, United States) and using an electrophoresis buffer (10 % (v/v) Tris-Glycine (10x), 1 % (v/v) SDS (10%) and 89 % (v/v) H_2O mili-Q). The current was of 120 V for 15 min and then 150 V until migration across the gel was finished. The gel was blotted onto P 0.45 polyvinylidene difluoride (PVDF) membrane GE Healthcare Life Science (Amersham Life Science, Arlington Heights, IL, USA) (previously activated with absolute methanol) for 90 min at 100 V in a Mini-trans blot electrophoretic transfer cell (Bio-Rad 170, Bio-Rad Laboratories, Inc., Hercules, CA, United States) and using a transfer buffer (20 % (v/v) absolute ethanol, 10 % (v/v) Tris-Glycine and 70% (v/v) H_2O mili-Q). The membrane was blocked with nonfat milk powder in 10 % Twin Tris Buffer Saline (TTBS) overnight at 4 °C. Gel was stained with Gel code™ blue safe protein stain, (Thermo Fisher Scientific Inc., Waltham, MA, USA)

to ensure that the transference and protein content were homogeneous. The membrane was washed three times for 5 min with 10 % TTBS, and then was incubated with specific primary antibody for each enzyme. After 1 hour of incubation, the membrane was washed three times during 5 minutes with 10 % TTBS and then the membrane was incubated with the secondary antibody. An anti-rabbit AP conjugated antibody (Sigma Chemical, Co., St. Louis, MO, United States) was used as a secondary antibody at a dilution of 1:20000. After 1 h of incubation, the membrane was washed three times during 5 minutes with 10 % TTBS and after that bands were identified using a BCIP/NBT kit which was amplified alkaline phosphatase immunoblot assay kit (1 % (v/v) color reagent A, 1 % (v/v) color reagent B, 4 % (v/v) color development (25x) and 94 % (v/v) H_2O mili-Q) (Bio-Rad 170-6412, Bio-Rad Laboratories, Inc., Hercules, CA, United States). Immunoblots were scanned using a GS-800 densitometer, and protein bands were quantified using Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, United States).

M.M.2.2.4. EPSPS specific parameters

Protein loaded for EPSPS enzyme determinations were 80 µg per well in GS samples and 15 µg per well in GR samples. EPSPS primary antibody was produced by a custom peptide facility (Agrisera AB, Vännäs, Sweden) against a sequence of residues (numbers 193–206) of *A. palmeri* EPSPS (GenBank accession no.FJ861242) (Fernández-Escalada et al. 2016). The primary EPSPS antibody dilution was 1:2000. The secondary antibody used is specified in section M.M.2.2.3.

M.M.2.2.5. DAHPS specific parameters

Protein loaded in each well were 40 µg in GS and GR samples for DAHPS enzyme determinations in chapters two and four. DAHPS primary antibody was produced by a custom peptide facility (Biogenes, Berlin, Germany) using a short, conjugated peptide as an antigen (C-QFAKPRSDS-FEEEKN) and the dilution used was 1:1000 (Orcaray et al. 2011). The secondary antibody used is specified in section M.M.2.2.3.

M.M.2.3. CM and AS enzymatic activities

Enzymatic activities of the enzymes CM and AS (Chapters one and two) were determined in leaf tissues of both populations.

Enzyme extraction for CM and AS activity assays was developed as described in Singh & Widholm (1974) with addition of 1 mM PMSF (Goers and Jensen 1984). Each sample (0.1 g) was extracted with 500 µL of extraction buffer (100mM Tris-HCL, 20 mM glutamine, 10 % (v/v) glycerol, 0.1 mM Na_2EDTA, 4 mM $MgCl_2$ and added in fresh 0.01 mM dithiothreitol (DTT) and 1 mM PMSF, pH 7.5). After vortexing and 10 min in ice, samples were centrifuged 20 min at 15000 *g* and 4 °C. The supernatant of each sample was desalted with desalting columns PD MiniTrapTM G-25 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein level of the samples was measured following Bradford protocol (Bradford 1976) as it was described in section M.M.2.2.2.

CM enzymatic activity was measured as described in Goers & Jensen (1984). 80 µL of reaction buffer and 100 µL of 2.7 mM chorismate (chorismic acid barium salt, Sigma-Aldrich Co., St. Louis, MO, USA) were added to 20 µL of desalted extracts and incubated for 30 min. Control for each sample was carried out using enzymatic extracts inactivated with 100 µL of 1 N HCl. After the first incubation, 100 µL of 1 N HCl were added to samples and the mix was incubated for 20 min more at 37 °C. After the second incubation, 700 µL of 2.5 N NaOH were added to the samples and 300 µL of this mix were charged in an ultraviolet (UV) plate Costar® (Corning Inc., Corning, NY, USA). Phenylpyruvate content was measured spectrophotometrically at 320 nm. The molar extinction (ε) of phenylpyruvate is 17500 $M^{-1}cm^{-1}$ (corrected with the absorbance values, height and volume of the sample and extinction coefficient). The units of CM activity were nkat mg^{-1} protein.

AS enzymatic activity was quantified as described in Ishimoto et al. (2010). They were added 100 µL of 2.7 mM chorismate to 100 µL of desalted extract and were incubated 30 min at 30 °C. Controls of each sample were performed using 5 min-boiled enzymatic extract (Matsukawa et al. 2002). After incubation, samples were boiled for 5 min and then centrifuged 10 min at 18000 *g*. In a fluorescence Black clini plate (Thermo Scientific, Thermo-Fisher Scientific, Vantaa, Finland). 150 µL of each supernatant were loaded. AS activity was measured by monitoring the formation of anthranilate. It was measured with an excitation filter at 330 nm and a fluorescence filter of emission at 400 nm AS activity was calculated as relative units of fluorescence mg^{-1} protein.

M.M.2.4. Free amino acid extraction and determination

Free amino acid content were determined in chapters one and two from ground leaf samples by adding 1.5 mL of 1 M HCl to about 0.1 g FW of plant tissues. After

incubating on ice for 10 min, they were centrifuged at 18000 *g* for 15 min at 4 °C. The supernatants were collected and transferred to new tubes, and were neutralized to a pH between 7 and 8 by adding NaOH and stored at -20 °C. Samples were derivatized with 1 mM fluorescein isothiocyanate (FITC) dissolved in acetone, and then samples were 5-fold diluted in 20 mM borate buffer (pH 10.0). Samples were finally incubated for 15 h at room temperature in the dark until amino acid content determination (Orcaray et al. 2011). After derivatization with FITC, amino acid content was measured with a Beckman Coulter capillary electrophoresis PA-800 (Beckman Coulter Inc., Brea, CA, USA) coupled to a laser-induced fluorescence detector (Argon laser at 488 nm). Separation was performed basically as described in Arlt et al. (2001), using a 45 mM α-cyclodextrin in 80 mM borax buffer (pH 9.2) except for Cys (its content was only determined in chapter one). Cys content was determined from the same acid extracts derivatized with 5-iodoacetamide fluorescein and reduced with tributylphosphine, as described previously (Zinellu et al. 2005) and the fluorescein was detected using the laser at 494 nm excitation and 518 nm emission. In Cys the separation is obtained by using this buffer: 20 mM of Na_3PO_4, 16.5 mM H_3BO_3, 100 mM Nmethyl N-glucamine (pH 11.2). Analyses were performed at 20 °C and at a voltage of +30 kV. For chapter one, an extra electropherogram was performed to determine Trp and Val contents, where voltage was reduced to +20 kV in order to improve separation.

M.M.2.5. Shikimate extraction and determination

In chapters one, two and three, shikimate content was determined in frozen leaf disks of treated plants. Shikimate was extracted as described before (Koger et al. 2005) by adding 100 µL of 0.25 N HCl per disk to each vial. Samples were incubated at room temperature for 90 min and mixed by vortexing. After incubation, the samples were placed in a freezer (-20 °C) for 24 h. A 25 µL volume of each sample were transferred to a 96-well microtiter plate.

In chapter four, each well contained one disk in 100 µL of the treatment solutions (Table M.M.4 and Figure M.M.5.A). Microplates were thrown at room temperature and shikimate was extracted from the frozen-thawed leaf disks by adding 25 µL of 1.25 N HCl and incubating the plates at 60 °C for 15 min and then 25 µL volume of each well was transferred to a 96 well microtiter plate.

Shikimate content was measured as described previously (Cromartie and Polge 2000). 100 µL 0.25 % (w/v) periodic acid / 0.25 % (w/v) metaperiodate was added to each well, which contained 25 µL of each sample, and incubated for 60 min in

darkness. After the periodic acid-metaperiodate incubation, 100 µL of 0.6 M sodium hydroxide with 0.22 M sodium sulfate solution were added. The optical density of the solution was determined spectrophotometrically at 380 nm. For absorbance measurements a SinergyTM HT Multi-Detection Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT, USA) was used. A shikimate standard curve was developed by adding known amounts of shikimate to wells (3, 6, 12, 25, 50, and 100 µg mL^{-1}).

M.M.2.6. Quinate extraction and determination

Quinate was extracted in trichloroacetic acid (TCA) as described before (Orcaray et al. 2010) by adding 1.5 mL of 5% (w/v) TCA to about 0.2 g FW of plant tissues. The mixture was centrifuged for 10 min at 1750 *g*, at 4 °C. The aqueous phase was washed three times with diethyl ether saturated with water. The ether was discarded and the aqueous phase was kept. This phase was purged with helium for 2 min and then filtered through a 0.45 µm syringe filter. Quinate levels were analyzed by ion chromatography in a 940 Professional IC Vario 2 (Metrohm AG; Herisau: Suiza) equipped with Metrosep A Supp16 150/4.0 (Metrohm AG; Herisau: Suiza) column at 45 °C. The solvents were ultrapure water (solvent A) and 60 mMNaOH (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; a 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; 90% in A and 10 % in B from 28 to 40 min. Detection was performed by conductivity.

M.M.2.7. Metabolomic profiling determination

In chapters two and three *Amaranthus palmeri* metabolomic profiling was determined. It was performed in the Leibniz Institute for Plant Biochemistry, in Halle (Saale), Germany.

M.M.2.7.1. Extraction

Each sample (0.1 g) was extracted with 500 µL of extraction solution (80 % methanol with 100 µM ribitol, 5 µM kinetin, 5 µM biochanin A and 5 µM IAA-Valine. All products were LCMS grade. Grinding beads were added to each Eppendorf tube and the samples were homogenized with a tissue homogenizer (Precellys 24, lysis and homogenization, Bertin Instruments, France) for 45 s at

6500 Hz. Samples were placed on a shaker (Ika® Vibrax VXR basic, IKA®-Werke GmbH & Co. KG, Germany) for 10 min at 18000 Hz, in ultrasonic bath (Sonorex digitec, Bandelin, Germany) in floaters for 10 min, and then replaced in the shaker for 10 min at 18000 Hz. Samples were centrifuged at maximum speed for 15 min. Pellets were reextracted again as before and the supernatants were transferred and mixed in a new Eppendorf tube. The mixture was dried in the vacuum centrifuge and resuspended in 250 µL.

M.M.2.7.2. GC-MS

Test derivatization and analyses showed, that specific samples demonstrated several very high peaks. For proper metabolite profiling with as many compounds as possible in the linear dynamic response range, all samples were analyzed with two dilutions according to Gorzolka et al. 2012: A) undiluted samples for the low abundant compounds and B) diluted samples for the high abundant compounds. For A) 25 µl sample were dried in an Eppendorf tube using a vacuum centrifuge at 40 °C. Afterwards, 25 µl methoxylamin-hydrochloride (40 mg/mL in pyridine, Sigma–Aldrich) were added and metabolites were derivatized at 37 °C, 1750 *g* for 90 min. Then, 25 µl N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Macherey–Nagel) with 10 % (v/v) spiked alkane retention time index mix (C12, C15, C19, C22, C28, C32, each 0.05 mg/mL in pyridine; Sigma Aldrich) were added and samples incubated at 37 °C, 1000 rpm for 30 min. Samples were transferred to GC-MS vials and analyzed within the next 30 hours. For B) samples were diluted 1:10 in 80 %, ethanol with 100 µM ribitol. 10 µl were dried in the vacuum centrifuge and underwent the same derivatization protocol as the undiluted samples (A).

The measurements were performed using a gas chromatograph (6890N GC; Agilent Technologies) equipped with a ZB-5 Zebron Guardian™ Capillary GC column (30 m + 10 m Zebron™, iD 0.25 mm, df 0.25 µm; Phenomenex) and coupled to mass spectrometer (5975 MSD; Agilent Technologies) with settings and method adapted to Gorzolka et al., 2012. Derivatized samples (2 µl) were injected automatically by multipurpose sampler (MPS 2XL; Gerstel) at 230 °C injector temperature and separated chromatographically with 1 mL/min flow (Helium as carrier gas) and the following oven program: 1 min 70 °C, ramp with 7 °C per minute up to 310 °C, 10 min 310 °C. The transfer line temperature was set at 300 °C and ion source at 230 °C. Mass spectra were recorded from m/z 50 – 800 at 2 Hz in the time range of 6.40 min to 45.00 min. The MS was calibrated and tuned on PFTBA. A chemical blank (derivatization agents without biological sample) was interspersed every five to six samples to check for a potential

carryover of metabolites during measurement. Each sample was analysed with one analytical replicate.

The raw data were converted to cdf-files by the Data Analysis software (Agilent Technologies) and uploaded to the MeltDB software. In MeltDB, peak detection with SN = 5 and FWHM = 5 using the warped-algorithm and metabolite profiling was done. Retention indices were annotated manually. Identification of metabolites by mass spectra similarity was performed in MeltDB based on customized spectral and index libraries from analytical standards, metabolite annotations are based on NIST11 and GMD database MS similarity search as well as on observations on typical MS fragmentation patterns. Each compound was reviewed for proper annotation and alignment. Metabolites that were not detected properly were quantified manually in DataAnalysis Qedit (ChemStation D0.2, Agilent Technologies).

Peaks were quantified on the peak area of characteristic ion traces and normalized on ribitol as internal standard to compensate for device performance variation. Data were exported to Excel for further statistics as well for Import in MetaboAnalyst (https://www.metaboanalyst.ca/).

M.M.2.7.3. LC-MS

Samples extractions were mixed 80 % of sample with 20 % of water with 0.1 % formic acid. Samples were stored overnight at -20 °C, centrifuged for 15 min before pipetting the supernatant into the LC-Vials. Ultra-performance liquid chromatography (Waters Acquity UPLC equipped with a HSS T3 column (100 × 1.0 mm)) coupled to electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC/ESI-QToF-MS) was performed using a high-resolution MicrOTOF-QII hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics). Data were acquired in centroid mode with the following MS instrument settings for positive mode: nebulizer gas: nitrogen, 1.6 bar; dry gas: nitrogen, 6 L/min, 190°C; capillary, 4000 V; end plate offset: 500 V; funnel 1 radio frequency (RF): 200 Volts peak-to-peak (Vpp); funnel 2 RF: 300 Vpp; in-source collision-induced dissociation (CID) energy: 0 eV; hexapole RF: 100 Vpp; quadrupole ion energy: 3 eV; collision gas: argon; collision energy: 5 eV; collision cell RF: 300 Vpp; transfer time: 70 µs; prepulse storage: 5 µs; pulser frequency: 10 kHz; and spectra rate: 3 Hz. Data were processed by MetaboScape (Bruker).

M.M.3. Statistical analysis

A detailed description of the statistical analysis performed will be included within each chapter.

CHAPTER 1

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

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

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1.1. OBJECTIVE

Quinate and glyphosate affect the shikimate pathway differently (quinate feeds the pathway and glyphosate blocks it), which can be taken advantage of by causing their interaction in the pathway in order to enhance the toxicity produced by glyphosate. If the efficacy of glyphosate were increased with quinate application, it would be possible to control *Amaranthus palmeri* with lower herbicide rates.

Before evaluating the potential enhancer effect of quinate on glyphosate efficacy, two questions had to be addressed. First, the effect of quinate alone on sensitive and resistant *A. palmeri* populations had to be described and evaluated. Second, the moment of application of quinate (before, simultaneously or after) in relation to glyphosate had to be stablished.

The general aim of this chapter was to study **whether the toxicity and physiological effects of glyphosate were affected by its combined application with quinate and whether the effects were similar in glyphosate sensitive and resistant *Amaranthus palmeri* plants.** This aim was approached by three specific objectives that were covered up in the three experiments performed.

Specific objectives:

-To determine the specific effect of the exogenous quinate application in the AAA pathway in GS and GR *A. palmeri* populations. To reach this aim **Experiment 1.A** was developed with a time-course research after quinate treatment evaluating quinate content, AAA content and expression of AAA pathway at transcriptional level.

-To establish the most suitable moment of application of quinate and glyphosate. To reach this aim, **Experiment 1.B** was developed analyzing the possible combinations of glyphosate and quinate in GS population.

-To determine the effects of the combined application of glyphosate and quinate in the toxicity of glyphosate and in the shikimate pathway. To achieve the objective it was developed the **Experiment 1.C**; where the response of GS and GR populations of *A. palmeri* was evaluated after glyphosate and/or quinate treatments at the level of visual symptoms, parameters of the AAA pathway and free amino acid content.

1.2. EXPERIMENTAL APPROACH

1.2.1. Experiment 1.A. Quinate applied exogenously. Time course experiment.

Plants of two populations (GS and GR) of *Amaranthus palmeri* were treated with surfactant or quinate as indicated before (Section M.M.1.2). Leaf samples were harvested 0, 1, 2 and 3 days after treatment application (Figure 1.1.) and immediately frozen in liquid nitrogen and stored at -80 °C for analytical determinations.

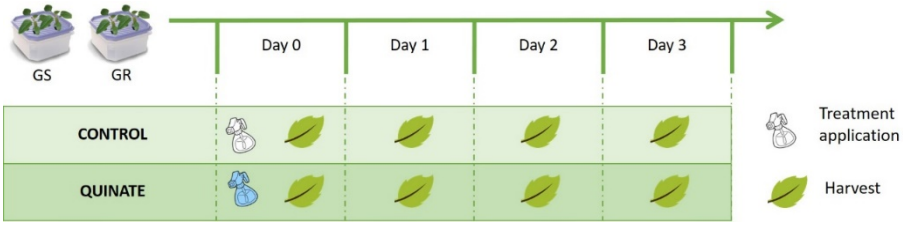

Figure 1.1. Overview of the treatment application and harvest in glyphosate sensitive (GS) and resistant (GR) *Amaranthus palmeri* plants in experiment 1.A. Plants were treated with the surfactant (Control) or with quinate (Quinate). Control and quinate plants were harvested 0, 1, 2 and 3 days after the treatment application.

1.2.1.1. Analytical determinations

The analytical determinations performed in the experiment 1.A. and the sections where they are included are indicated in the Table 1.1.

Table 1.1. Analytical determinations realized for the experiment 1.A.

Metabolite content	
Quinate	Section M.M.2.6
Amino acids	Section M.M.2.4
Relative gene expression	
Transcript level (AAA biosynthetic pathway)	Section M.M.2.1.2

1.2.2. Experiment 1.B. Determination of the moment of application of quinate in combination with glyphosate.

To determine the most suitable moment to apply the quinate in combination with the herbicide, an experiment with the GS population with the following combinations was developed (Figure 1.2): quinate applied 24 h before glyphosate treatment (A); quinate and glyphosate applied simultaneously (B); and quinate applied 24 h after glyphosate treatment (C). Plants of *Amaranthus palmeri* were treated with quinate and glyphosate as indicated before (Section M.M.1.2.).

Table 1.2. Overview of the glyphosate treatments applied in Experiment 1.B to glyphosate sensitive (GS) population of *Amaranthus palmeri* plants. RD = Recommended field dose (Culpepper et al. 2006).

	Amaranthus palmeri
	GS
Glyphosate	0.25 RD = 0.21 kg ha^{-1}

In this study, a sub lethal dose was used (Table 1.2) in order to determine the effect of the combination of both compounds, because too high doses of glyphosate would have covered up the effect of quinate in the combined treatments. In this experiment, only visual effects were analyzed, evaluating the lethality 7 days after the treatment application.

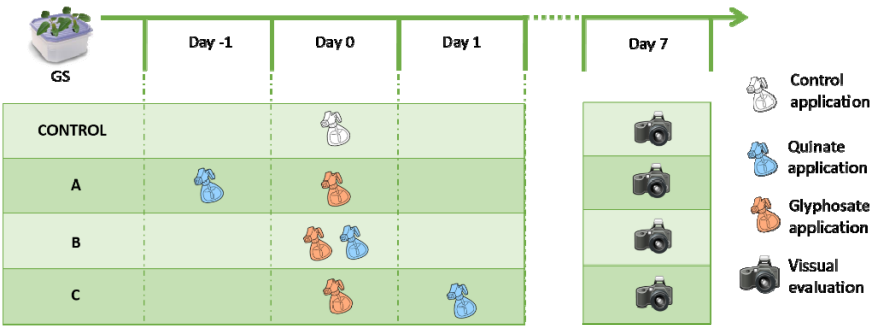

Figure 1.2. Overview of the treatment application in glyphosate sensitive (GS) *Amaranthus palmeri* plants in experiment 1.B. Plants were treated with the surfactant (Control) or with the combination of glyphosate and quinate. Quinate was applied 24 h before (A), simultaneously (B) and 24 h after glyphosate application (C). Visual evaluation was performed 7 days after glyphosate treatment.

1.2.3. Experiment 1.C. Effects of the combined application of glyphosate and quinate on sensitive and resistant populations

To determine the effects of the combined application of glyphosate and quinate in the toxicity of glyphosate and in the AAA biosynthetic pathway expression, it was developed the **Experiment 1.C.** The doses applied (Table 1.3) were adapted to each population due to the resistance of the GR, in order to obtain comparable response in each one, and in both cases, a sub lethal dose of glyphosate was used in order to allow a detection of an increased effect. Quinate and glyphosate were applied alone or combined (Figure 1.3). Quinate application was performed 24 h after glyphosate treatment. The experiment was stablished as described in section M.M.1.2. Samples were harvested 3 days after glyphosate treatment, and 2 days after quinate application, and immediately frozen in liquid nitrogen and stored at -80 °C for analytical determinations.

Table 1.3. Overview of the glyphosate treatments applied in Chapter 1 (Experiment 1.C) to two populations of *Amaranthus palmeri* plants, glyphosate sensitive (GS) and glyphosate resistant (GR). RD = Recommended field dose (Culpepper et al. 2006).

	Amaranthus palmeri	
	GS	**GR**
Glyphosate	0.25 RD = 0.21 kg ha^{-1}	0.5 RD = 0.42 kg ha^{-1}

Figure 1.3. Overview of the treatment application and harvest in glyphosate sensitive (GS) and resistant (GR) *A. palmeri* plants in experiment 1.C. Plants were treated with the surfactant (Control), quinate, glyphosate or with the combination of both compounds. Quinate was applied 24 h after glyphosate application (G+Q). A different glyphosate dose was used for each population: 0.25 recommended field dose (RD) for GS and 0.5 RD for GR. Plants from all treatments were harvested 3 days after glyphosate application.

1.2.3.1. Analytical determinations

The analytical determinations performed in the experiment 1.C. and the sections where they are included are indicated in the Table 1.4.

Table 1.4. Analytical determinations realized for the experiment 1.C.

Metabolite content	
Shikimate	Section M.M.2.5
Quinate	Section M.M.2.6
Amino acids	Section M.M.2.4
Enzymatic activities	
Chorismate Mutase	Section M.M.2.3
Anthranilate Synthase	Section M.M.2.3
Enzyme content	
DAHPS	Section M.M.2.2
EPSPS	Section M.M.2.2

1.2.4. Statistical analysis

In the experiment investigating the time-course of quinate (Experiment 1.A), 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 Experiment 1.C the experimental data of the untreated plants of each population were compared using Student's t test, showing no significant differences. After that, for each population one-way ANOVA with a multiple-comparison adjustment (Tukey) at $p \leq 0.05$ was used to determine significant differences. Analyses were performed using at least 4-6 biological replicates using samples from different individual plants.

1.3. RESULTS

1.3.1. Quinate applied exogenously. Time course experiment (Experiment 1.A).

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 (Figure 1.4 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 (Figure 1.4 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 hours of quinate treatment, while Trp content was not modified (Figure 1.4 C, D, E).

The relative expression level of the enzymes from the AAA biosynthetic pathway was determined. Transcript level of *DAHPS, DHQS, DQSD, SK* as pre-EPSPS pathway (Figure 1.5); and *EPSPS, CS* and *AS* (Figure 1.6); and *CM2, CM1-3, ADHα* and *ADH β* (Figure 1.7) as post-EPSPS pathway were measured and expressed normalized to the *β-tubulin* expression level as housekeeping gene. A different pattern in the transcript level of the AAA biosynthetic pathway enzymes was found for each population (Figures 1.5, 1.6 and 1.7). Quinate provoked a general decrease in the relative mRNA level of all the genes of the shikimate pathway only in GS population. A slight decrease in the relative gene expression of the pre-EPSPS genes was detected, only with transient changes at day 2 or 3 in *DHQS, DHSD* and *SK* expression. Relative mRNA level of *DAHPS* gene showed a very strong decrease from day 2 onwards after quinate application (Figure 1.5.A). The effect of the quinate was higher in the post-EPSPS pathway, detecting a general decrease in all the enzymes of this part of the pathway (Figures 1.6 and 1.7). Indeed, the relative mRNA level of *AS* gene was the most affected in this population, showing a significant decrease after 1, 2 and 3 days of quinate application.

Contrary to the pattern detected in the GS population, the relative transcript level of the genes of the AAA biosynthetic pathway was almost not affected in the GR population (Figures 1.5, 1.6 and 1.7). Only a decrease of the relative transcript level of the *AS* one day after treatment application (Figure 1.6.C) and of the *ADH β* two days after quinate application (Figure 1.7.D) were detected.

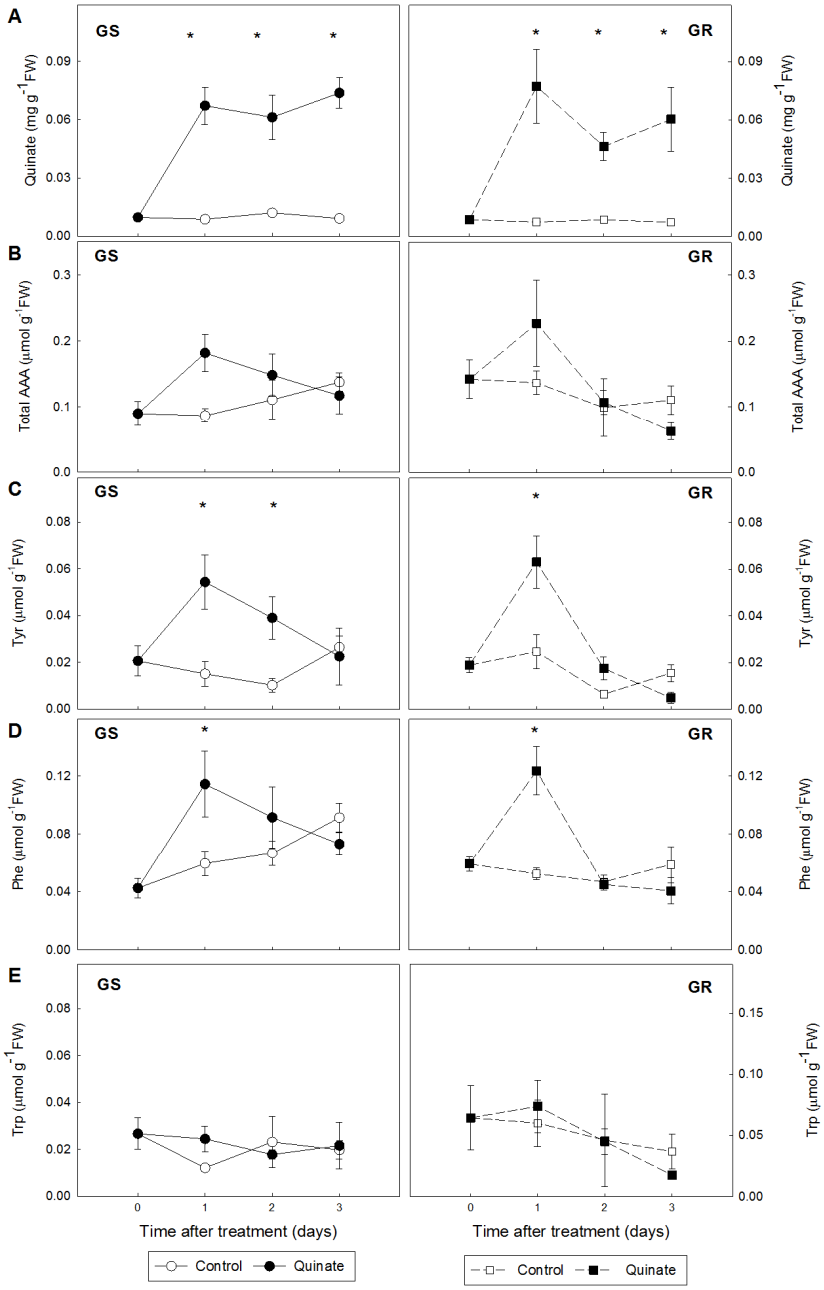

Figure 1.4. Quinate and aromatic amino acid content throughout four days of control (represented in white) or quinate (represented in bold) treatment in glyphosate-sensitive (circles, left; GS) and glyphosate-resistant (squares, right; GR) *Amaranthus palmeri* plants. Quinate (A), total aromatic amino acid content (B), tyrosine (Tyr; C), phenylalanine (Phe; D) and tryptophan (Trp; E) were measured (Mean ± SE; n=4). The symbol * indicates significant differences between treatment and control for each harvest time in each population (p-value≤0.05).

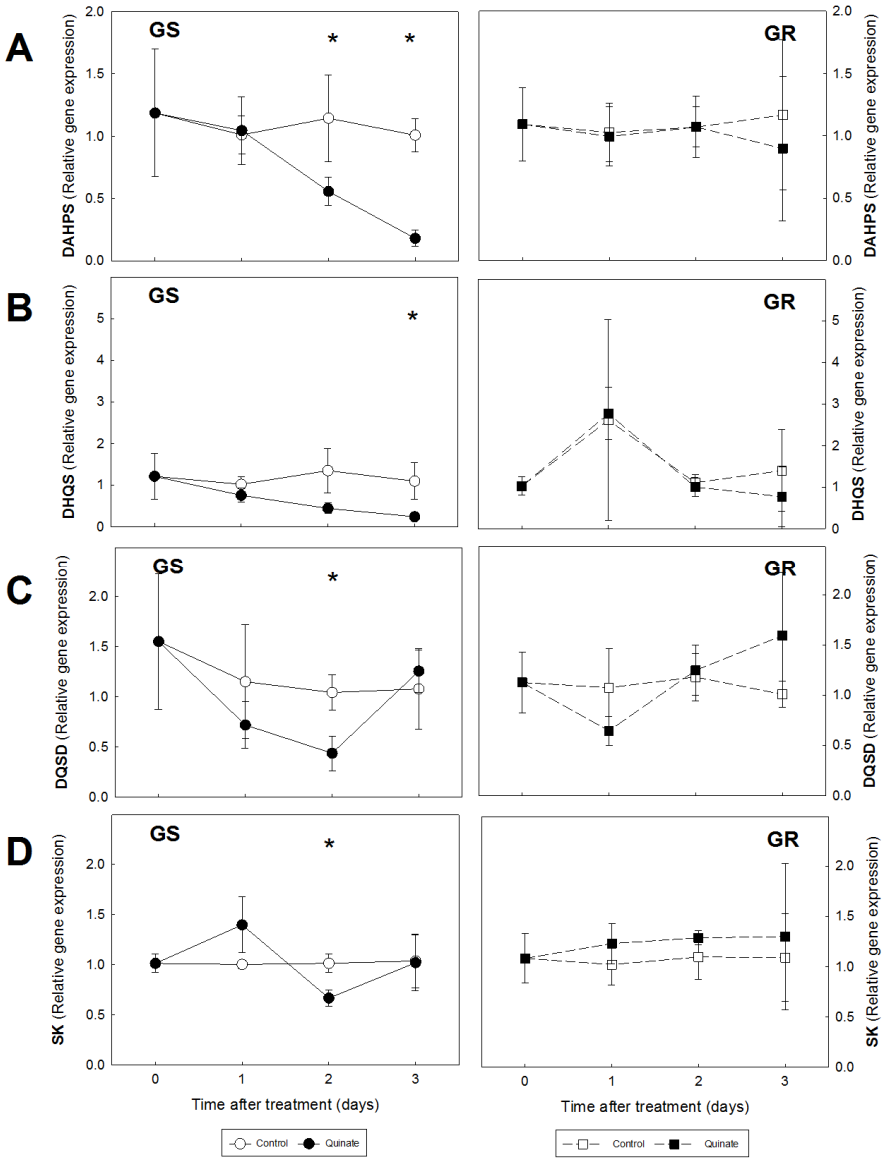

Figure 1.5. Transcript abundance of genes in the pre-EPSPS aromatic amino acid (AAA) biosynthetic pathway throughout four days of control (represented in white) or quinate (represented in bold) treatment in glyphosate-sensitive (circles, left; GS) and glyphosate-resistant (squares, right; GR) *Amaranthus palmeri* plants. Relative transcript abundance was normalized using the normalization gene *beta tubulin* and each population to its own daily control of 3-Deoxy-d-arabino-heptulosonate-7-phosphate synthase (*DAHPS*; A), dehydroquinate synthase (*DHQS*; B), 3-dehydroquinate dehydratase/shikimate dehydrogenase (*DQSD*; C), shikimate kinase (*SK*; D) (Mean ± SE; n=4). The symbol * indicates significant differences between treatment and control for each harvest time in each population (p-value≤0.05).

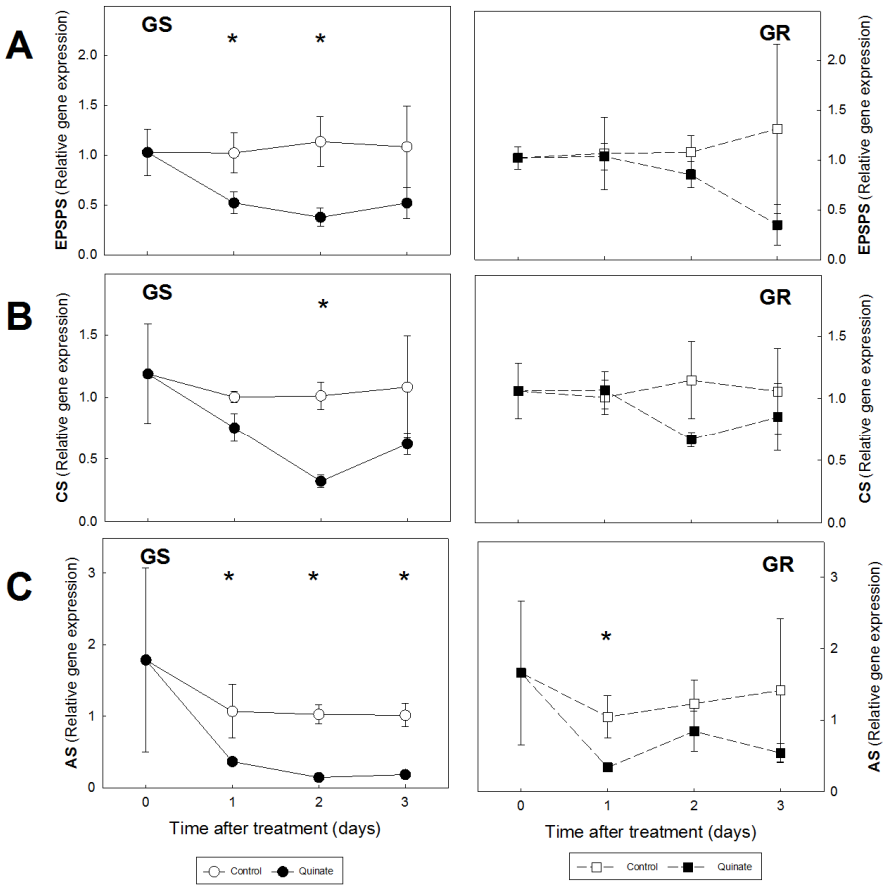

Figure 1.6. Transcript abundance of genes in the post-EPSPS aromatic amino acid (AAA) biosynthetic pathway throughout four days of control (represented in white) or quinate (represented in bold) treatment in glyphosate-sensitive (circles, left; GS) and glyphosate-resistant (squares, right; GR) *Amaranthus palmeri* plants. Relative transcript abundance was normalized using the normalization gene *beta tubulin* and each population to its own daily control of 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*; A), chorismate synthase (*CS*; B), anthranilate synthase (*AS*; C) (Mean ± SE; n=4). The symbol * indicates significant differences between treatment and control for each harvest time in each population (p-value≤0.05).

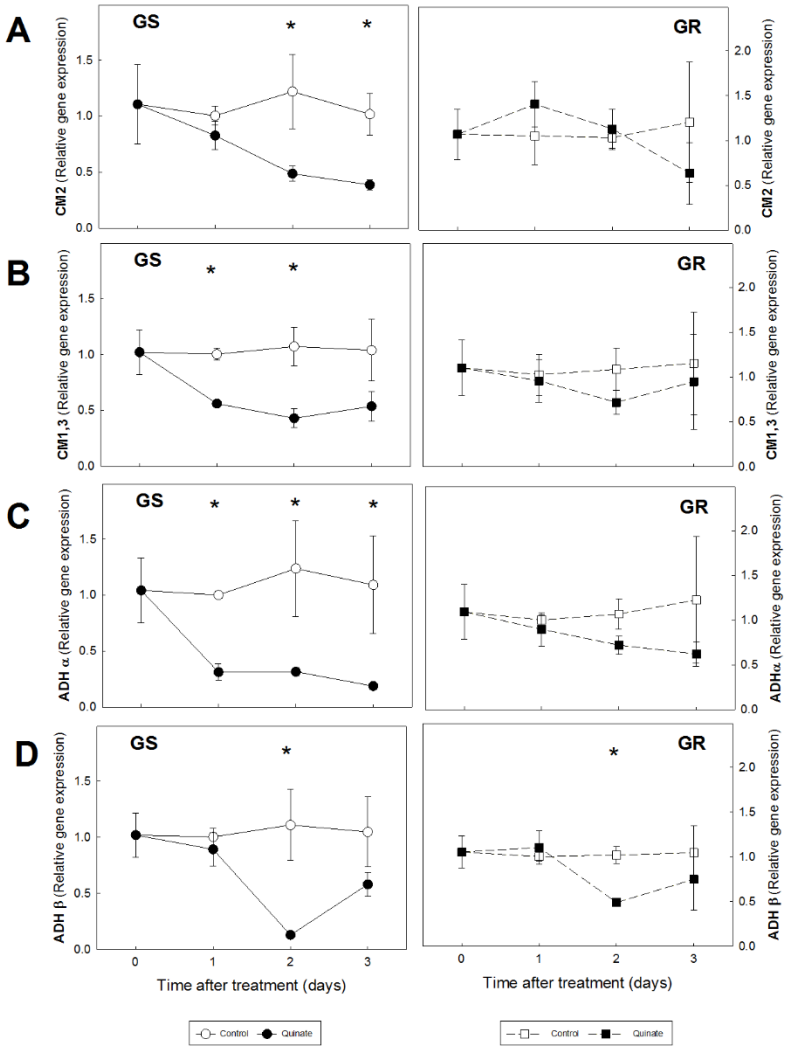

Figure 1.7. Transcript abundance of genes in the post-chorismate aromatic amino acid (AAA) biosynthetic pathway throughout four days of control (represented in white) or quinate (represented in bold) treatment in glyphosate-sensitive (circles, left; GS) and glyphosate-resistant (squares, right; GR) *Amaranthus palmeri* plants. Relative transcript abundance was normalized using the normalization gene *beta tubulin* and each population to its own daily control of chorismate mutase isoform 2 (*CM2*; A), chorismate mutase isoforms 1 and 3 (*CM1-3*; B), arogenate dehydrogenase isoform α (*ADHα*; C) and arogenate dehydrogenase isoform β (*ADHβ*; D) (Mean ± SE; n=4). The symbol * indicates significant differences between treatment and control for each harvest time in each population (*p*-value≤0.05).

1.3.2. Determination of the moment of application of quinate in combination with glyphosate (Experiment 1.B).

Experiment 1.B was performed to select the order of application of the compounds in the combined treatment. To this end, exogenous application of quinate was assayed 1 day before, 1 day after and simultaneously with glyphosate application. Effects were assessed visually 7 days after glyphosate treatment application. The greatest visual effects (Figure 1.8) were found when quinate was applied 24 hours later than glyphosate; therefore, this is the order that was selected to perform the full physiological approach.

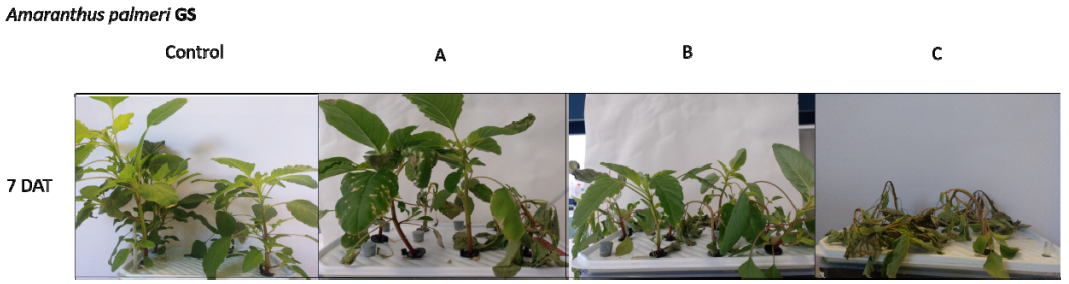

Figure 1.8. Visual appearance of the *Amaranthus palmeri* plants in glyphosate sensitive population (GS) 7 days after the treatment (DAT). Plants were untreated (Control) or treated with quinate and glyphosate 0.25RD (recommended dose) in different order: quinate applied 24 h before glyphosate (A); quinate and glyphosate applied simultaneously (B); and quinate applied 24 h after glyphosate (C).

1.3.3. Effects of the combined application of glyphosate and quinate on sensitive and resistant populations (Experiment 1.C).

1.3.3.1. Visual symptoms and lethality

Glyphosate was applied at one-quarter or half of the recommended field dose for *A. palmeri* to GS and GR populations, respectively. These sublethal doses allowed to observe increasing effect in the cases of combined treatments. The visual status of the plants was monitored for 10 days after treatments application (Figures 1.9 and 1.10).

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) (Figure 1.9). Interestingly, when this dose of glyphosate was combined with quinate and applied 24 h after, it was more effective and caused plant death in approximately 7-10 days (Figure 1.9).

No treatment was lethal in the case of the GR population. Nevertheless, as shown in Figure 1.10, 10 days after treatment, GR plants treated with the combined treatment were more affected than were plants treated with glyphosate alone.

It should be pointed out that the complete study of AAA biosynthetic pathway and AA profile in these plants was performed with samples harvested 3 days after glyphosate treatment, when no visual symptoms were detected after any of the treatments in either of the populations (Figures 1.9 and 1.10).

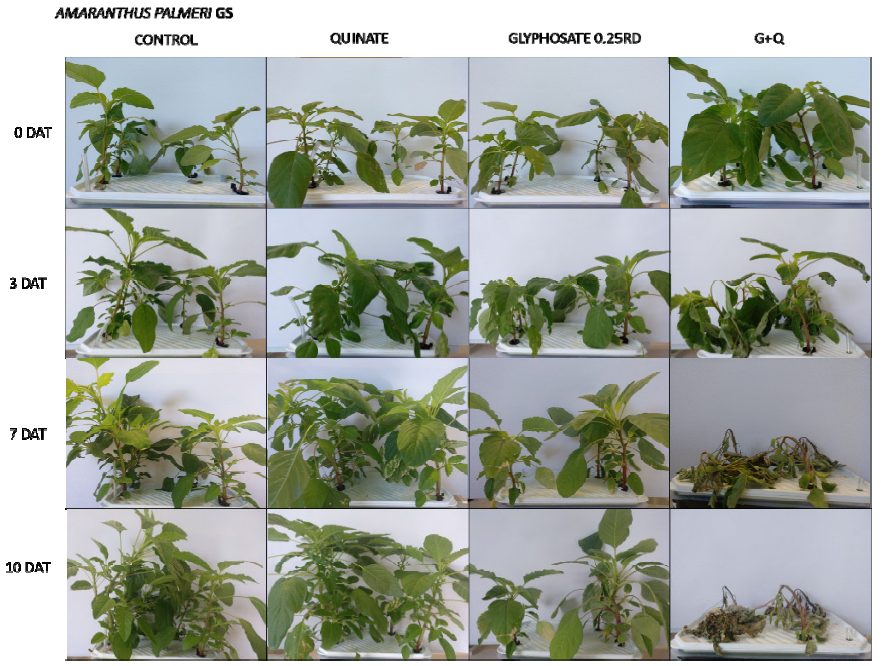

Figure 1.9. Visual appearance of the glyphosate-sensitive (GS) *Amaranthus palmeri* plants 0, 3, 7 and 10 days after the treatment (DAT). Plants were untreated (Control) or treated with quinate, glyphosate 0.25RD (recommended dose) or the combination of 0.25RD of glyphosate and quinate (G+Q).

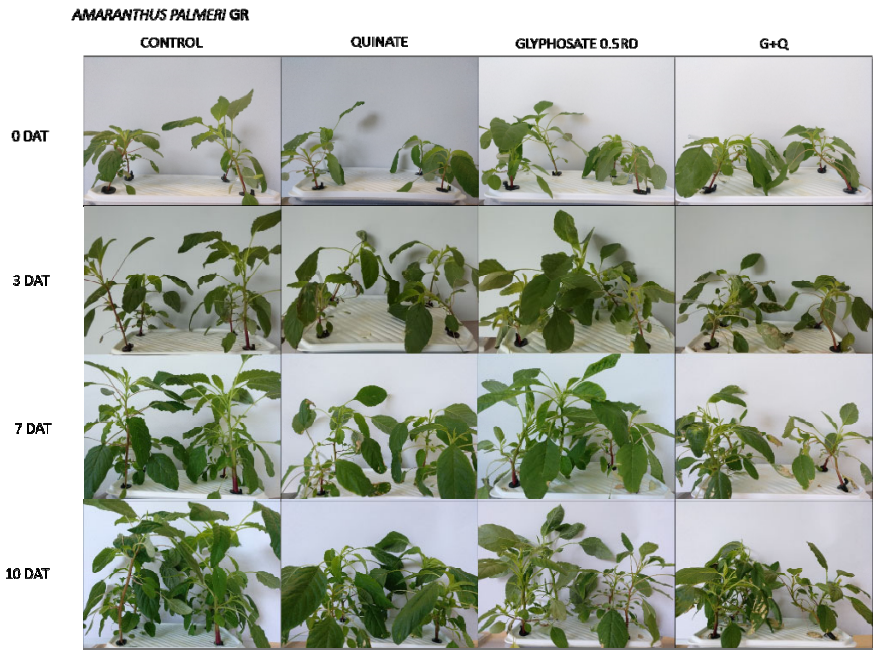

Figure 1.10. Visual appearance of the glyphosate-resistant (GR) *Amaranthus palmeri* plants in 0, 3, 7 and 10 days after the treatment (DAT). Plants were untreated (Control) or treated with quinate, glyphosate 0.5RD (recommended dose) or the combination of 0.5 RD of glyphosate and quinate (G+Q).

1.3.3.2. Pattern of the AAA biosynthetic pathway

Quinate and glyphosate were applied alone or in combination, and their effects on the AAA pathway and the amino acid pattern were measured for both populations 3 days after glyphosate treatment application. In the combined treatment, quinate was applied 1 day after glyphosate; thus, in concordance with the elapsed time of the combined treatment, quinate treatments were evaluated after 2 days in both treatments: applied alone or in combination with glyphosate; and treatments including glyphosate were evaluated after 3 days in both cases: applied alone or in combination with quinate.

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 (Figure 1.11.A). 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.

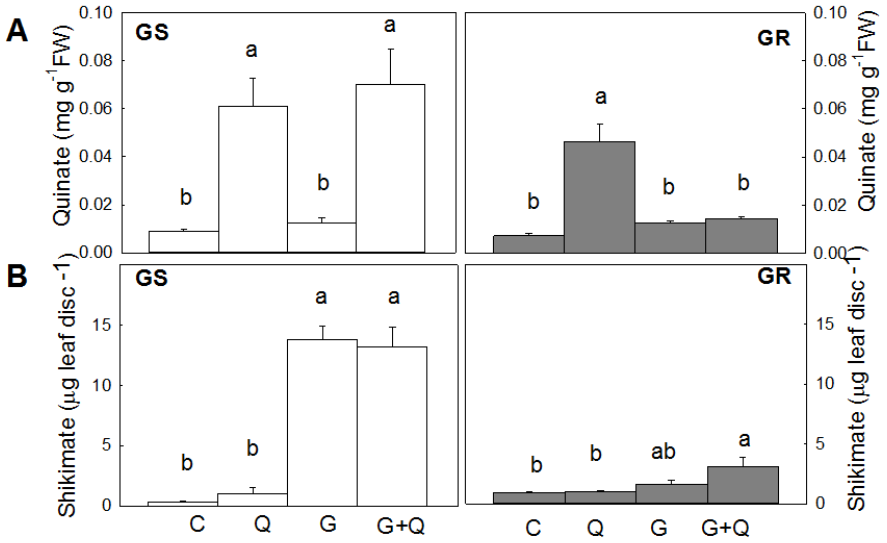

Figure 1.11. Quinate (A) and shikimate (B) content. Glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations were untreated (Control, C) or treated with quinate (Q), glyphosate (G) or a combination glyphosate with quinate (G+Q) (Mean ± SE; n=4). Different letters within each population indicate significant differences between treatments (p value ≤ 0.05, Tukey).

Shikimate content (Figure 1.11.B) 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 a very low shikimate accumulation (approximately 1.8-fold) in the GR population. The higher shikimate accumulation in GS than in GR after glyphosate treatment (even though the dose was higher in GR) confirms the inhibition of the EPSPS by glyphosate in GS and the 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 in this population (approximately 3.3-fold that in the untreated plants), greater than the induced by glyphosate alone (Figure 1.11.B).

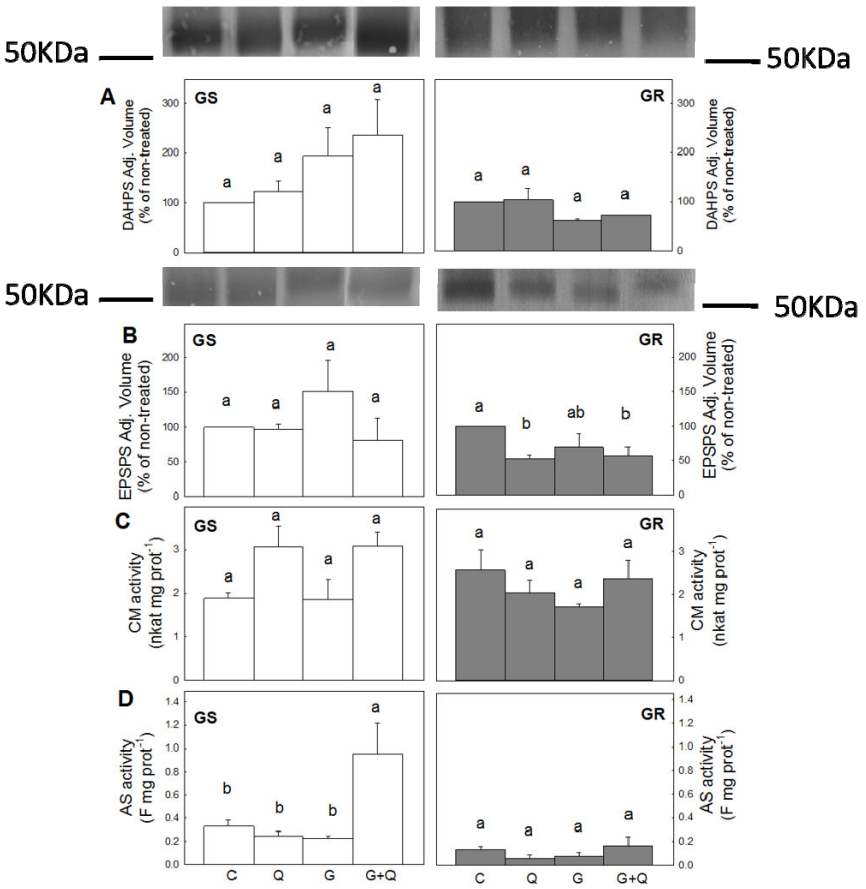

Figure 1.12. Enzymes of the aromatic amino acid biosynthetic pathway. (A) Normalization of the quantity of 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (DAHPS) (mean ± SE; n=3). Top: Representative immunoblots for DAHPS are plotted, and lanes contained 40 µg of total soluble proteins. (B) Normalization 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), or treated with quinate (Q), glyphosate (G) or a combination

glyphosate with quinate (G+Q). Different letters within each population indicate significant differences between treatments (p value ≤ 0.05, Tukey).

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 (Figure 1.12.A, 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 (Figure 1.12.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 (Figure 1.12.C).

1.3.3.3. Amino acid profile

The effect of quinate and/or glyphosate treatments on the free amino acid content of both populations was monitored (Figures 1.13, 1.14 and 1.15). 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 (Figure 1.13). Moreover, three other groups previously reported to be affected by glyphosate were studied: branched-chain (valine, (Val); leucine (Leu) and isoleucine (Ile)) (Figure 1.14); acidic (aspartate (Asp) and glutamate (Glu)); and amide (glutamine (Gln) and asparagine (Asn) (Figure 1.15.B, 1.15.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 (Figure 1.13). 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 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 (Figure 1.14 and 1.15). The total free amino acid content was measured in both

populations (Figure 1.15.A). 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 (Figure 1.14.A) and glyphosate alone or combined provoked accumulation of acidic amino acids (Figure 1.15.C).

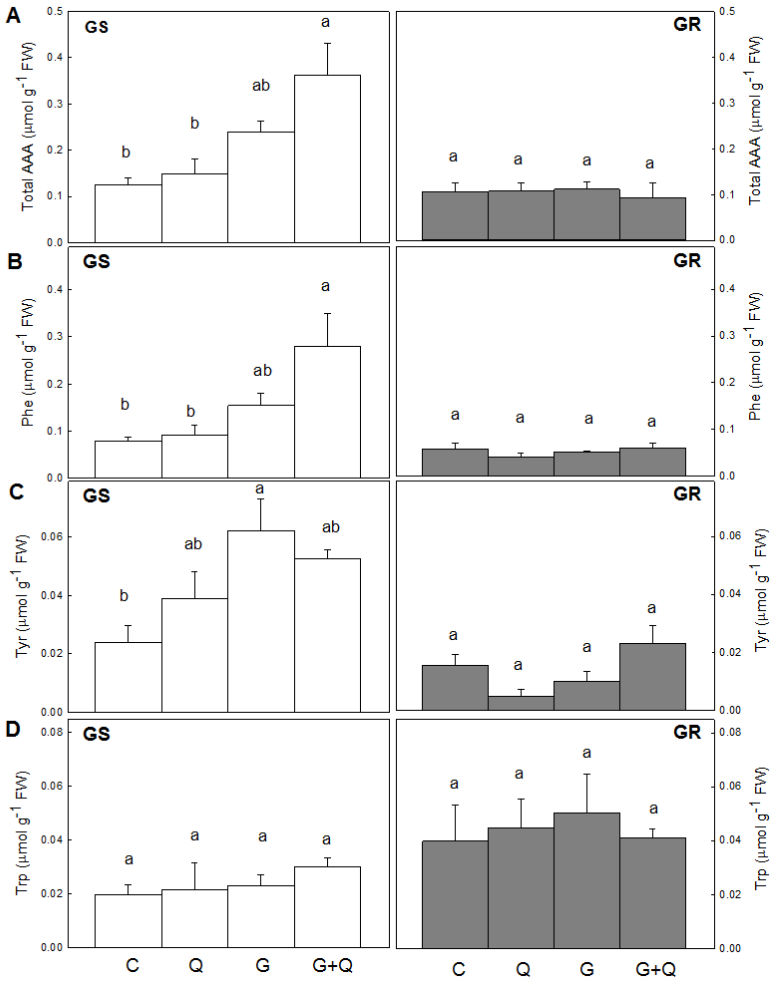

Figure 1.13. Aromatic amino acid profile. Total aromatic amino acid (A), phenylalanine (Phe; B), tyrosine (Tyr; C), and tryptophan (Trp; D) contents were measured in leaves of plants of glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations. Plants were untreated (Control, C), or treated with quinate (Q), glyphosate (G) or a combination glyphosate with quinate (G+Q) (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 ≤ 0.05, Tukey).

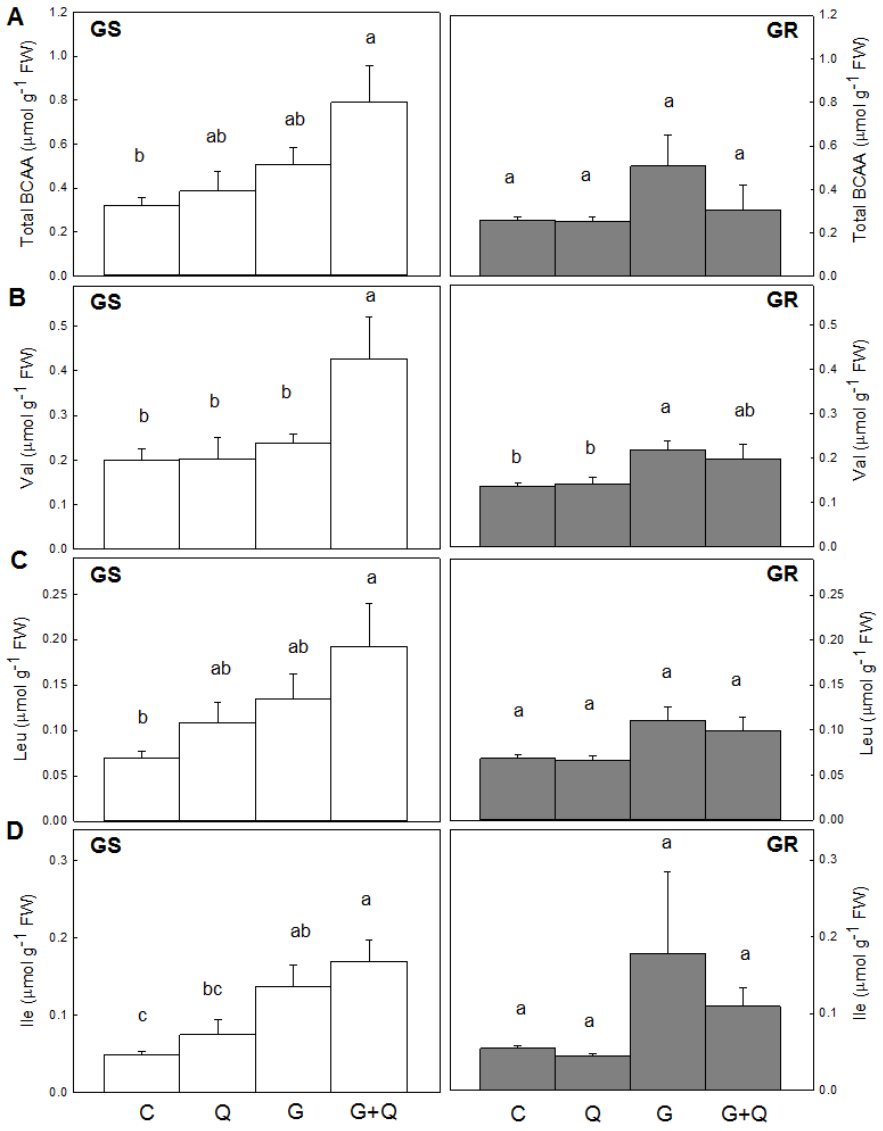

Figure 1.14. Branched-chain amino acid profile. Total branched-chain amino acids (A), valine (Val; B), leucine (Leu; C) and isoleucine (Ile; D) contents were measured in leaves of plants of glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations. Plants were untreated (Control, C), or treated with quinate (Q), glyphosate (G) or a combination glyphosate with quinate (G+Q) (Mean ± SE; n=4). Different letters within each population indicate significant differences between treatments (p value ≤ 0.05, Tukey).

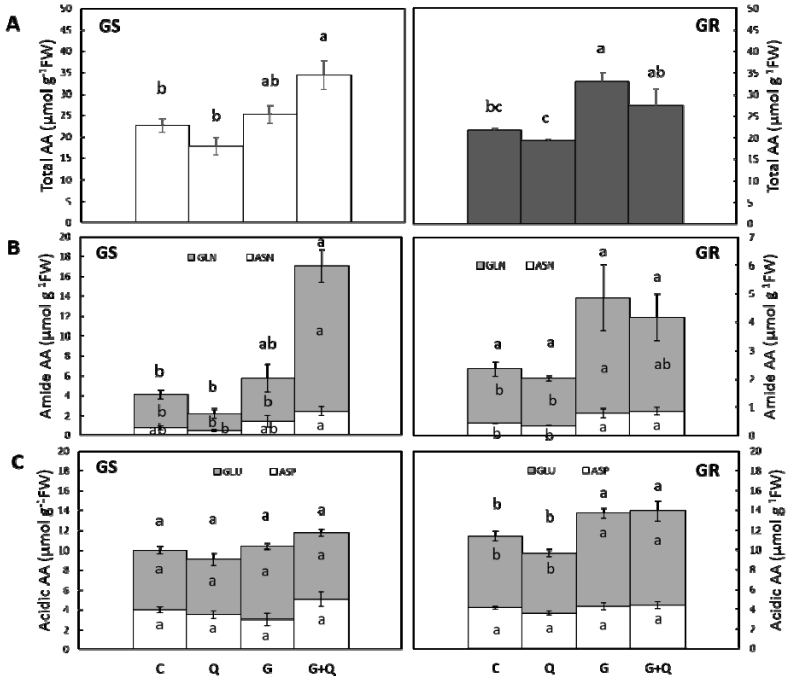

Figure 1.15. Total free amino acid (A), acidic amino acid (aspartate (Asp) and glutamate (Glu); B), and amide amino acid (glutamine (Gln) and asparagine (Asn); E) contents were measured in leaves of plants of glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations. Plants were untreated (Control, C), or treated with quinate (Q), glyphosate (G) or a combination glyphosate with quinate (G+Q) (Mean ± SE; n=4). Different letters within each population indicate significant differences between treatments (p value ≤ 0.05, Tukey).

1.4. DISCUSSION

1.4.1. Quinate applied exogenously. Time course experiment (Experiment 1.A).

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 and 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 and Haslam 1990; Leuschner et al. 1995; Guo et al. 2014).

Exogenous spraying of quinate onto the leaves of *A. palmeri* induced 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 and Schultz 1991a, b), 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 (Figure 1.4), 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 and Dudareva 2012). The accumulation of Tyr and Phe occurred during the first 24 hours after the treatment and was detected in both populations. This result suggests that, after quinate supply, carbon flux increases in the post-chorismate pathway through Tyr and Phe biosynthesis, independent of the EPSPS overexpression by gene amplification in 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 and Galili 2010a). 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).

In GS population, exogenous quinate application to the *A. palmeri* plants modified the expression of the enzymes of the pathway, provoking a decrease in all the transcripts of the shikimate pathway after 2 or 3 days of quinate application. Interestingly, this general decrease of gene expression (2-3 days) (Figures 1.5, 1.6 and 1.7) occurred 1 day after the detected accumulation of Tyr and Phe (Figure 1.4). After quinate application, the incorporation of the quinate in the pathway would increase the carbon flux through it and the expected result would be the accumulation of Tyr and Phe, the final products of the shikimate pathway, detected 24 and 48 h after quinate treatment in this population (Figure 1.4. C, D). AAA accumulation could have affected the expression of the enzymes catalyzing their biosynthesis, reducing them. Interestingly, the expression of all the genes previous to the Phe and Tyr synthesis decrease significantly, but the most susceptible gene seems to be *AS*, which is the first gene of the Trp biosynthesis pathway after the branch point. The fact that Trp content after quinate would indicate that there would be other signal affecting the *AS* relative expression independent to the Trp content. There are limited studies about the effect of the content of AAAs on the expression of the shikimate pathway genes performed in plants. It has been described that reduced levels of AAAs or their downstream products may act as a signal to induce the expression or activity of the shikimate pathway genes or enzymes and reestablish the carbon flux through the pathway in plants (Maeda and Duradeva, 2012). Indeed, it has been described that reduced levels of Phe increased the relative gene expression of the genes from the AAA biosynthetic pathway in some mutant lines of petunia (Maeda et al. 2010). In this same way, it can be proposed that the increase of the AAAs, Tyr and Phe detected after quinate application (Figure 1.4) would act as a signal to reduce gene expression. Another hypothesis is that the decrease of gene expression after quinate treatment can be countered as a physiological response to a stress situation, as changes in the gene expression of the AAA biosynthetic pathway have been reported after biotic or abiotic stresses (Dyer et al. 1989; Keith et al. 1991).

In post-translational regulation, it has been described that the activity of the enzymes DAHPS, CM, AS, CM1 and 3, and ADHβ were feedback inhibited by the final products of the shikimate pathway. DAHPS, the enzyme that catalyzes the first step of the shikimate pathway, would be feedback inhibited by Trp or Tyr (Graziana and Boudet 1980; Reinink and Borstlap 1982); but a DAHPS sensitive to Phe has not been described in plants (Maeda and Dudareva 2012). Enzymes CM and AS, the first enzymes of the two different branches of the post-chorismate pathway, would be inhibited by Trp, and Tyr and Phe, respectively, the final products of their belonging branch (Romero et al. 1995; Maeda and Dudareva 2012). The activities of the isoforms CM1 and CM3, located in the plastids, have been previously described in *Arabidopsis* to be generally inhibited by Phe and Tyr (Tzin and Galili 2010a; Maeda and Dudareva 2012). Although in this study the enzymatic activities were not determined, the *AS* gene expression was the most inhibited after quinate supply, which could be related to the increase detected in Tyr and Phe contents. Although it has been previously described in *Arabidopsis* that the activity CM2, the isoform located in the cytosol, is insensitive to allosteric regulation by AAAs (Benesova and Bode 1992; Eberhard et al. 1996), the expression of this enzyme was decreased after quinate treatment in GS population while no effect would be expected after the treatment. This result suggests a different regulation at transcriptional or posttranslational level of CM2.

Previous studies showed that the transcript levels of the shikimate pathway were very similar between the two populations (Fernández-Escalada et al. 2017), except for the expression of *EPSPS* gene. Interestingly, this study shows an important different effect in the response of the relative gene expression to exogenous quinate between GS and GR populations, even though the Tyr and Phe accumulation was similar in both of them (Figure 1.4). The proposed inhibitory effect of the accumulation of the final products of the pathway on the AAAs relative expression detected in GS plants after quinate treatment did not occur in GR plants (Figures 1.5, 1.6 and 1.7), with the exception of the *AS* gene. The reduction on the *AS* relative gene expression matched with Tyr and Phe accumulation, so these accumulations could act as the signals to reduce the relative gene expression of *AS* gene. It seems that some characteristic present only in GR population, probably due to the overexpression of the *EPSPS*, would avoid the effect of quinate on the gene expression of the pathway.

1.4.2. The moment of application of quinate in combination with glyphosate (Experiment 1.B.).

The application of both glyphosate and quinate in different order and moment resulted on different effect on *A. palmeri* plants. In this experiment quinate was applied 24 h before (A), 24 h after (B), or simultaneously (C) to glyphosate treatment. The mayor visual effects were detected in 'C' treatment, which was lethal 7 days after glyphosate treatment application (Figure 1.8).

'A' treatment, in which quinate was applied 24 h before glyphosate, it is incorporated before the inhibition of the enzyme EPSPS by the glyphosate. The Tyr and Phe content would increase, as detected in the study of quinate applied alone, in Experiment 1.A (Figure 1.4), and the effect of glyphosate applied later would be attenuated. When both compounds were applied simultaneously (B), the effect on the plants was similar to 'A' treatment.

In treatment 'C' the incorporation of the quinate in the pathway happens when the EPSPS is already blocked by the glyphosate. The perturbation in the pathway would be then more intense than in the other treatments due to the increase of the carbon flux though a previously blocked shikimate pathway. It has been reported that glyphosate toxicity could be related to the accumulation of the metabolites upstream EPSPS, due to the inhibition of the enzyme (de María et al. 2006). In this treatment, glyphosate toxicity would be exacerbated after fueling the pathway with quinate.

1.4.3. Effects of the combined application of glyphosate and quinate on sensitive and resistant populations (Experiment 1.C).

1.4.3.1. 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 and Schultz 1991a, b) or by blocking the shikimate pathway at the level of EPSPS (Steinrücken and 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 (Figure 1.4) 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 (Pinto et al. 1988; Fernández-Escalada et al. 2016) and accumulation of the metabolites upstream of the enzyme EPSPS, such as shikimate and quinate (Lydon and Duke 1988; Hernandez et al. 1999; 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 (Figure 1.11.B). 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 (Vivancos et al. 2011; Orcaray et al. 2011; Zulet et al. 2013a, 2015; 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 (Orcaray et al. 2010; Fernández-Escalada et al. 2016, 2017; Maroli et al. 2018b) and amides (Orcaray et al. 2010) and a higher relative increase in branched-chain amino acids (Orcaray et al. 2010; Maroli et al. 2015; Fernández-Escalada et al. 2016). 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 (Figures 1.13, 1.14 and 1.15).

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 lethal (Figure 1.9).

1.4.3.2. 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. 2016, 2017). 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 (Figure 1.14.B), acidic amino acids (Figure 1.15.C) and total free amino acid pool (Figure 1.15.A). A decrease in the amount of EPSPS protein was not detected after glyphosate treatment (Figure 1.12.B), 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 observed after quinate applied alone and enhance shikimate accumulation typical after glyphosate treatment. 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.

1.5. CONCLUSIONS

-Exogenous quinate application produced a perturbation in the AAA biosynthetic pathway, and Phe and Tyr were accumulated in GS and GR *Amaranthus palmeri* populations. A different pattern was observed in the response of the gene expression, detecting a decrease of the transcripts of the genes in GS population while in GR no decrease was observed, except the *AS* gene.

-Previously reported physiological effects of glyphosate on AAA biosynthetic pathway and amino acid profile were not evident in any of the populations due to the low and sublethal doses employed in the present study.

-The combination of quinate with glyphosate was more effective if quinate was applied 24 h after herbicide. Quinate would incorporate into a previously blocked AAA biosynthetic pathway by glyphosate, exacerbating the herbicide effects by accumulating the metabolites upstream EPSPS. This combination would increase the phytotoxicity of the herbicide, becoming lethal a sublethal glyphosate dose.

-The combined treatment induced the most striking changes in both populations, increasing the phytotoxicity of the herbicide in GS plants and provoking a deregulation of the pathway in GR. In GS population, only the combined treatment was lethal and provoked changes in amino acid profile previously reported as herbicide markers: total and specific free amino acid accumulation. In GR, metabolic perturbations were detected at the level of shikimate pathway, where can be suggested that carbon influx from quinate was increased.

-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.*

CHAPTER 2

Metabolite content and relative gene expression of the shikimate pathway after the combined treatment of quinate and glyphosate

2.1. OBJECTIVE

The efficacy of glyphosate was enhanced by quinate and the sub-lethal dose of a quarter part of the recommended dose in combination with quinate provoked the death of GS plants and altered the metabolism in GR. After checking the increase of the efficacy of the combined treatment, the physiological causes remain to be elucidated.

In chapter 1 it was proposed that the combined treatment would be more toxic in GS because quinate would incorporate into a previously blocked AAA biosynthetic pathway by glyphosate, exacerbating the toxic herbicide effects by an over-accumulation of metabolites upstream EPSPS. In the GR population, metabolic perturbations at the shikimate pathway were proposed. Nevertheless, a specific study of the pattern of shikimate pathway after the application of both compounds remains to be performed.

The aim of this chapter **was to unravel the pattern of the shikimate pathway after applying quinate and glyphosate together,** to check if it could explain the increase of the efficacy detected in GS population and the altered metabolism detected in GR population.

To this end, the effects of both compounds applied individually and the combined treatment were evaluated on the shikimate pathway of GS and GR *Amaranthus palmeri* plants. In addition, an additional herbicide dose that provoked the same visual effects than the combined treatment in GS population and in GR population were added in the experiment in order to elucidate possible similarities on toxicity patterns.

The pattern of the shikimate pathway after the treatments was approached at metabolic and transcriptional level:

-Some intermediate metabolite content of the AAAs biosynthetic pathway were determined by GC-MS: quinate and derivatives, metabolites upstream EPSPS, gallic acid, AAAs and phenylpropanoids.

-Relative mRNA level of the genes of the enzymes of the AAA biosynthetic pathway were determined

2.2. EXPERIMENTAL APPROACH

2.2.1. Plant material and treatment application

The experiment was developed as described in the section M.M.1.2. The doses applied (Table 2.1) were adapted to each population due to the resistance of the GR, in order to obtain comparable response in both of them. Sub-lethal glyphosate dose was adapted from 0.5 RD to RD in this chapter for GR due to the low effect observed in Chapter 1. An extra treatment was applied in this chapter for each population, a higher glyphosate dose that would provoke a similar visual aspect than the combined treatment. The high glyphosate doses applied in this chapter (Table 2.1) were chosen according to dose effects on shikimate accumulation and dry weight obtained in previous studies (Fernández-Escalada et al. 2016). The high doses were RD for GS and 3 RD for GR. Quinate was applied alone and combined with the sublethal dose of glyphosate (Figure 2.1). Quinate application was performed 24 h after glyphosate treatment. Samples were harvested 3 days after glyphosate treatment, and 2 days after quinate application, and immediately frozen in liquid nitrogen and stored at -80 °C for analytical determinations.

Table 2.1. Overview of the glyphosate treatments applied in Chapter 2 to two populations of *Amaranthus palmeri* plants, glyphosate sensitive (GS) and glyphosate resistant (GR). RD = Recommended field dose (Culpepper et al., 2006)

		Amaranthus palmeri	
		GS	***GR***
Glyphosate	Sub lethal	0.25 RD = 0.21 kg ha^{-1}	RD = 0.84 kg ha^{-1}
	High dose	RD = 0.84 kg ha^{-1}	3 RD = 2.52 kg ha^{-1}

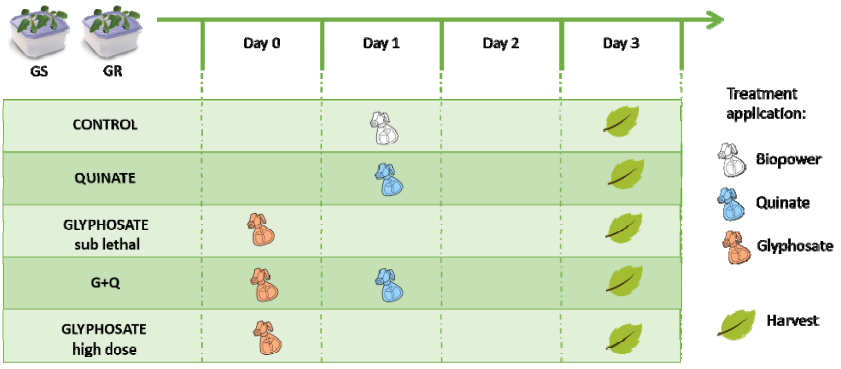

Figure 2.1. Overview of the treatment application and harvest in glyphosate sensitive (GS) and resistant (GR) *Amaranthus palmeri* plants in Chapter 2. Plants were treated with the surfactant

(Control), quinate, glyphosate or with the combination of both compounds. Quinate was applied 24 h after glyphosate application (G+Q). A different glyphosate dose was used for each population: 0.25 recommended field dose (RD) and RD for GS; and RD and 3 RD for GR. Plants from all treatments were harvested 3 days after glyphosate application.

2.2.2. Analytical determinations

The analytical determinations performed in Chapter 2 and the sections where they are included are indicated in Table 2.2.

Table 2.2. Analytical determinations realized for the Chapter 2 and their sections in this document.

Metabolite content	
Shikimate	Section M.M.2.5
GCMS	Section M.M.2.7
Relative gene expression	
Transcript levels (AAA biosynthetic pathway)	Section M.M.2.1.2

2.2.3. Statistical analysis

Analyses were performed using at least 4-6 biological replicates of samples from different individual plants. Firstly, the difference between untreated plants of each population was evaluated using Student's t test ($p \leq 0.05$). After that, in each population, one-way ANOVA with a multiple-comparison adjustment (Tukey) at $p \leq 0.05$ was used to determine significant differences between treatments.

2.3. Results

In the combined treatment, G+Q, quinate was applied 24 h after glyphosate, and plants were harvested 3 days after glyphosate application. Then, at harvest moment, plants of the combined treatment had been treated with quinate for only 2 days. For comparing the combined treatment with the treatments in which the compounds were applied alone, plants were harvested in concordance with the former; quinate treated-plants were harvested 2 days after spraying, and glyphosate-treated plants after 3 days.

2.3.1. Visual symptoms and lethality

The visual status of *A. palmeri* plants was followed up to 15 days after treatments application. The visual status of *A. palmeri* plants 7 and 10 days in GS and GR plants, respectively, is shown in Figure 2.2. Glyphosate was applied at two different doses for each population due to the resistance of the GR and in order to be able to compare the doses in both populations: one-quarter or the RD for GS plants; and one or three times the RD (3RD) for GR plants. The sub-lethal dose of glyphosate was used when the herbicide was applied in combination with quinate, in order to observe the potential increasing effect in the case of combined treatments.

According to the results observed in Chapter 1 (Figure 1.9), *A. palmeri* GS plants treated with 0.25 the recommended field glyphosate dose did not die during the course of the experiment. However, the same dose combined with quinate applied one day after reassembled the effects of the recommended field dose, and the plants died after 7 days (Figure 2.2.A), increasing the toxicity of the herbicide.

Plants of *A. palmeri* of the GR population treated with the RD and 3 RD showed growth arrest but neither of the treatments was lethal, although, as expected, the effect was more intense in the highest dose (Figure 2.2.B). The RD applied in combination with quinate one day after resembled the effects induced by the higher glyphosate dose, as plants showed similar visual aspect and growth was affected in a similar way.

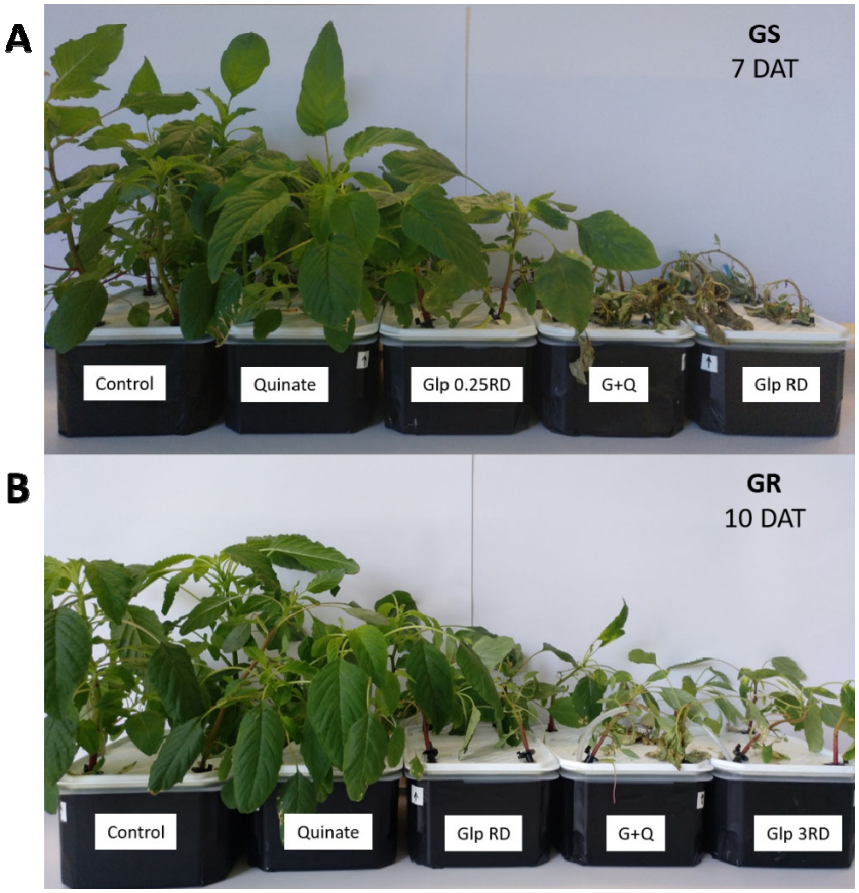

Figure 2.2. Visual appearance of *Amaranthus palmeri* plants in glyphosate sensitive (GS) and resistant population (GR) 7 and 10 days after glyphosate treatment (DAT), respectively. Plants of GS population were untreated (Control) or treated with quinate, glyphosate 0.25RD, RD (recommended dose) or with 0.25RD and quinate (G+Q). Plants of GR population were untreated (Control) or treated with quinate, glyphosate RD, 3RD or with RD and quinate (G+Q).

2.3.2. Effects on the metabolites of the AAA biosynthetic pathway

A non-targeted metabolic profiling by GM-MS was performed in the Leibniz Institute for Plant Biochemistry in Halle (Saale), Germany, as described in Section M.M.2.7. Non-targeted metabolomics detect as many metabolites as possible in the sample and they can be structurally detected and identified afterwards by comparing the results with Data Bases. In this chapter, detected and identified metabolites related to AAA biosynthesis are shown. Metabolite content is expressed as peak intensity normalized to ribitol, internal standard of the

samples, and are organized as following: Quinate and derivates; metabolites belonging the shikimate pathway; gallic acid; and phenylpropanoids.

For each metabolite content, before evaluating the effect of the treatments on each population, differences between untreated plants of both populations were compared. Significant differences are indicated in the figures and mentioned in the text.

2.3.2.1. Quinate and derivatives

Quinate was highly accumulated in both populations after all quinate treatments (Figure 2.3), confirming that the compound was absorbed by the plant, as observed in Chapter 1 (Figures 1.4 and 1.11 A). Sub-lethal doses of glyphosate did not provoke quinate accumulation in neither GS nor GR population (Figure 2.3). However, a significant quinate accumulation was detected in GS plants treated with the RD glyphosate dose.

In GS population, the greatest quinate accumulation was observed after the combined treatment, where the accumulation of this compound in this treatment was significantly higher than when quinate was applied alone. In GR plants, quinate accumulation was similar in both quinate treatments (Figure 2.3), and it was not detected any enhancer effect of the combined treatment.

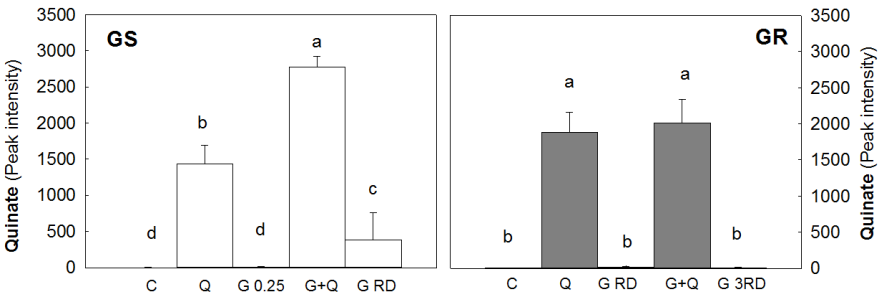

Figure 2.3. Quinate content in *Amaranthus palmeri* plants expressed as normalized peak intensity. Glyphosate-sensitive (white bars, left; GS) plants were untreated (C) or treated with quinate (Q), glyphosate 0.25RD, RD (recommended dose) or with 0.25RD and quinate (G+Q). Glyphosate-resistant (gray bars, right; GR) plants were untreated (Control) or treated with quinate, glyphosate RD, 3RD or with RD and quinate (G+Q). (Mean ± SE; n=6). Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tukey).

Four different quinate derivatives were detected in *A. palmeri* GS and GR plants (Figure 2.4). The four derivatives were accumulated after quinate treatments in GS and GR plants while no changes were detected with glyphosate alone. Quinate

derivative 1 in GS plants (Figure 2.4.A) and derivatives 1, 2, 3 and 4 in GR plants (Figure 2.4.A, B, C and D) were similarly accumulated in plants treated with quinate alone or with the combined treatment. However, in GS population, the accumulation of quinate derivatives 2, 3 and 4 was enhanced after the combined treatment (Figure 2.4.B, C and D) so the accumulation detected when quinate was applied alone was exacerbated with the presence of glyphosate.

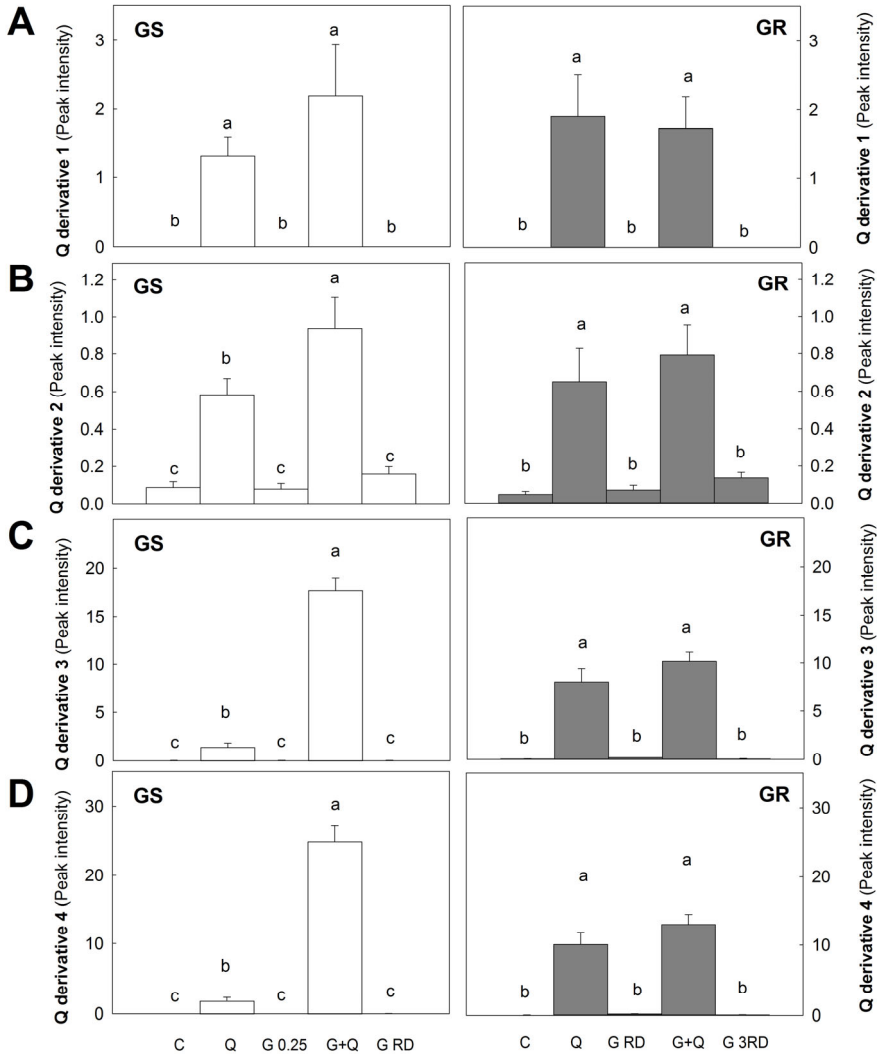

Figure 2.4. Quinate (Q) derivatives 1 (A), 2 (B), 3 (C), and 4 (D) content in *Amaranthus palmeri* plants expressed as normalized peak intensity. Glyphosate-sensitive (white bars, left; GS) plants were untreated (C) or treated with quinate (Q), glyphosate 0.25RD, RD (recommended dose) or with 0.25RD and quinate (G+Q). Glyphosate-resistant (gray bars, right; GR) plants were untreated (Control) or treated with quinate, glyphosate RD, 3RD or with RD and quinate (G+Q). (Mean ± SE; n=6).

Different letters within each population indicate significant differences between treatments (*p*-value≤0.05, Tukey).

2.3.2.2. Metabolites in the shikimate pathway and phenylpropanoids

Several metabolites of the shikimate pathway (Figure 2.5), gallic acid (Figure 2.6) and the amino acids Tyr and Phe (Figure 2.7) were determined in GS and GR *Amaranthus palmeri* plants. 3-dehydroshikimate, shikimate and S3P are intermediate metabolites from the shikimate pathway, in the pre-chorismate pathway, the first part of the pathway (Boudet 2012; Maeda and Dudareva 2012); and anthranilate is an intermediate metabolite from the post-chorismate pathway, the first metabolite of the Trp biosynthesis branch. Gallic acid is a secondary compound, formed in a lateral branch from the 3-dehydroshikimate in the main trunk (Figure GI.15), and Tyr and Phe are two of the AAA.

Quinate treatment alone did not affect any metabolite of the pre-chorismate pathway or gallic acid in any population. On the contrary, in both populations glyphosate treatment provoked an important and dose dependent accumulation of the metabolites upstream EPSPS (Figure 2.5 A, B, and C) and gallic acid (Figure 2.6). The greatest accumulation detected in GS was after the highest dose of glyphosate, confirming the inhibition of the EPSPS by glyphosate. The accumulation was less intense in GR, and it was only detected after the 3 RD dose, confirming the resistance of the population. Combined treatment did not exacerbate the effect of the herbicide applied alone on the content of these compounds in neither of the populations.

In non treated plants, anthranilate content was significantly higher in GS than GR plants. No effect of the treatments was observed in anthranilate content (Figure 2.5 D) in GS. However, it was detected an increase in anthranilate content in the lower dose of glyphosate treatment in GR.

Tyr and Phe content (Figure 2.7) were highly increased in GS population after glyphosate treatment, and in both cases the highest accumulation (and only significant in the case of Phe) was observed after the highest glyphosate dose applied. Quinate did not modify their content when it was applied alone. AAA accumulation was not exacerbated in the combined treatments in GS. Indeed, it was similar (in the case of Phe) or even less (in the case of Tyr) than the accumulation detected after the sub lethal dose of the herbicide alone. In GR, the content of the AAAs was not affected by quinate and/or glyphosate.

The phenylpropanoids are metabolites derived of the AAA biosynthetic pathway. The first committed step of the phenylpropanoid pathway is mediated by PAL enzyme, which catalyzes the conversion from Phe to cinnamic acid. Phenylpropanoids presented in this chapter are caffeic acid, ferulic acid, vanillic acid and salicylic acid (Figure 2.8).

Non-treated plants of GS population showed higher content of vanillic and caffeic acid than non-treated GR plants. Caffeic acid, ferulic acid and vanillic acid content (Figure 2.8 A, B and C), were not modified after any treatment in any of the populations. Only salicylic acid content (Figure 2.7.D) was affected by the treatments. In GS, the sub-lethal dose of glyphosate but not the lethal dose induced a significant accumulation of salicylic acid and the combined treatment did not modify the response after the sub-lethal dose. In GR, only the high dose of glyphosate (3RD) increased the salicylic acid content.

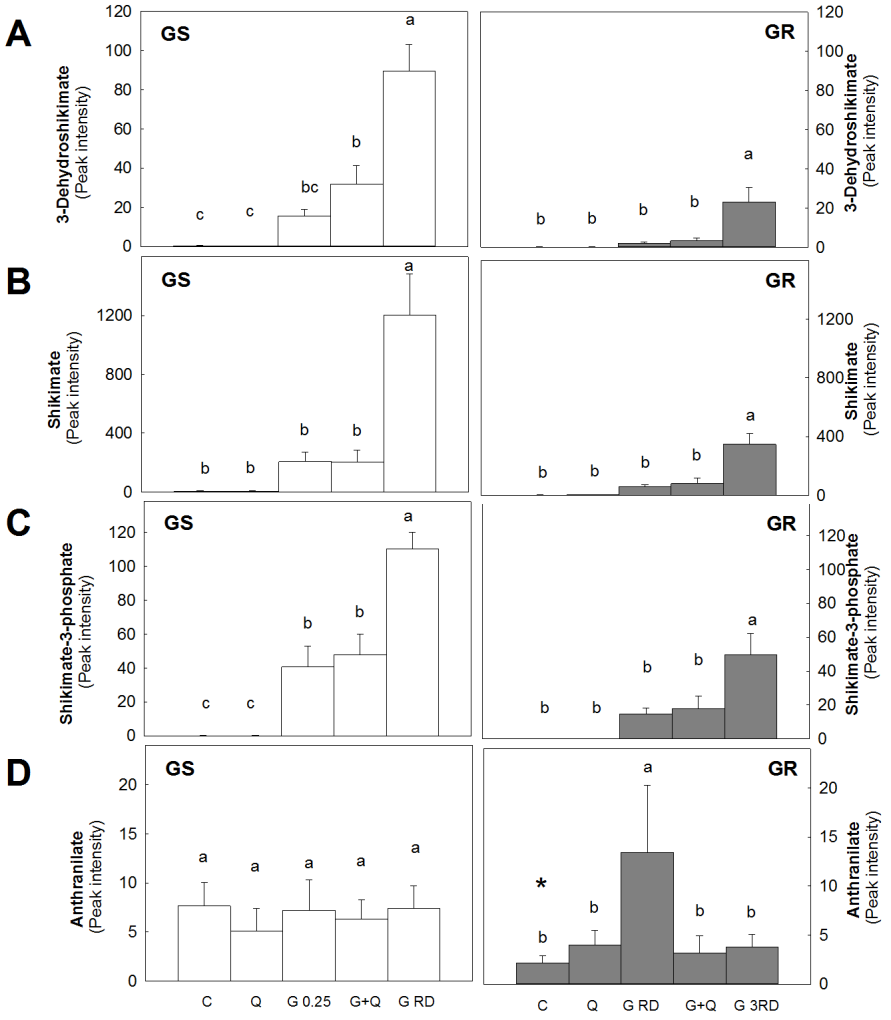

Figure 2.5. 3-Dehydroshikimate (A), shikimate (B), shikimate-3-phosphate (C) and anthranilate (D) content in *Amaranthus palmeri* plants expressed as normalized peak intensity. Glyphosate-sensitive (white bars, left; GS) plants were untreated (C) or treated with quinate (Q), glyphosate 0.25RD, RD (recommended dose) or with 0.25RD and quinate (G+Q). Glyphosate-resistant (gray bars, right; GR) plants were untreated (Control) or treated with quinate, glyphosate RD, 3RD or with RD and quinate (G+Q). (Mean ± SE; n=6). Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tukey). The symbol * indicates significant differences between untreated plants of each population (p-value≤0.05).

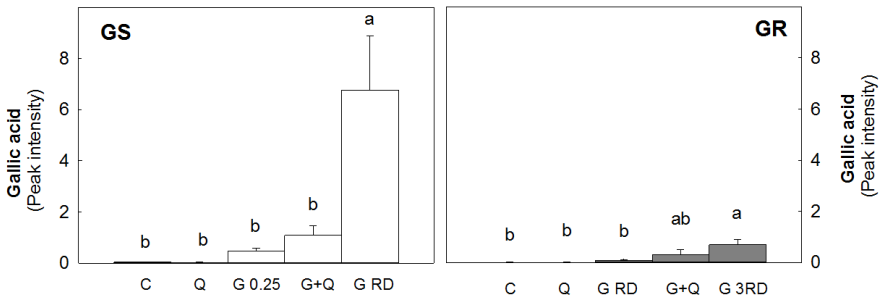

Figure 2.6. Gallic acid content in *Amaranthus palmeri* plants expressed as normalized peak intensity. Glyphosate-sensitive (white bars, left; GS) plants were untreated (C) or treated with quinate (Q), glyphosate 0.25RD, RD (recommended dose) or with 0.25RD and quinate (G+Q). Glyphosate-resistant (gray bars, right; GR) plants were untreated (Control) or treated with quinate, glyphosate RD, 3RD or with RD and quinate (G+Q). (Mean ± SE; n=6). Different letters within each population indicate significant differences between treatments (*p*-value≤0.05, Tukey).

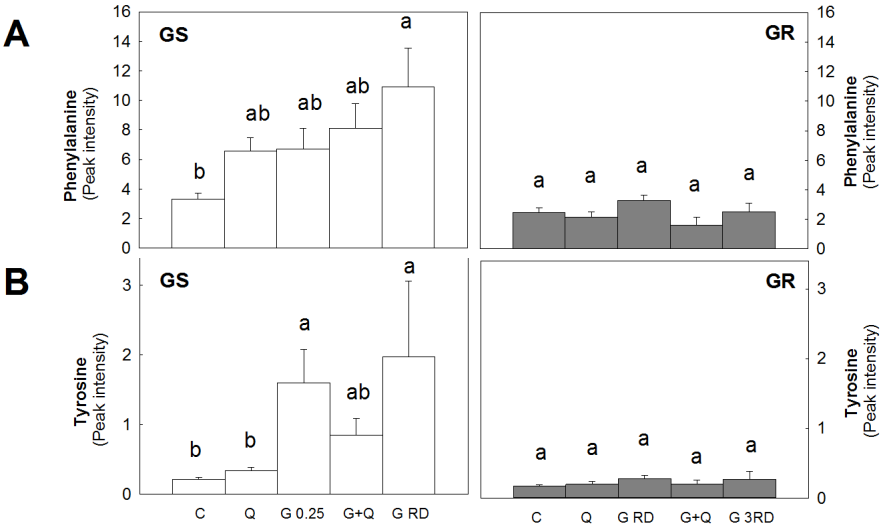

Figure 2.7. Phenylalanine (A) and tyrosine (B) content in *Amaranthus palmeri* plants expressed as normalized peak intensity. Glyphosate-sensitive (white bars, left; GS) plants were untreated (C) or treated with quinate (Q), glyphosate 0.25RD, RD (recommended dose) or with 0.25RD and quinate (G+Q). Glyphosate-resistant (gray bars, right; GR) plants were untreated (Control) or treated with quinate, glyphosate RD, 3RD or with RD and quinate (G+Q). (Mean ± SE; n=6). Different letters within each population indicate significant differences between treatments (*p*-value≤0.05, Tukey).

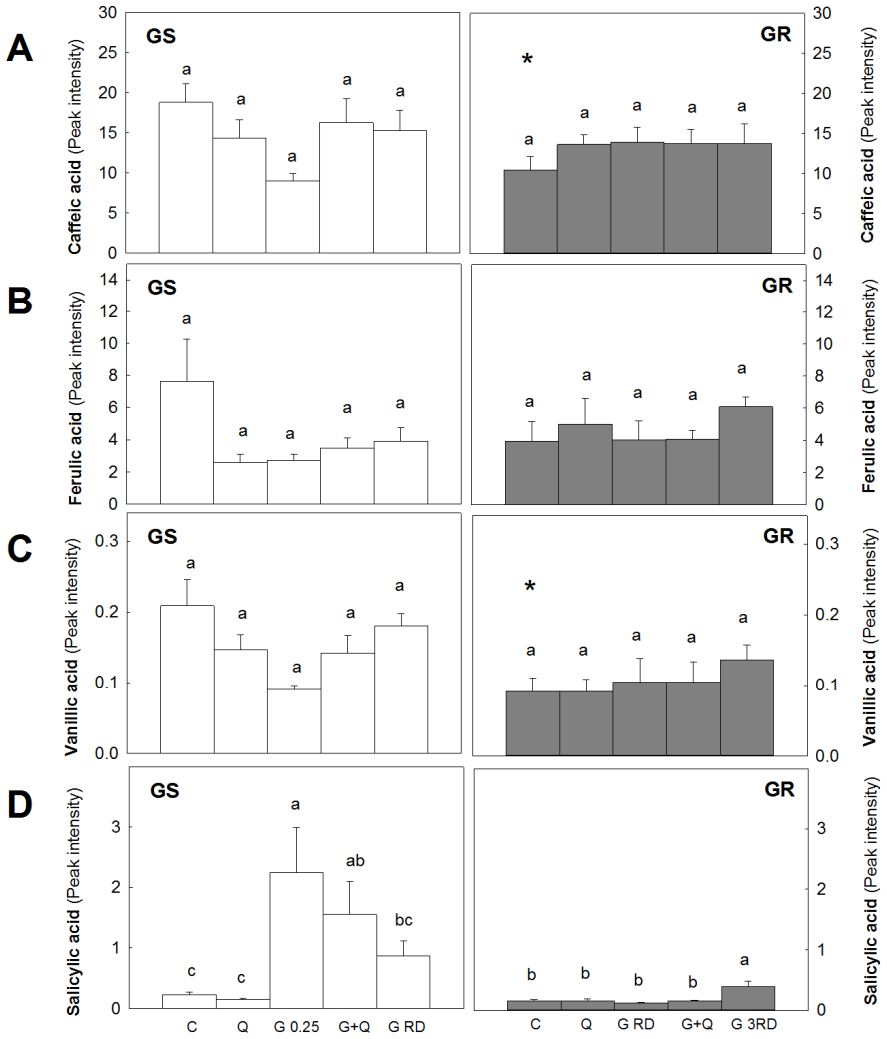

Figure 2.8. Caffeic acid (A), ferulic acid (B), vanillic acid (C) and salicylic acid (D) content in *Amaranthus palmeri* plants expressed as normalized peak intensity. Glyphosate-sensitive (white bars, left; GS) plants were untreated (C) or treated with quinate (Q), glyphosate 0.25RD, RD (recommended dose) or with 0.25RD and quinate (G+Q). Glyphosate-resistant (gray bars, right; GR) plants were untreated (Control) or treated with quinate, glyphosate RD, 3RD or with RD and quinate (G+Q). (Mean ± SE; n=6). Different letters within each population indicate significant differences between treatments (*p*-value≤0.05, Tukey). The symbol * indicates significant differences between untreated plants of each population (*p*-value≤0.05).

2.3.3. Effects on the relative gene expression of the AAA biosynthetic pathway

Transcript levels of 11 genes related to the AAA biosynthetic pathway were determined by qPCR in GS and GR *A. palmeri* plants. In absence of any treatment, with the exception of *EPSPS* transcript level (which was increased by 36.2 ± 5.7 fold in GR), no differences were observed between populations, confirming the results of Fernández-Escalada et al. (2016 and 2017).

As previously described in Chapter 1 (Figures 1.5, 1.6 and 1.7), quinate treatment provoked a decrease in the expression of all the genes of the AAA biosynthetic pathway in GS population (Figures 2.9, 2.10 and 2.11). In Chapter 1, differences were significant due to the statistical analyses performed (t-Student test) while in Chapter 2 ANOVA post-hoc Tukey test considering all treatments do not allow to detect significant differences, with the exception of *ADHα* and *β*, where significant differences were also detected between non treated and quinate-treated plants. On the contrary, in GR population, no changes were detected after quinate treatment, with the exception of *ADHβ* gene (Figure 2.11), result also observed in Chapter 1 (Figure 1.7).

In general, glyphosate provoked an induction of the transcript level of the AAA biosynthetic pathway genes (Figures 2.9, 2.10 and 2.11). The pattern of this induction was different in each population being more evident in the GS population.

In GS population, the induction in relative gene expression by glyphosate was detected even with the lowest dose and, with the exception of *DAHPS* and *DHQS*, similar gene expression increase was detected independently of the dose applied, evidencing that a higher glyphosate dose did not increase the effect on gene expression (Figure 2.9). The induction in the relative gene expression was only dose dependent in *DAHPS* gene, observing a higher effect on the relative expression level after the highest glyphosate dose. On the contrary, *DHQS* gene showed the opposite pattern, showing less induction in the relative gene expression after the highest than the lowest herbicide dose. This is the first study reporting the relative gene expression of the genes of the post-chorismate part of the pathway *CM2, CM1-3, ADHα* and *ADHβ* after glyphosate treatment in *A. palmeri* (Figure 2.11). Glyphosate treatment did not provoke any change in the relative gene expression of these post-chorismate genes after any dose in any of the populations.

In GR population, the lower glyphosate dose did not modify the relative gene expression of the shikimate pathway genes. In this population, only the highest glyphosate dose applied, 3 RD, provoked significant upregulation of the expression. A significant induction on the transcript levels of *DAHPS* and *DQSD* on the pre-EPSPS pathway (Figure 2.9 A and C) of *EPSPS* and *AS* on the post-EPSPS pathway (Figure 2.10. A and C) was observed. The most responsive gene was *AS* with upregulation over 4 fold with the highest dose (Figure 2.10.C).

The combination of glyphosate with quinate did not modify the gene expression pattern detected after the herbicide alone in any of the populations, with the exception of *AS* relative gene expression in the GS population (Figure 2.9, 2.10 and 2.11). The combined treatment abolished the increase in *AS* relative gene expression detected with glyphosate alone, and plants treated with the combined treatment showed similar expression levels than untreated plants (Figure 2.10).

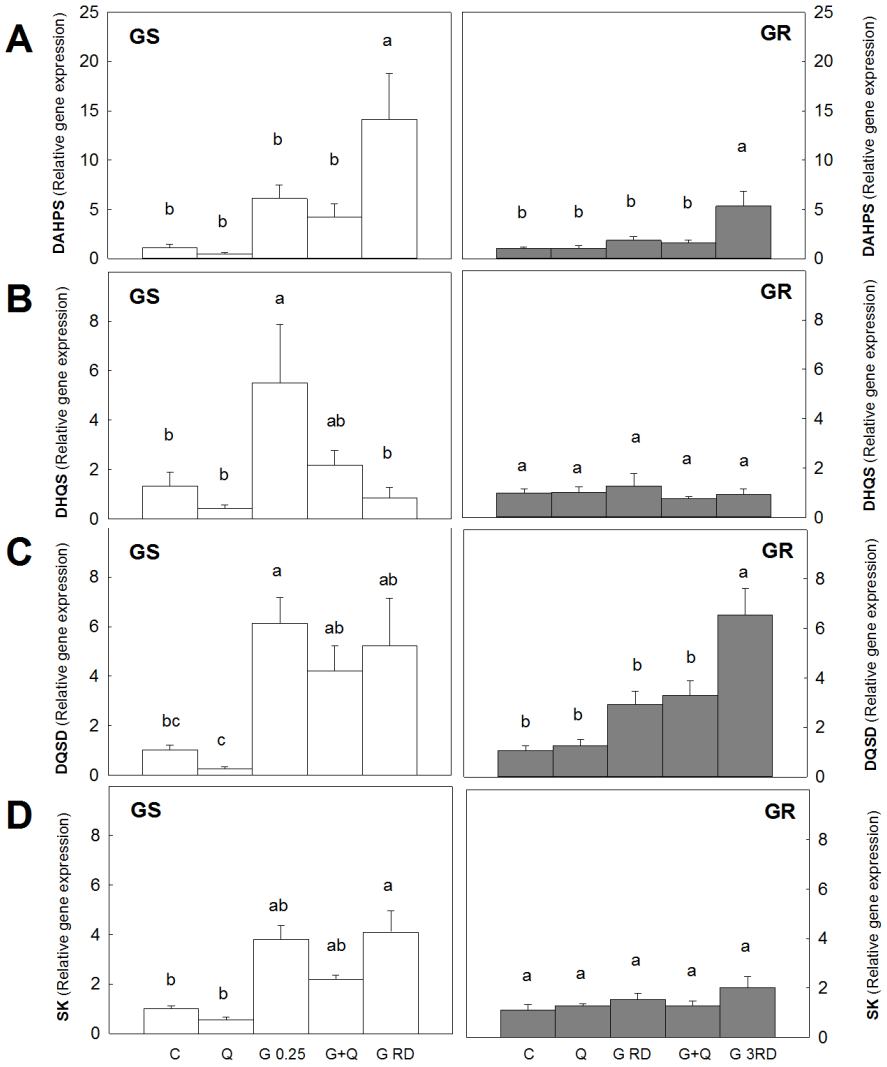

Figure 2.9. Transcript abundance of genes in the pre-EPSPS aromatic amino acid (AAA) biosynthetic pathway was measured in *Amaranthus palmeri* plants. Glyphosate-sensitive (white bars, left; GS) plants were untreated (C) or treated with quinate (Q), glyphosate 0.25RD, RD (recommended dose) or with 0.25RD and quinate (G+Q). Glyphosate-resistant (gray bars, right; GR) plants were untreated (Control) or treated with quinate, glyphosate RD, 3RD or with RD and quinate (G+Q). Relative transcript abundance was normalized using the normalization gene *beta tubulin* and each population to its own daily control of 3-Deoxy-d-arabino-heptulosonate-7-phosphate synthase (*DAHPS*; A), dehydroquinate synthase (*DHQS*; B), 3-dehydroquinate dehydratase/shikimate dehydrogenase (*DQSD*; C), shikimate kinase (*SK*; D) (Mean ± SE; n=6). Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tukey).

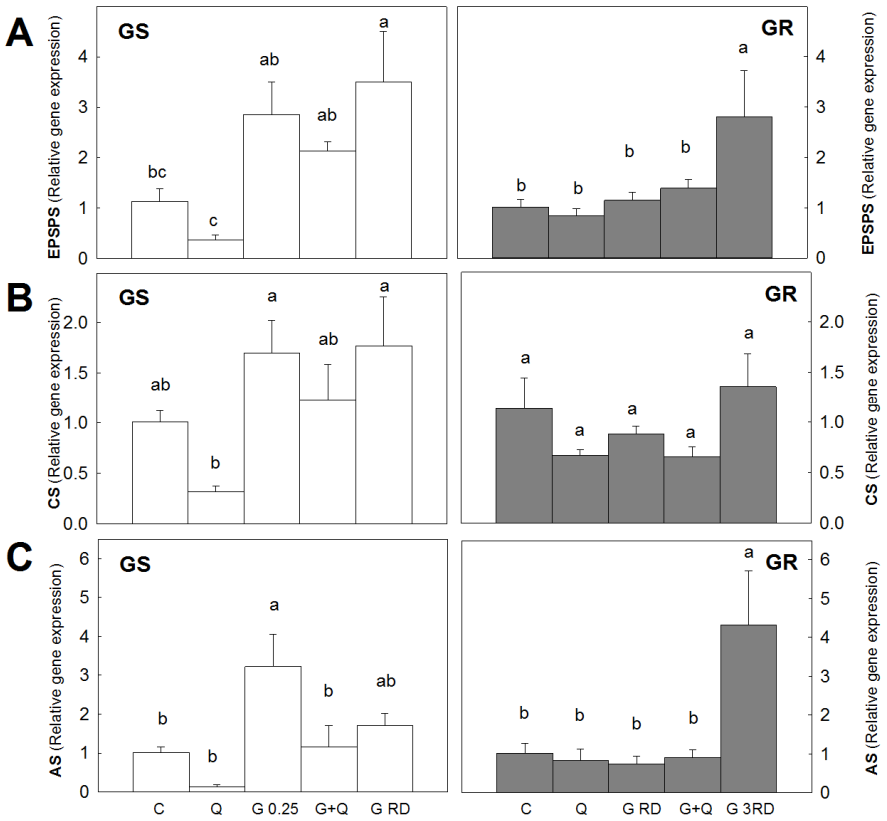

Figure 2.10. Transcript abundance of genes in the post-EPSPS aromatic amino acid (AAA) biosynthetic pathway was measured in *Amaranthus palmeri* plants. Glyphosate-sensitive (white bars, left; GS) plants were untreated (C) or treated with quinate (Q), glyphosate 0.25RD, RD (recommended dose) or with 0.25RD and quinate (G+Q). Glyphosate-resistant (gray bars, right; GR) plants were untreated (Control) or treated with quinate, glyphosate RD, 3RD or with RD and quinate (G+Q). Relative transcript abundance was normalized using the normalization gene *beta tubulin* and each population to its own daily control of 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*; A), chorismate synthase (*CS*; B), anthranilate synthase (*AS*; C) (Mean ± SE; n=6). Different letters within each population indicate significant differences between treatments (*p*-value≤0.05, Tukey).

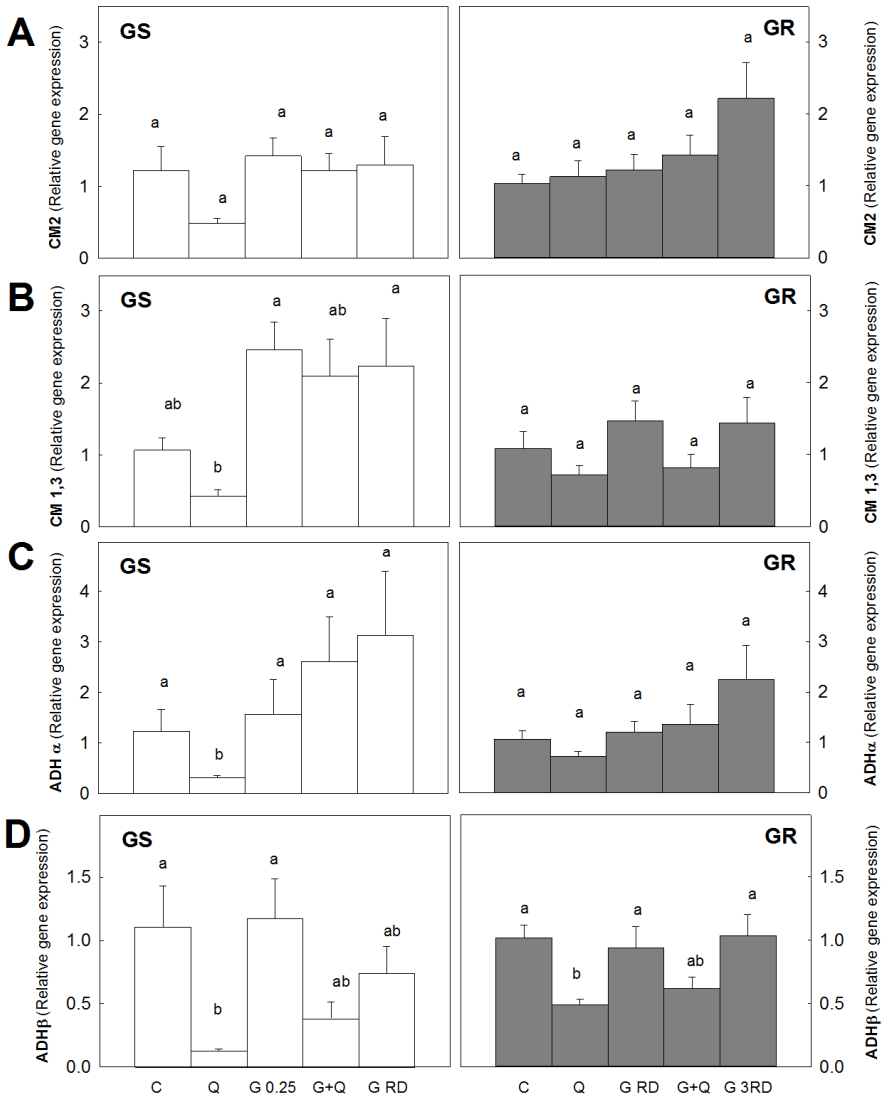

Figure 2.11. Transcript abundance of genes in the post-chorismate aromatic amino acid (AAA) biosynthetic pathway was measured in *Amaranthus palmeri* plants. Glyphosate-sensitive (white bars, left; GS) plants were untreated (C) or treated with quinate (Q), glyphosate 0.25RD, RD (recommended dose) or with 0.25RD and quinate (G+Q). Glyphosate-resistant (gray bars, right; GR) plants were untreated (Control) or treated with quinate, glyphosate RD, 3RD or with RD and quinate (G+Q). Relative transcript abundance was normalized using the normalization gene *beta tubulin* and each population to its own daily control of chorismate mutase isoform 2 (*CM2*; A), chorismate mutase isoforms 1 and 3 (*CM1-3*; B), arogenate dehydrogenase isoform α (*ADHα*; C) and arogenate dehydrogenase isoform β (*ADHβ*; D) (Mean ± SE; n=6). Different letters within each population indicate significant differences between treatments (*p*-value≤0.05, Tukey).

2.4. DISCUSSION

2.4.1. Combined treatment provokes similar visual effects as the higher dose of glyphosate

Plants of GS and GR populations of *A. palmeri* were treated with quinate, a sub-lethal dose of glyphosate or the combination of both compounds, and a high glyphosate dose. According with the results obtained in Chapter 1 (Figures 1.9 and 1.10), where the toxicity of the sub-lethal dose of the herbicide was increased in GS if quinate was applied 24 h after, the compounds were applied with the same lapse of time.

Two new high doses of glyphosate, RD and 3 RD, were applied additionally in GS and GR, respectively (Figure 2.2). These two new treatment were included in order to compare the results of the combined treatment with an herbicide treatment with similar visual effects. The high glyphosate doses were determined for each population based on previous studies (Fernández-Escalada et al. 2016) , according to the dose-response effects on shoot biomass and shikimate content.

The sub-lethal doses applied in GS and GR plants (0.25 RD and RD respectively) provoked growth arrest but were not lethal. As described in Chapter 1, the same sub-lethal dose applied in combination with quinate was lethal in GS plants (Figure 2.2.A) and increased the toxicity in GR plants (Figure 2.2.B). The visual appearance of the plants treated with the combined treatment were similar to the plants treated with the highest dose of glyphosate in both populations (Figure 2.2). Moreover, in GS population death was caused at the same period (7 days) after the combined treatment and the highest herbicide dose.

2.4.2. The content of quinate derivatives increases when quinate is applied after glyphosate in GS population

Quinate is a secondary metabolite formed in a secondary branch from the shikimate pathway, very closely related to shikimate (Boudet 2012). It has been reported that quinate is accumulated in plants after glyphosate treatment (Orcaray et al. 2010; Barroso et al. 2018), showing that quinate accumulation is an important physiological effect in the mode of action of the herbicide glyphosate. The accumulation of quinate content observed in GS plants after applying the highest (lethal) dose of herbicide (Figure 2.3) confirms the results

obtained previously in other species (Orcaray et al. 2010; Barroso et al. 2018) and the role of quinate accumulation after lethal glyphosate doses.

Four different quinate derivatives were detected and quinate and quinate-derivatives were accumulated in both populations after quinate treatment (alone or combined treatments) (Figures 2.3 and 2.4). Quinate, quinate-derivative 2, 3 and 4 were significantly more accumulated when quinate was combined with glyphosate in GS plants (Figures 2.3, 2.4.B, C and D) and such pattern was more intense for quinate derivatives 3 and 4 (Figure 2.4.C and D). The EPSPS inhibition by glyphosate in the sensitive plants before the supply of quinate 24 h after would minimize quinate incorporation through the shikimate pathway and would lead to the metabolization of the compound through a lateral branch of the shikimate pathway, increasing the quinate and its derivative content. Then, this accumulation could be related with the increased toxicity of this treatment in which GS plants treated died 7 days after treatment application (Figure 1.9 and 2.2.A). In the case of RD, the high glyphosate dose applied in GS, it also provoked the death of the plants, but only quinate and no quinate derivatives was accumulated, suggesting that lethality would be related to different or additional causes in the case of the combined treatment and in the highest dose.

In GR population, no significant differences in the quinate and quinate-derivatives accumulation were detected after quinate alone or combined. The EPSPS overexpression of the GR population would cover the exacerbation on quinate and 2, 3 and 4 derivatives in this population, and the differential accumulation detected in GS was not detected in this population (Figure 2.4), confirming that the accumulation after the combined treatment in GS is due to the EPSPS inhibition. So, the suggested blockage of the shikimate pathway and later accumulation of quinate and its derivatives after fueling the pathway with quinate were not detected in this population mainly because EPSPS is overexpressed.

2.4.3. The inhibition of EPSPS provokes a dose dependent accumulation of the metabolites upstream the enzyme

It has been previously described that shikimate is accumulated after glyphosate treatment due to the EPSPS inhibition (Lydon and Duke 1988; Becerril et al. 1989; Hernandez et al. 1999; Orcaray et al. 2010, 2012; Fernández-Escalada et al. 2016), and it is usually used to differentiate sensitive from resistant plants (Koger et al. 2005). Shikimate accumulation was dose dependent in GS population and only detected with the highest dose in the GR population (Figure 2.5.A), due to

the level of inhibition of the EPSPS enzyme after each dose applied. The other two metabolites detected upstream EPSPS (Figure 2.5.A and C) and gallic acid (Figure 2.6) showed the same pattern than shikimate content in both populations. The accumulation of the metabolites upstream EPSPS was more intense in GS than in GR due to the less sensitivity to glyphosate because of EPSPS overexpression. Interestingly, accumulation of intermediates upstream EPSPS in the GS population was similar after the sub lethal dose alone or combined with quinate and was much higher after the highest dose, suggesting that the accumulation provoked is glyphosate dose-dependent. However, the toxicity observed after the combined or RD treatment was similar, proposing that toxicity after each treatment would be mediated by different/additional physiological effects to metabolites upstream accumulation.

Gallic acid is a 3-dehydroshikimate derivative and it has been widely reported an accumulation of this metabolite after glyphosate treatments (Lydon and Duke 1988; Becerril et al. 1989; Hernandez et al. 1999; de María et al. 2006; Zabalza et al. 2017). An important increase was detected in this study in both populations after the treatment with the highest dose of glyphosate, especially in GS population, where the gallic acid content increased up to 7 fold while no changes were detected after other treatments (Figure 2.6). The inhibition of the EPSPS by glyphosate would redirect the carbon flux though lateral branches of the shikimate pathway upstream the enzyme, such as the synthesis of gallic acid.

The 3-dehydroshikimate and S3P (Figure 2.5. A, C) are not easily detectable intermediate compounds, and interestingly they were also accumulated after glyphosate treatment. An increase in 3-dehydroshykimate was previously detected in *Amaranthus palmeri* GS plants after glyphosate treatment (Maroli et al. 2015), and confirmed in this study. Although the accumulation of shikimate is the most common marker after glyphosate treatment, S3P is the substrate of the EPSPS enzyme, so the inhibition of EPSPS enzyme also provokes an increase in the S3P content (Siehl 1997). The detection of this compound is not as easy as the detection of shikimate, due to the chemical properties that make S3P more unstable to be accumulated and/or detected than shikimate after glyphosate.

Anthranilate, a metabolite of the post-chorismate pathway, located downstream EPSPS enzyme, was not accumulated after glyphosate treatment, suggesting that intermediate accumulation in the GS population was restricted to metabolites located upstream EPSPS (Figure 2.5.D). In the GR population, a high increase in its content was observed after the lower glyphosate dose. Unfortunately, this anthranilate content increase can not be directly related to an upregulation of

the *AS* gene, because the induction of the *AS* relative gene expression was only detected after the highest dose, and not after the lower dose (Figure 2.10.C).

Tyr accumulation was detected after both doses of glyphosate and Phe accumulation was detected only after the highest dose of glyphosate in the GS population (Figure 2.7), and pattern was not modified in the combined treatment. AAAs accumulation after glyphosate in sensitive plants has been reported before (Orcaray et al. 2010; Zulet et al. 2013a; Fernández-Escalada et al. 2016) and it has been related to an increased turnover rate of the existing proteins. The AAA content in GR plants was largely unaffected by glyphosate treatment.

2.4.4. Non lethal doses of glyphosate induce salicylic acid accumulation

Plant phenolic secondary metabolites and their precursors are synthesized via shikimate pathway and its numerous branch points (Tohge et al. 2013a, b). Many of these compounds play important roles in plant defense against different stresses and stress interactions (Maeda and Dudareva 2012). In this study, no significant differences were observed after treatments in the phenylpropanoid content downstream EPSPS with the exception of salicylic acid after glyphosate treatment.

The effect of glyphosate treatment in phenylpropanoid content is not clear, as both accumulation or no effect have been reported. Several studies have described that glyphosate treatment causes an accumulation of some hydorxybenzoic acids in plants (Lydon and Duke 1988; Becerril et al. 1989; Hernandez et al. 1999; de María et al. 2006). Indeed, it was reported the specific increase of two metabolites included in this study, caffeic and vanillic acids (Zabalza et al. 2017). On the contrary, results previously reported in other *A. palmeri* population (Maroli et al. 2015) showed no differences in phenylpropanoid content after glyphosate treatment. In concordance with the latter, no differences were observed in this study after glyphosate treatment in the content of the detected phenylpropanoids (Figure 2.8), supporting that the herbicide has no effect on secondary metabolites synthesized from AAAs.

The only change detected was in the content of salicylic acid, where an increase was detected after the lower dose of glyphosate in GS population plants and after the higher in GR population. Salicylic acid is a molecule that plays an important role in controlling plant growth and development after numerous biotic and

abiotic stresses (Hayat et al. 2010; Aldesuquy 2015). The increase of salicylic acid content observed after glyphosate treatment in this study would be a defense response to herbicide stress. On one hand, glyphosate has been described to provoke oxidative stress (Ahsan et al. 2008; Miteva et al. 2010). On the other hand, salicylic acid content has been related with the oxidative stress (Pan et al. 2017) and would boost the activities of the antioxidant enzymes (Kaya and Yigit 2014). Indeed, salicylic acid applied exogenously alleviated the oxidative stress after paraquat treatment (Strobel and Kuc 1995). In this study, it can be suggested that salicylic acid accumulation could be related with the changes in the oxidative status elicited by the glyphosate. This response would be independent of the EPSPS overexpression and elicited only after sub lethal doses of the herbicide, such as the lower dose of glyphosate in GS and the 3 RD in GR population. A previous report (Maroli et al. 2015) showed a stronger antioxidant status in another *A. palmeri* GR population compared to sensitive plants, so it would be necessary a highest dose of glyphosate to induce the oxidative-mediated salicylic acid accumulation in GR population than in GS population.

2.4.5. Glyphosate upregulates the expression of pre-chorismate AAA biosynthetic pathway in both populations but it is only downregulated by quinate in the GS population

Exogenous quinate application provoked a decrease in all the relative transcript level evaluated of the shikimate pathway only in GS population (Figures 2.8, 2.9 and 2.10), as described in Chapter 1 (Figures 1.5, 1.6 and 1.7). As discussed in Chapter 1 (Section 1.4.1.), the transient accumulation of the final products of the pathway, Tyr and Phe, one day after quinate treatment would act as a signal to reduce the expression of the genes of the pathway one day after.

The exposure of plants to various stresses generally induces the expression of genes encoding shikimate pathway and AAA metabolism enzymes (Tzin and Galili 2010a). It has been previously described that glyphosate treatment provokes an accumulation of the transcripts of the enzymes of the shikimate pathway (Baerson et al. 2002; Yuan et al. 2002; Chen et al. 2015b; Mao et al. 2016; Fernández-Escalada et al. 2017). Previous studies with these GS and GR populations of *A. palmeri* described that the gene induction after glyphosate treatment was observed in both populations, suggesting that the induction would be provoked independently of EPSPS overexpression (Fernández-Escalada et al. 2017). These previous results match with the results obtained in this study, where glyphosate provoked a general dose-response increase in the gene transcription of the pre-chorismate pathway and *AS* gene in both populations.

Reduction in AAA levels does not appear to elicit the increased relative gene expression of AAA pathway genes, because Tyr and Phe levels were not affected or increased by glyphosate in GR and GS populations, respectively.

To the author knowledge, this is the first study reporting an upregulation of the genes located before chorismate in the pathway and *AS* after a sub-lethal glyphosate does in GS population. Moreover, the results obtained in this study suggest that of most the genes of the pathway showed no dose-dependent upregulation after glyphosate treatment, while *DAHPS* gene upregulation was dose-dependent. Relative gene expression of the shikimate pathway would be a very sensitive marker, with significant changes detected even though the treatment was not lethal.

In GS population, the relative gene expression of *DHQS* gene showed a different pattern than the rest of the genes evaluated, with a relative transcript level after RD, the highest glyphosate dose, similar to control values (Figure 2.9.B). Interestingly, this was the only treatment that caused quinate accumulation (Figure 2.3), suggesting a feedback regulation on the expression of the enzyme, as accumulated quinate would abolish the potential effect of the RD in the relative *DHQS* expression due to an increase of the product of the enzyme (Figure 2.9.B).

In GR plants it was detected an increase in the relative gene expression of *DAHPS*, *EPSPS*, *DQSD* and *AS* after the highest glyphosate dose, 3 RD (Figure 2.9 and 2.10), as it has been reported before (Fernández-Escalada et al. 2017). The most sensitive enzyme expression to glyphosate in GR was *AS* (Figure 2.10), result also observed previously (Fernández-Escalada et al. 2017).

Induction of gene expression after glyphosate treatment was restricted to enzymes located upstream chorismate and to AS enzyme after chorismate, as the expression of *CM* and *ADH* was not affected by the presence of the herbicide in any of the populations. Glyphosate has been described to provoke a preferential carbon flux through the Trp biosynthetic pathway (Fernández-Escalada et al. 2017), and this preference would be affecting more that part of the pathway, not modifying the relative gene expression of *CM* nor *ADH,* genes belonging the Tyr and Phe biosynthetic pathway.

In GS population, the pattern of the gene expression after the combined treatment was a mix of the effects of each independent treatment. Expression was upregulated in the presence of glyphosate and downregulated with quinate, so after the combined treatment effects were counteracted and relative

expression level after the combined treatment was in most cases similar to control values (Figures 2.9, 2.10 and 2.11). In the GR population, no changes in mRNA levels were detected after the compounds applied alone or combined.

2.4.6. Lethality caused in GS population by the high dose of glyphosate and the low dose combined with quinate are mediated by different patterns of the shikimate pathway

In GS population, the relative gene expression after glyphosate treatment was more sensitive than the metabolite content, because after the lower glyphosate significant differences were observed in the expression of all the genes of the shikimate pathway located before chorismate and *AS* while no effects on metabolite content was reported. Significant differences in metabolite content were only detected after the highest glyphosate dose.

The combination of the sub-lethal glyphosate dose with quinate, G+Q, provoked an enhancement of glyphosate toxicity that caused the death of the plants in GS in the same period of time than the high dose applied. Although both treatments were lethal, it was not possible to describe a common pattern of the metabolites or gene expression level of the shikimate pathway that would explain the common toxicity.

On one hand, the results obtained in this experiment suggest that the increase in the toxicity observed in the combined treatment would be explained by the significantly increase in quinate and quinate-derivative content, as it was the most remarkable difference between the low dose of glyphosate and its combination with quinate. There were no significantly changes neither in phenylpropanoid content nor relative mRNA level that would explain the enhanced toxicity. On the other hand, an important upregulation of the relative mRNA content of the genes of the shikimate pathway and accumulation of quinate and metabolites upstream EPSPS were detected in the GS plants treated with the highest dose of glyphosate, suggesting that the death of the plants belonging this treatment was caused by different/additional causes of the combined treatment. These results suggest that there would not be a clear pattern of the lethality, and it would be related to different metabolic disturbances.

2.4.7. No significant metabolic perturbations of the shikimate pathway were detected in the GR population after the combined treatment

Although in Chapter 1 it was suggested an altered shikimate pathway in GR plants treated with the combination of quinate of glyphosate, the exhaustive study of the pattern of the shikimate pathway performed in this chapter reveals no significant metabolic perturbations. GR plants treated with quinate, the RD of glyphosate or their combination showed no changes on the metabolite content nor on gene expression. Changes in the shikimate pathway were detected only after the treatment with the highest (but not lethal) dose of glyphosate (3DR). After this treatment, upregulation of shikimate pathway genes and accumulation of metabolites upstream *EPSPS* were detected although the intensity of the effects was lower than in the sensitive population. The detection of these previously well-known effects of the herbicide evidences that these changes are dose-response representative effects of the herbicide, that occur independently of the EPSPS overexpression of the GR population.

2.5. CONCLUSIONS

-In GS population, the RD provoked quinate accumulation, effect previously described in other species. Glyphosate treatment also provoked a dose-dependent accumulation of the metabolites upstream EPSPS in both populations.

-Salicylic acid was the only phenylpropanoid accumulated after sub lethal doses of glyphosate in both populations. Salicylic acid signaling could be related with possible changes in the oxidative status, as has been reported in other species treated with glyphosate.

-Glyphosate treatment caused an upregulation of the relative gene expression of the genes of the AAA biosynthetic pathway genes before chorismate and *AS* in GS even after the lowest dose applied, while in the GR population it was only detected an upregulation of *DAHPS*, *DQSD*, *EPSPS* and *AS* after the highest herbicide dose. *CM* and *ADH* relative gene expression were not affected by the herbicide.

-The combined treatment provoked a higher increase of quinate and quinate derivative content than glyphosate alone in GS population, suggesting that the increase of the phytotoxicity in the combined treatment would be related to a higher carbon flux to quinate and its derivatives. The exacerbation of the phytotoxicity of the combined treatment was not related to any remarkable change in the relative gene expression, where the effect was a mix between the decrease induced by quinate alone and the increase induced by glyphosate alone.

-The shikimate pathway after treatment with the lethal dose of glyphosate showed in GS plants an important increase in the relative mRNA transcript level and accumulation of metabolites upstream EPSPS and quinate. The lethality caused in GS by the RD and the combined treatment were provoked by additional/different causes, because that it was not possible to detect a common pattern of the shikimate pathway after the two lethal treatments.

- The shikimate pathway of the GR population was not altered after the treatment with the low dose of glyphosate, quinate or their combination. Nevertheless, GR plants treated with the highest dose of glyphosate showed the previously well-known effects on f the shikimate pathway.

CHAPTER 3

Metabolomic profiling of glyphosate-sensitive and resistant plants treated with glyphosate and/or quinate

3.1. OBJECTIVES

Metabolomics is a functional tool that has been previously successfully applied to describe the mode of action of herbicides and to characterize physiological responses after certain herbicide treatment, by drawing a general overview of the metabolic consequences of the application of the herbicides.

In chapter 1, it was stablished an increase of the glyphosate efficacy after its combination with exogenous quinate and in Chapter 2 the physiological reasons of such efficacy enhancement were studied at the level of shikimate pathway, because it is the pathway specifically affected by glyphosate and quinate. In order to study the physiology beyond shikimate pathway, metabolomics seems to be an attractive tool to obtain a general overview of the physiology of treated plants.

The aim of this chapter was **to characterize the metabolic profiling of sensitive and resistant populations of *Amaranthus palmeri* after applying quinate, glyphosate or their combination.** To this end, a non-targeted metabolomic profiling was performed by GC-MS and LC-MS of *A. palmeri* plants treated both compounds applied individually and the combined treatment. Relative intensity of GC-MS data and LC-MS data were used to determine the pattern of metabolites from AAA biosynthetic pathway, primary metabolism, phenylpropanoid and flavonoid biosynthetic pathways. Metabolome comparison was approached by:

-Metabolic comparison between untreated plants of both populations.

- Comparing the effect of glyphosate on the metabolic profile of both populations by comparing the pattern of plants treated with two different doses of glyphosate, one of them sub-lethal and a high one

-In order to identify a metabolic profile that would explain the increased efficacy of the combined treatment, the metabolome of plants treated with quinate, the sub-lethal glyphosate dose and the combined treatment was studied.

3.2. EXPERIMENTAL APPROACH

Plants of two populations (GS and GR) of *Amaranthus palmeri* were treated with quinate and/or glyphosate, as performed in Chapter 2. An additional treatment containing the sub lethal dose of glyphosate and the surfactant used in the control and quinate treatments was applied (Glyphosate sub lethal + Biopower) was applied in this Chapter. This new treatment was included in order to ensure the control treatment in the study with the combined treatment, to focus on the metabolite changes elicited by quinate and glyphosate and no by the surfactant.

Figure 3.1. Overview of the treatment application and harvest in glyphosate sensitive (GS) and resistant (GR) *Amaranthus palmeri* plants in experiment 3. Plants were treated with the surfactant (Control), quinate, or with two doses of glyphosate (sub lethal or high dose), and an additional treatment of the sub lethal dose with surfactant. Plants were harvested 3 days after the treatment application.

Leaf samples were harvested 3 days after glyphosate application (Figure 3.1) and immediately frozen in liquid nitrogen and stored at -80 °C for analytical determinations. The experiment was repeated twice.

Table 3.1. Overview of the glyphosate treatments applied in Chapter 3 to two populations of *Amaranthus palmeri* plants, glyphosate sensitive (GS) and glyphosate resistant (GR). RD = Recommended field dose (Culpepper et al. 2008)

		Amaranthus palmeri	
		GS	***GR***
Glyphosate	Sub lethal	0.25 RD = 0.21 kg ha^{-1}	RD = 0.84 kg ha^{-1}
	High dose	RD = 0.84 kg ha^{-1}	3 RD = 2.52 kg ha^{-1}

3.2.1. Analytical determinations

A non-targeted metabolic profiling was performed in the Leibniz Institute for Plant Biochemistry in Halle (Saale), Germany. The analytical determinations performed in this chapter and the sections where they are included are indicated in the Table 3.2.

Table 3.2. Analytical determinations realized in Chapter 3.

Metabolomic profiling determination	
GC-MS	Section M.M.2.7
LC-MS	Section M.M.2.7

3.2.2. Statistical analysis

The normalized intensities of metabolites identified by GC-MS were analyzed by multivariate statistical analysis using MetaboAnalyst software (available in https://www.metaboanalyst.ca). The metabolomics data were auto scaled before multivariate analysis and only metabolites identified were used. First, differences between GC-MS values of untreated plants of both populations were evaluated using principal component analysis (PCA) and Student's t test ($p \leq 0.05$). Second, PCA was applied to study the effect of the herbicide and the effect of the combined treatment. In both cases, the top loadings of the principal components 1 and 2 were selected, and for these metabolites, differences between treatments within each population were evaluated performing one-way ANOVA with a multiple-comparison adjustment (Tuckey) at $p \leq 0.05$ with IBM SPSS Statistics. After that, hieratical cluster analysis of all the significantly different metabolites (ANOVA) were performed and visualized using heat maps, using Euclidean distance, of the metabolite responses to the treatments.

PCA and Student's t test ($p \leq 0.05$) were performed in metabolites identified in the positive mode of LC-MS to study the differences between untreated plants of both populations. For LC-MS identified metabolites, the effect of glyphosate and the combined treatment focused on phenylpropanoid and flavonoid biosynthetic pathway. In both cases, differences between treatments within each population were evaluated using one-way ANOVA with a multiple-comparison adjustment (Tuckey) at $p \leq 0.05$.

Analysis were performed using 6 biological replicates using samples from different individual plants.

3.3. RESULTS AND DISCUSSION

In this study, non-targeted metabolic profiling was performed in plants of *A. palmeri* untreated or treated with two doses of glyphosate, quinate and the combination of quinate with a sub lethal dose of glyphosate. GC-MS analysis positively identified more than 80 metabolites across all samples by their mass-spectral fingerprints and retention-index matches (Supplemental Table 1). Metabolites detected by GC-MS were analyzed by PCA. After identifying factor loadings in both populations, the more significant parameters were selected and they were subjected to separate one-way ANOVA analysis in each population. LC-MS detected around 8500 mass spectra features in positive mode (More than 4800 mass spectra features in negative mode –data not shown-), but positively identified around 250 (Supplemental Table 2). Among all metabolites identified in LC-MS, metabolites related to phenylpropanoid and flavonoid pathways were used in this study, in order to focus on the secondary metabolism pattern.

All data were used to compare the metabolic profile between untreated plants of both populations. Afterwards, two approaches were developed, on one hand to evaluate a possible dose-response of metabolites after glyphosate treatment and on the other to compare the metabolic profile of the combination of quinate and glyphosate with the individual treatments.

3.3.1. Metabolite profile of untreated plants of GS and GR populations

The availability of a biotype with overexpression of the EPSPS enzyme provides an opportunity to analyze how overexpression of ESPS affects metabolite content by comparison with a sensitive population. In order to perform a general comparison, PCA was performed with untreated plants (control), observing that both populations were indistinct from the other, in both GC-MS (Figure 3.2) and LC-MS identified metabolites (Figure 3.3).

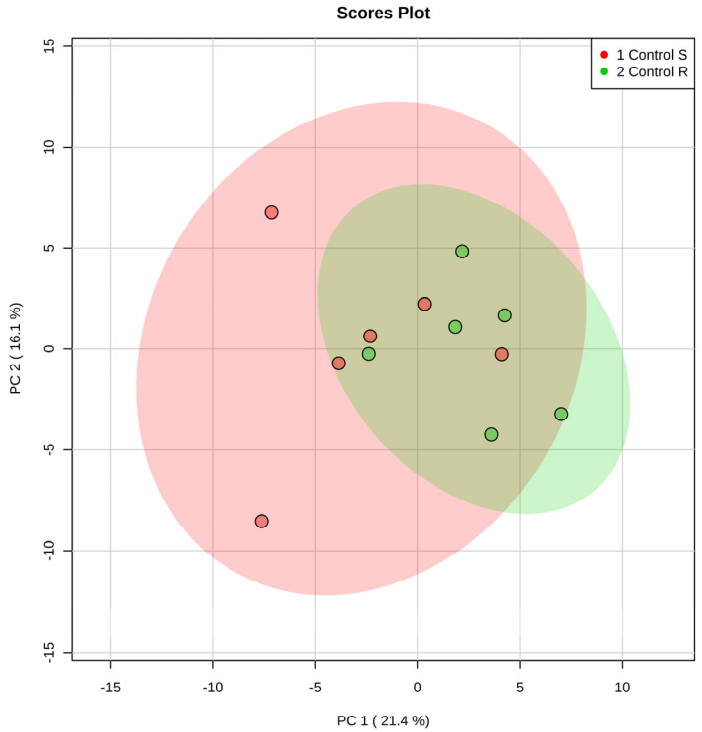

Figure 3.2. Principal component analysis (PCA) of metabolites detected by GC-MS in untreated (control) glyphosate-sensitive (GS; red) and glyphosate-resistant (GR; green) *Amaranthus palmeri* plants.

PC1 and PC2 explained about 37.5% of the variance in GC-MS metabolites and about 32% in LC-MS metabolites. None of the metabolites detected by GS-MS or LC-MS showed significant differences after t-Test (*p*-value ≤0.05). Thus, those results suggest that the two populations present similar characteristics at metabolome level, which implies that pleiotropic effects due to shikimate pathway perturbation are not apparent. The lack of pleiotropic effects on shikimate pathway was reported before for these two populations (Fernández-Escalada et al. 2017), but the results of this study broadens the lack of pleiotropic effects beyond shikimate pathway to metabolite profile. Moreover, untreated glyphosate-sensitive and resistant *A. palmeri* plants were very closely associated in previous reports after performing GC-MS analysis (Maroli et al. 2015). These results suggest that the overexpression of the EPSPS in GR population would not affect the metabolite pool, and that under control conditions the increase in EPSPS protein would not increase the carbon flux through the pathway.

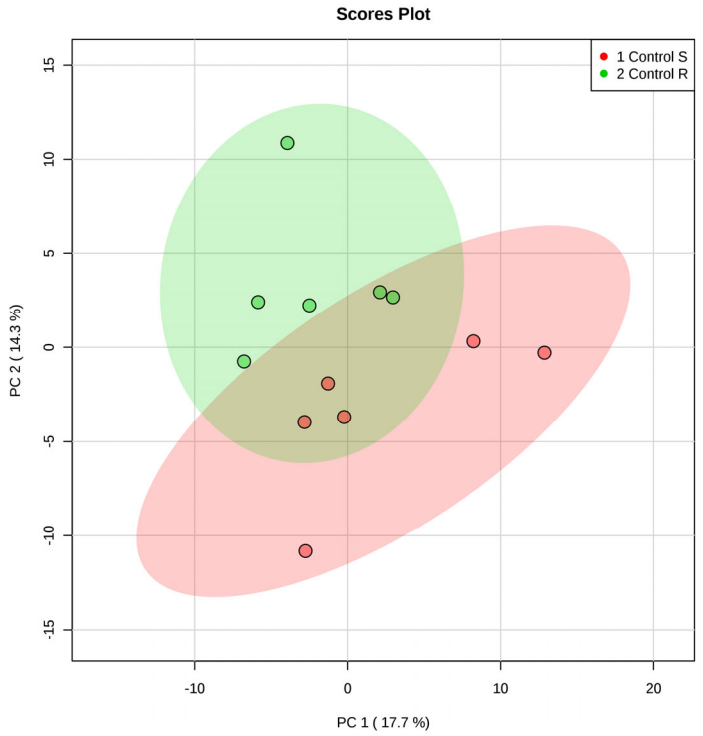

Figure 3.3. Principal component analysis (PCA) of metabolites detected by LC-MS in Control glyphosate-sensitive (GS; red) and glyphosate-resistant (GR; green) *Amaranthus palmeri* plants.

3.3.2. Effect of glyphosate treatment in metabolic profile

3.3.2.1. General overview and most significant metabolites detected by GC-MS

PCA was performed within each population (Figure 3.4) using the metabolites positively identified by GC-MS after treated with two different doses of glyphosate. In GS population, the doses were the sub lethal 0.25 RD and the lethal RD while GR population was treated with two sub-lethal doses (RD and 3RD).

In GS population, different glyphosate doses showed a clear discrimination from one another when the plot of component 1 was represented against component 2 (Figure 3.4.A). The component 1 seems to be related to the effect of the herbicide toxicity, and clearly discriminates the highest and lethal glyphosate dose, RD, from the other non-lethal treatments. The component 2 clearly

discriminates the presence or absence of glyphosate in each treatment, showing a clear separation between control treatment and the treatments of the two glyphosate doses.

In contrast, in GR population different treatments did not show a clear discrimination between them (Figure 3.4.B). The plot of component 1 against component 2 showed very closely the treatments Control and the lower glyphosate dose, RD. The highest glyphosate dose applied in this population neither showed a clear discrimination from the other two treatments.

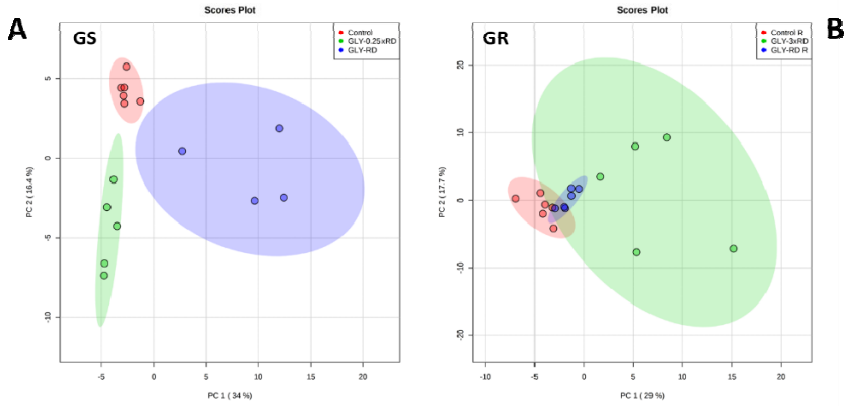

Figure 3.4. Principal component analysis (PCA) of metabolites detected by GC-MS in glyphosate-sensitive (GS; A; left) and glyphosate-resistant (GR; B; right) *Amaranthus palmeri* plants. Plants were untreated (control; red) or treated with glyphosate in two different doses: GS population with 0.25 RD (recommended dose) (green) or RD (blue); GR population with RD (blue) or 3 RD (green).

After identifying factor loadings in both populations, the metabolites more significant were selected to perform a more exhaustive analysis. In GS population, the pattern of selected metabolites after glyphosate treatments is shown in Figures 3.5 and 3.7 and are classified according to their relation with the shikimate pathway (shikimate pathway, quinate derivatives, AAAs or phenylpropanoids) and their characteristics: amino acids, fatty acids, carbohydrates or Krebs cycle´s metabolites. Interestingly, in GR some of the top loadings of PC1 were similar to the ones obtained for GS population: shikimate, S3P, dehydroshikimate and β-alanine; in addition to threonine. Similar to GS, aspartate and tyr were also principal loadings of PC2, including also lysine and malate. The pattern of these representative metabolites in GR population is shown in Figures 3.6 and 3.8.

In GS population, the detected metabolites directly related to the shikimate pathway were located upstream EPSPS (Figure 3.5.A) and showed a dose-

dependent response, evidencing the highest increase after the highest glyphosate dose, RD. Actually, in general, the sub-lethal glyphosate dose did not provoke this accumulation in those metabolites, and the detection level was significantly similar to control plants. Three of the four quinate derivatives detected (1-3; Figure 3.5.A) did not show a dose-dependent pattern. In fact, one of them, quinate derivative 1, was not detectable after those treatments by GC-MS method.

AAAs detected, tyr and phe, (Figure 3.5.B.1), as well as many AAs detected, also showed the same dose-dependent pattern observed in the metabolites upstream EPSPS of the shikimate pathway. For example, Val, Leu (BCAAs), β-alanine, asparagine, glutamine isoform 1, threonine, lysine and serine (Figure 3.5.B.2). Only the sinapic acid derivative showed an increase after the RD (Figure 3.5.C). Two derivatives of sinapic acid and benzoate were also accumulated in sensitive plants after the lethal dose of glyphosate. Primary metabolites such as carbohydrates, fatty acids and metabolites belonging to the Krebs cycle were also affected after glyphosate treatment in GS population (Figure 3.7). Carbohydrates myo-inositol, fructose (isoform 1) and glucose (isoforms 1 and 2) relative content were increased after glyphosate treatment. (Figure 3.7.A). Malate, succinate, citrate/isocitrate, and fumarate also were affected when glyphosate was applied (Figure 3.7.B). Interestingly, primary metabolism seem to be more affected after the sub-lethal glyphosate dose and not after the RD. It was detected a dose-response increase in the relative content of the stearic fatty acid (Figure 3.7.C).

In GR population, the effect of the herbicide was less intense than in GS. In this population, only the 3 RD of glyphosate provoked an increase in the relative content of some metabolites: Shikimate, S3P, 3-dehydroshikimate, quinate derivative 4 (Figure 3.6.A); the AAs valine, β-alanine, glutamine (isoform 2), and lysine (Figure 3.6.B.2); the carbohydrates xylitol, glucose (isoforms 1 and 2) (Figure 3.8.A); malate, citrate/isocitrate, glycerol-3-phosphate and fumarate (Figure 3.8.B) and the fatty acid stearic acid (Figure 3.8.C). However, the AAs asparagine, methionine and aspartate decreased their relative content when the doses increased (Figure 3.6.B.2.).

Previous studies have also reported changes in almost all the metabolites determined by GC-MS (Böttcher et al. 2008a, b; Trenkamp et al. 2009; Maroli et al. 2015). Moreover, as in this study, most of them show a dose response effect of the herbicide on the elicited changes (Böttcher et al. 2008a). The increase observed in the content metabolites of the shikimate pathway was higher in GS than in GR population. This effect has been previously observed in chapters 1 and

2 and widely reported before (Gaines et al. 2010; Maroli et al. 2015; Fernández-Escalada et al. 2016, 2017). The EPSPS overexpression reduces glyphosate effect in these GR plants, provoking a lower effect of the herbicide.

As expected, among the most highly affected components were the amino acids, in concordance with the known effects of the glyphosate. Increase in AA content have also been widely described after glyphosate treatment (Böttcher et al. 2008a; Zulet et al. 2013a; Fernández-Escalada et al. 2016; Zabalza et al. 2017). Böttcher et al. (2008a) also detected changes in all the AAs detected by metabolomics, with the exception of aspartate and serine. As observed in the metabolites related to the shikimate pathway, the increase provoked by the herbicide in AAs was dose dependent and higher in GS than in GR population. The observed accumulation of free amino acids in treated plants has been attributed to increased protein turnover (Zulet et al. 2013a) and, indeed, a decrease in the content of total soluble protein was recently reported in glyphosate-treated *A. palmeri* populations (Maroli et al. 2015).

The blockage of the shikimate pathway by the herbicide leads to many adverse secondary effects on other pathways and processes. Glyphosate can also affect carbon metabolism after the application of the treatment. The accumulation of the metabolites of the Krebs cycle was also previously observed after glyphosate treatment (Trenkamp et al. 2009; Maroli et al. 2015), confirming that primary metabolism is affected by the herbicide. In this study, quite similar pattern of sugar accumulation was provoked in both populations, as observed previously in the same populations for total soluble sugars and starch (Fernández-Escalada et al. 2016). It has been described that carbohydrates accumulation would be related to growth arrest, decreasing the carbohydrate transport trough the plant (Armendáriz et al. 2016). However, the fact that carbohydrate accumulation was detected in both populations after glyphosate treatment, suggests that this physiological response would be a secondary effect of the treatment. In plants, the energy producing stage is regulated by different mechanisms, and a step of the glycolysis is inhibited by PEP, of which glyphosate is the competitive inhibitor for binding EPSPS. It can be suggested that, in glyphosate treated plants, the PEP available level is higher, inhibiting the glycolysis process leading to the increase in sugars due to impaired carbon metabolism (Maroli et al. 2015).

The content of saturated stearic acid with 18 carbons (18:0) was increased in both populations, and the increase was dose-dependent. This result shows an effect of glyphosate on lipid composition, and suggest that this effect can be viewed as a physiological response to the stress induced by the herbicide. Indeed,

the response of membrane lipids to different abiotic stresses can be considered as crucial for improving plant acclimatization ability to different environmental adversities (Liu et al. 2019).

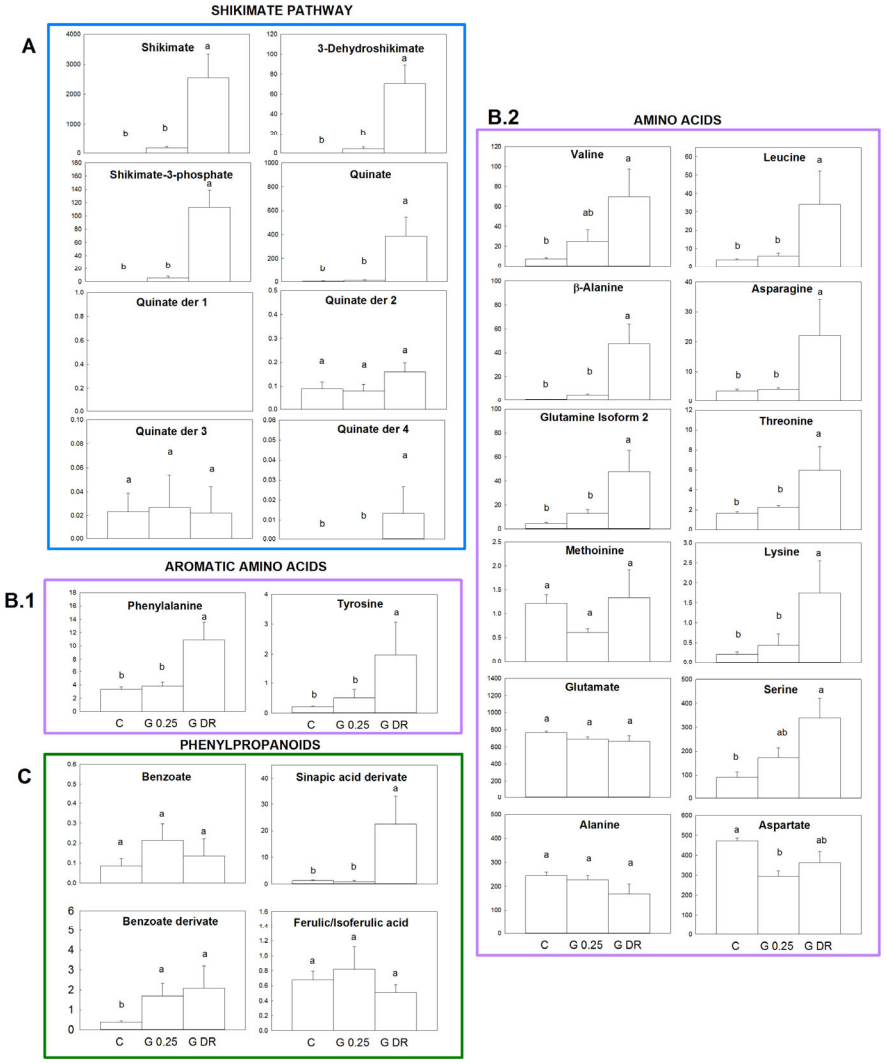

Figure 3.5. Principal metabolites detected by GC-MS in glyphosate-sensitive *Amaranthus palmeri* plants expressed as normalized peak intensity. Shikimate pathway (A; blue), aromatic amino acids (B.1, purple) and other amino acids (B.2; purple) and phenylpropanoids (C, green). Plants were untreated (control) or treated with glyphosate in two different doses: 0.25 RD (recommended dose) or RD. Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tuckey).

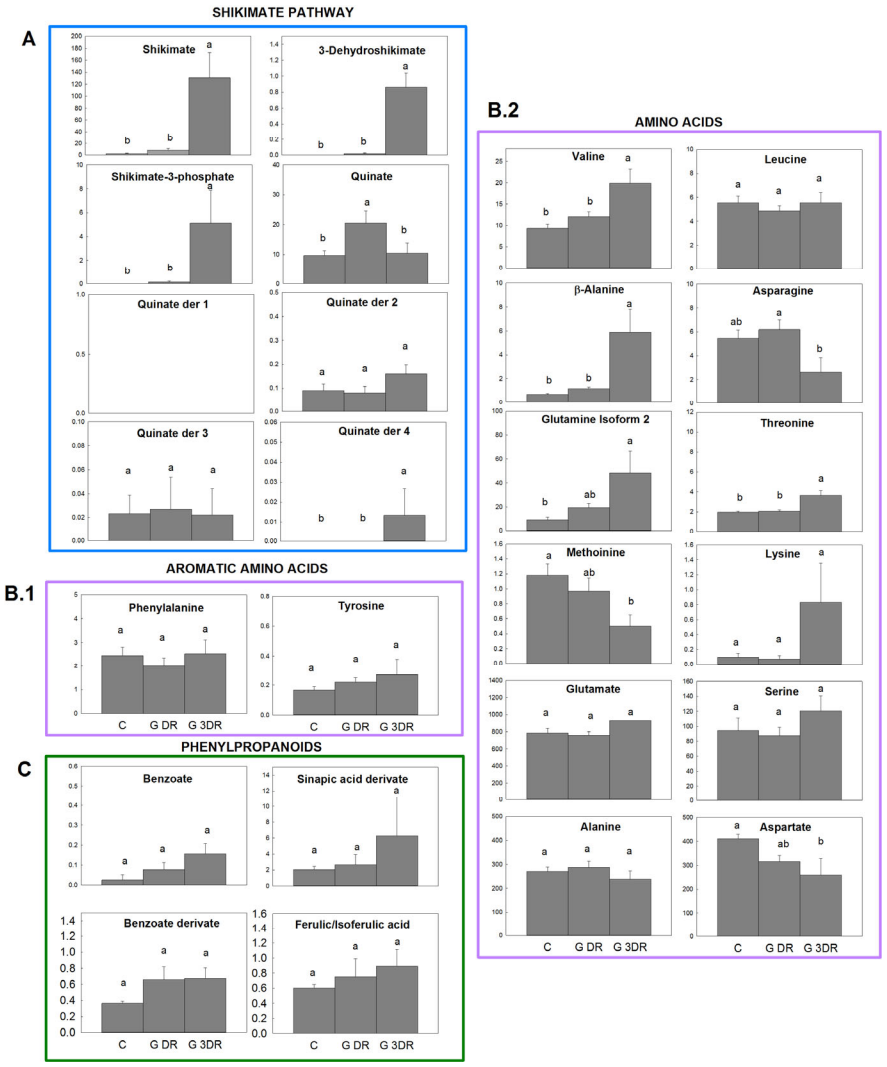

Figure 3.6. Principal metabolites detected by GC-MS in glyphosate-resistant *Amaranthus palmeri* plants expressed as normalized peak intensity. Shikimate pathway (A; blue), aromatic amino acids (B.1, purple) and other amino acids (B.2; purple) and phenylpropanoids (C, green). Plants were untreated (control) or treated with glyphosate in two different doses: RD (recommended dose) or 3 RD. Different letters within each population indicate significant differences between treatments (*p*-value≤0.05, Tuckey).

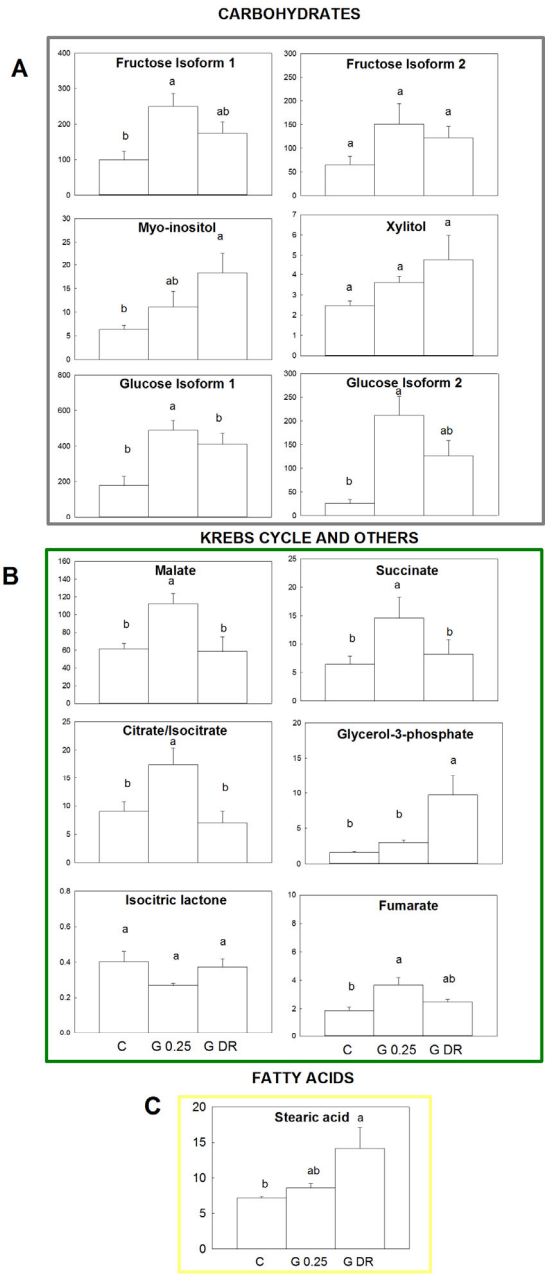

Figure 3.7. Principal metabolites detected by GC-MS in glyphosate-sensitive *Amaranthus palmeri* plants expressed as normalized peak intensity. Carbohydrates (A; grey), Krebs cycle and others (B; green) and fatty acids (C; yellow). Plants were untreated (control) or treated with glyphosate in two different doses: 0.25 RD (recommended dose) or RD. Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tuckey).

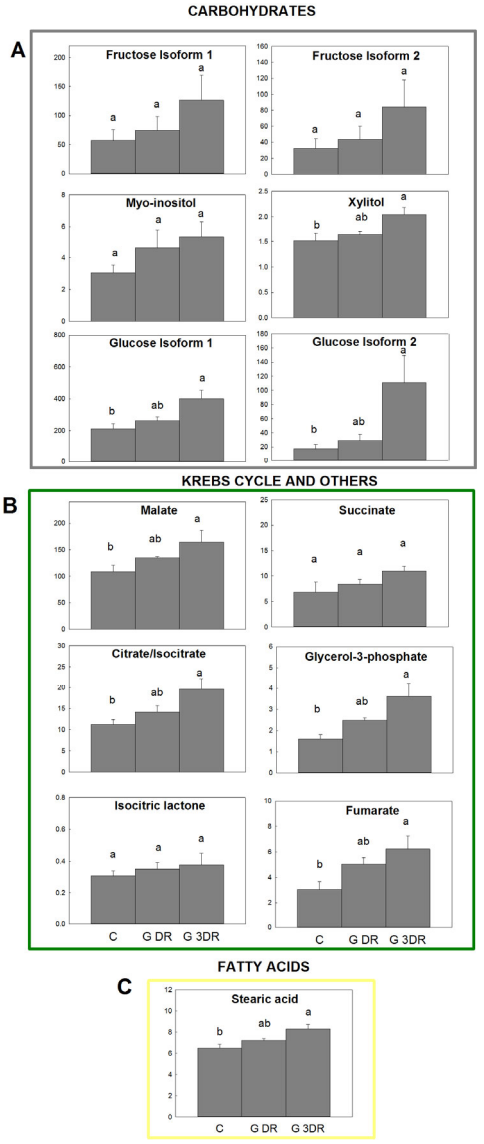

Figure 3.8. Principal metabolites detected by GC-MS in glyphosate-resistant *Amaranthus palmeri* plants expressed as normalized peak intensity. Carbohydrates (A; grey), Krebs cycle and others (B; green) and fatty acids (C; yellow). Plants were untreated (control) or treated with glyphosate in two different doses: RD (recommended dose) or 3 RD. Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tuckey).

A heat map with the metabolites that were signifincantly different in GS and GR after ANOVA (Tuckey) was perfomed (Figure 3.9). The 60 metabolites represented in the heat map were clustered and divided in 4 clusters according to their pattern affter the treatments. Heat map clearly showed differences between treatments.

The metabolites belonging to clusters 1 and 2 clearly showed a different pattern from metabolites of clusters 3 and 4. Metabolites that preferentially changed when no lethal dosis were applied, were included in clusters 1 and 2. Metabolites included in cluster 1, which included the two isoforms of glucose and fructose, and another two carbohydrates, were induced in 0.25DR in GS and 3RD in GR population. Also, cluster 2 metabolite content was increased in those two treatments, and also slightly increased in the lower dose in GR population. In this cluster, also was formed by carbohydrates and two amino acids: alanine and aspartate, suggesting that primary metabolism would be altered when the toxicity is not very high.

However, metabolites more intensely accumulated after the lethal glyphosate dose (RD in GS population) were clustered separately, and named as clusters 3 and 4. In this treatment, cluster 1 was also slightly affected, but the most remarkable effects were observed in clusters 3 and 4. Metabolites included in cluster 3 were metabolites related to the shikimate pathway: S3P, shikimate, dehydroshikimate and gallic acid. Cluster 4 is mainly constituted by AAs and carbohydrates.

The metabolite pools of DR treatment in GS population would be related to the lethality of the treatment, presenting the most altered metabolomic profile. The doses 0.25 RD in GS and 3 RD in GR provoked a very similar effec in metabolite pool, suggesting that the sublethal glyphosate doses would cause an effect in the primary and shikimate pathway metabolism.

3.3.2.2. Phenylpropanoid metabolites and metabolites of flavonoid bioynthetic pathways.

Plant phenolics are secondary metabolites, one of the most common substances in plants, are structurally diverse and exhibit a large and diverse array of biological properties. Besides a bulk of phenolic substances having cell wall structural roles, a great diversity of non-structural constitutents have been also found, having various roles as defending plants, establishing flower colour and contributing to certain flavours. The phenolic accumulation in plants tissues is considered a common adaptive response of plants to adverse environmental

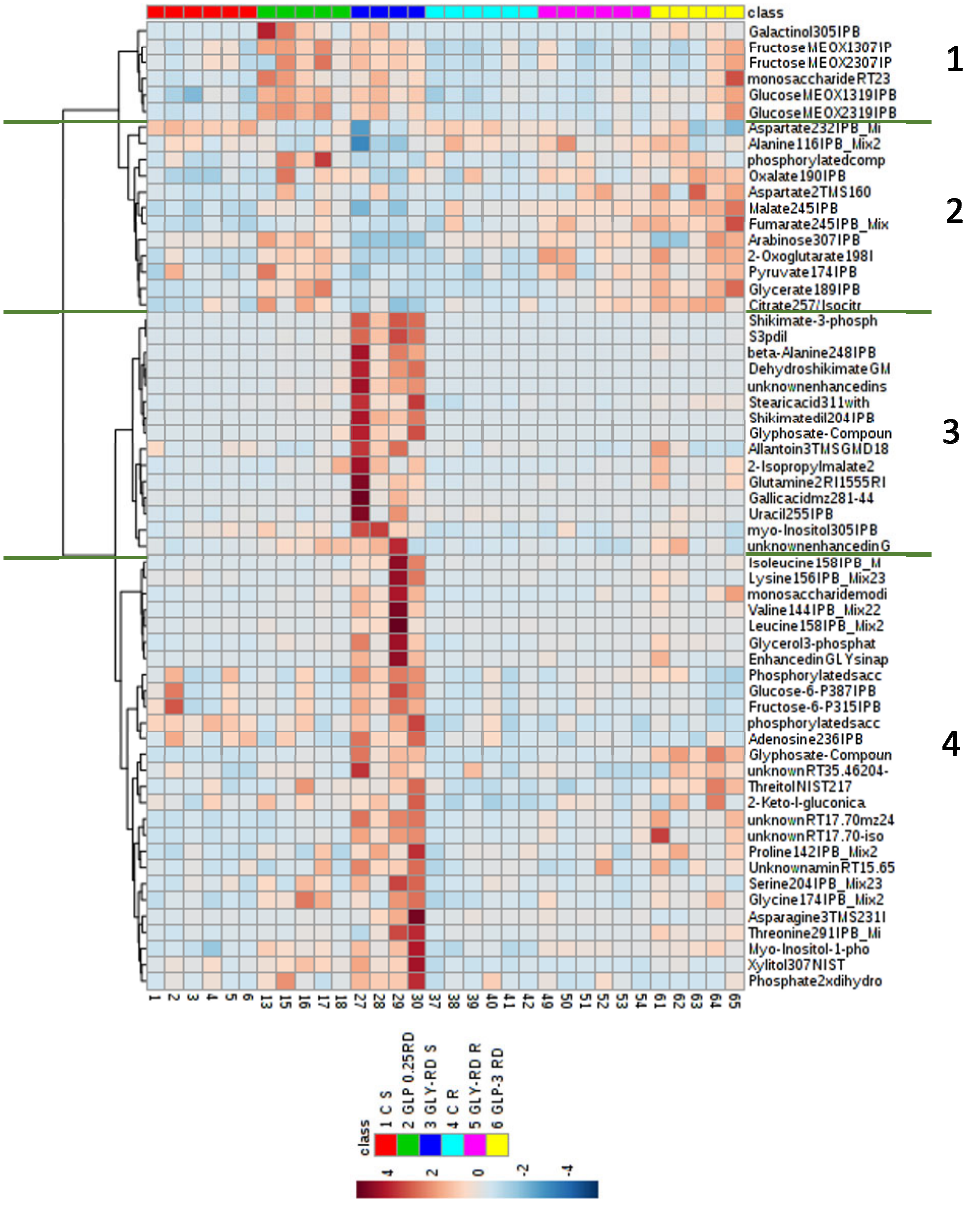

Figure 3.9. Heat map one-way hierarchical clustering of the ANOVA (Tuckey) significantly different polar metabolites detected by GC-MS in glyphosate-sensitive (S) and glyphosate resistant (R) *Amaranthus palmeri* plants. Plants were untreated (CS, CR) or treated with glyphosate in two different doses: GS population with 0.25 RD (recommended dose) (GLP 0.25RD) or RD (GLP RD S); GR population with RD (GLY RD R) or 3 RD (GLP 3 RD). The algorithm for heat map clustering was based on the Euclidean distance measure for similarity.

conditions, increasing evolutionary fitness (Buchanan et al. 2015). Increases in the amount of phenolic compounds can occur in stressed plants, and are

considered to have a key role as defense compounds when stresses such as bright light, low temperatures, pathogen infection or herbicides.

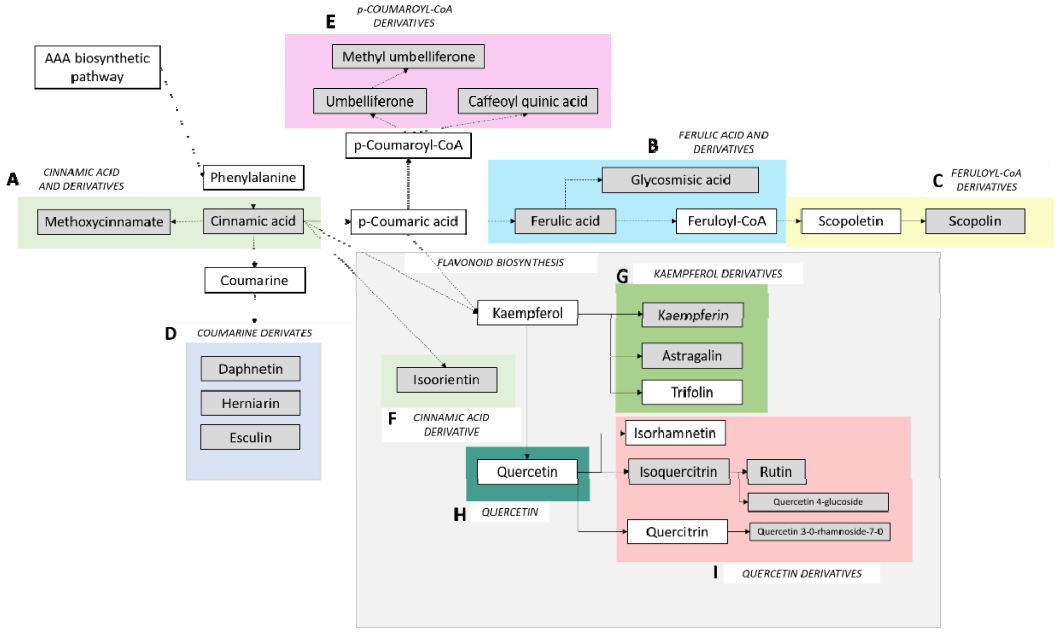

Figure 3.10. Simplification of the phenylpropanoid and flavonoid (squared in light grey) biosynthetic pathways. Metabolites in grey shows the metabolites identified by LC-MS; metabolites in white representes the metabolites no identified. One-step reactions are represented by arrows with continous line. Reactions containing more than one step are represented by arrows with discontinous line. The letters A-E and matched color code will be ploted in Figures 3.11.1 and 3.11.2; letters F-I and matched color code will be ploted in Figure 3.12.

Most of the phenolic compounds originate from the phenylpropanoid and phenylpropanoid-acetate pathways. Phenylpropanoid biosynthetic pathway leads to the synthesis of compounds with a C_6-C_3 core, like lignins and coumarins. Phenylpropanoid-acetate pathway is derived from the *p*-coumaric acid, metabolite from phenylpropanoid pathway, and leads to the synthesis of C_6-C_3-C_6 core, like flavonoids and isoflavonoids. Flavonoids are derived from phenylpropanoids and have been described to have multitude of important biological functions, like color pigments, signal molecules or/and plant protection (Böttcher et al. 2008b).

Metabolites related to the phenypropanoid and flavonoid biosynthetic pathways that were identified by LC-MS in the positive mode were chosen to plot them. The metabolites plotted were located and classified within the biosynthetic pathway in Figure 3.10, where a simplified pathway is represented. The first part of the figure was formed by cinnamic acid and derivatives (A), ferulic acid and

derivative (B), feruloyl-CoA derivative (C), coumarine derivatives (D) and p-coumaroyl-CoA derivatives (E) and were represented in 3.11.1 for GS and in 3.11.2 for GR population. The second part of the figure, named as 3.12, was formed by metabolites of the flavonoid biosynthetic pathway: cinnamic acid derivative (F), kaempferol derivatives (G), quercetin (H) and quercetin derivatives (I) for GS and GR population. Another figure including the signaling molecules detected in this experiment, abscisic and jasmonic acid, was represented(Figure 3.13).

It was not possible to establish a dose-response effect of the herbicide on the content of secondary metabolites of any of the populations, as it has not been observed a clear pattern in the phenylpropanoids or flavonoids after glyphosate treatment, because both increases and decreases in the content of some metabolites were detected in both populations.

In GS population, (Figures 3.11.1 and 3.12) few metabolites followed a dose-response effect, and they were cinnamic acid, glycosmisic acid, scopolin and herniarin (Figure 3.11.1), kaempferin (Figure 3.12) and abscisic acid (Figure 3.13). Moreover, other metabolites only increased after the sub-lethal glyphosate dose, like ferulic acid and metyl umbelliferone (Figure 3.11.1) and Isoorientin, quercetin derivative and quercetin deoxyhex+hex (Figure 3.12).

There were also detected changes in the metabolite content in plants of the GR population, where an increase in some metabolites was observed after the treatment application. It was detectd an increase in the content of: cinnamic acid, ferulic acid, glycosmisic acid, scopolin, daphnetin and methyl umbelliferone (Figure 3.11.2), kaempferin (Figure 3.12), and jasmonic and abscisic acids (Figure 3.13). Interestingly, other metabolites followed the contrary pattern, decreasing their relative content when the dose of herbicide was increased. This was detected in the case of caffeoyl quinic acid and umbelliferone (Figure 3.11.2.E) and 5 quercetin derivatives (Figure 3.12. H and I).

Limited studies have employed metabolomics to characterize weed physiology in response to herbicide application and herbicide resistance mechanisms (Maroli et al. 2018a). Although metabolomics can provide a high amount of information, metabolic processes are highly interconnected and dynamic, with rapid turnover rates that difficult the interpretation of the results. As detected in this study, no general or significant differences in phenylpropanoid content were observed after 80 hours of glyphosate application in GS and GR *Amaranthus palmeri* plants (Maroli et al. 2015), where even less effect was detected in GR population, due to the less effect of the herbicide caused by the EPSPS overexpression.

The perturbation caused by the glyphosate would affect not only the shikimate pathway but also the biosynthetic pathways deriving from it. Phenypropanoids have been described as defense and protection metabolites, particularly against various abiotic and biotic stresses (Maeda and Dudareva 2012). The increase in the content of the metabolites described might be related as part of a defense system against glyphosate action. In both populations it has been detected a dose-response increse of ferulic acid and derivatives, suggesting that would be related to the response to herbicide effect. However, this result does not match with Zabalza et al. (2017) where no changes were observed after glyphosate treatment.

The effect in the signaling molecules after glyphosate, in which an increase in the content of the metabolites in both populations, would be part of the proposed regulatory system. Indeed, in Chapter 2, an increase in the content of salicylic acid, another signaling molecule, was detected. The content of two signalling molecules jasmonic acid and abscisic acid was monitorized. While jasmonic acid content was only increased in GR population treated with the highest dose of the herbicide, abscisic acid was accumulated in both populations. Beyond the regulatory well known functions of abscisic acid, this signaling molecule also regulates the flavonoid biosynthesis, and recent studies suggest that also quercetin derivatives may also regulate the abscisic acic pathway (Brunetti et al. 2019), and would be regulating the glyphosate effect in both populations.

As pointed out before, while no general pattern of phenylpropanoids or signalling molecules could not be outlined in the response to the herbicide several metabolites were affected in a similar way (dose-response increase) in both populations: abscisic acid and ferulic acid and its derivatives. Interestingly, the pattern of flavonoids and metabolites derived from them was different between populations. While in GS population no significant change was detected, in GR population the content of quercetin and quercetin derivatives was lower in the presence of glyphosate. Quercitines have been described as very efective against reactive oxygen species (Brunetti et al. 2019) and the varations observed in quercetin derivatives after glyphosate in GR population could be related in some way with the response to the stress response caused by the herbicide. Indeed, it has been proposed that the oxidant quenching efficiency could potentially complement the glyphosate resistance in another glyphosate-resistant *A. palmeri* population (Maroli et al. 2015).

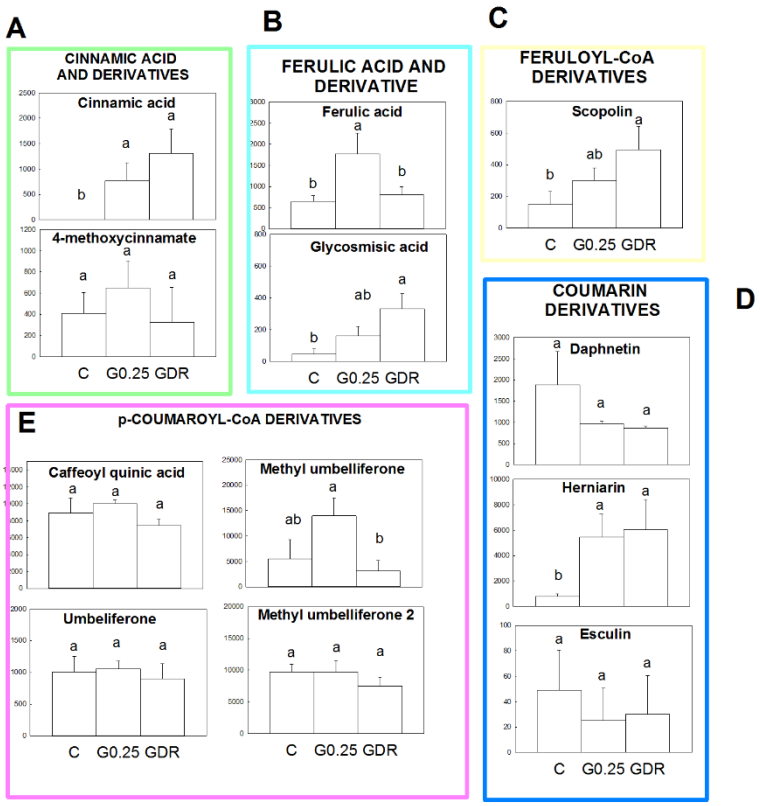

Figure 3.11.1. Phenylpropanoids identified by LC-MS in glyphosate-sensitive *Amaranthus palmeri* plants expressed as normalized peak intensity. Cinnamic acid and derivatives (A; green), ferulic acid and derivatives (B; light blue) and feruloyl-CoA derivative (C; yellow), coumarin derivatives (D; blue) and p-coumaroyl-CoA derivatives (E;pink). Plants were untreated (control) or treated with glyphosate in two different doses: 0.25 RD (recommended dose) or RD. Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tuckey).

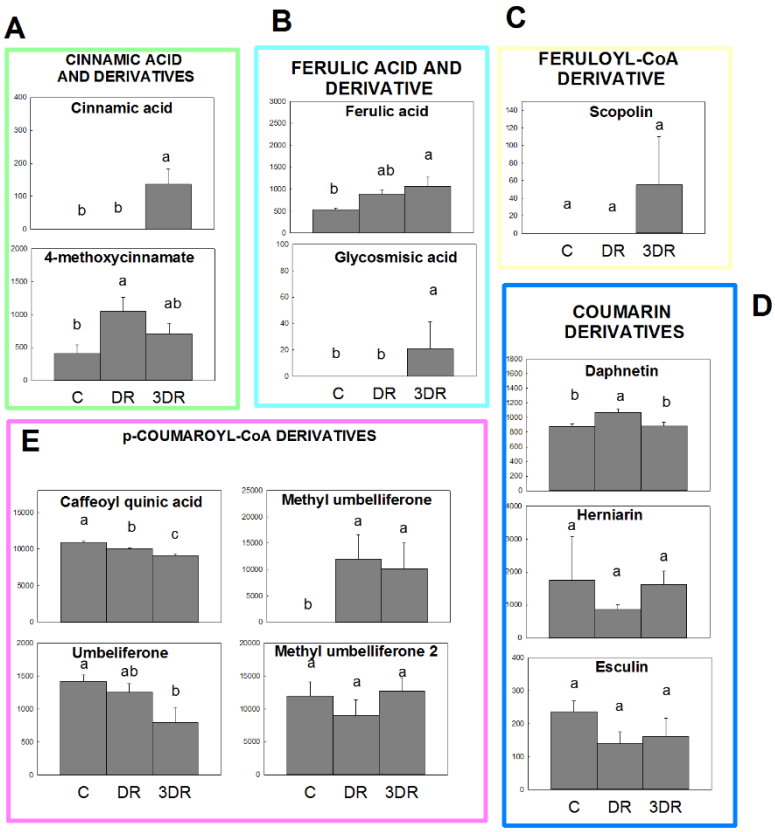

Figure 3.11.2. Phenylpropanoids identified by LC-MS in glyphosate-resistant *Amaranthus palmeri* plants expressed as normalized peak intensity. Cinnamic acid and derivatives (A; green), ferulic acid and derivatives (B; light blue) and feruloyl-CoA derivative (C; yellow), coumarin derivatives (D; blue) and p-coumaroyl-CoA derivatives (E;pink). Plants were untreated (control) or treated with glyphosate in two different doses: RD (recommended dose) or 3 RD. Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tuckey).

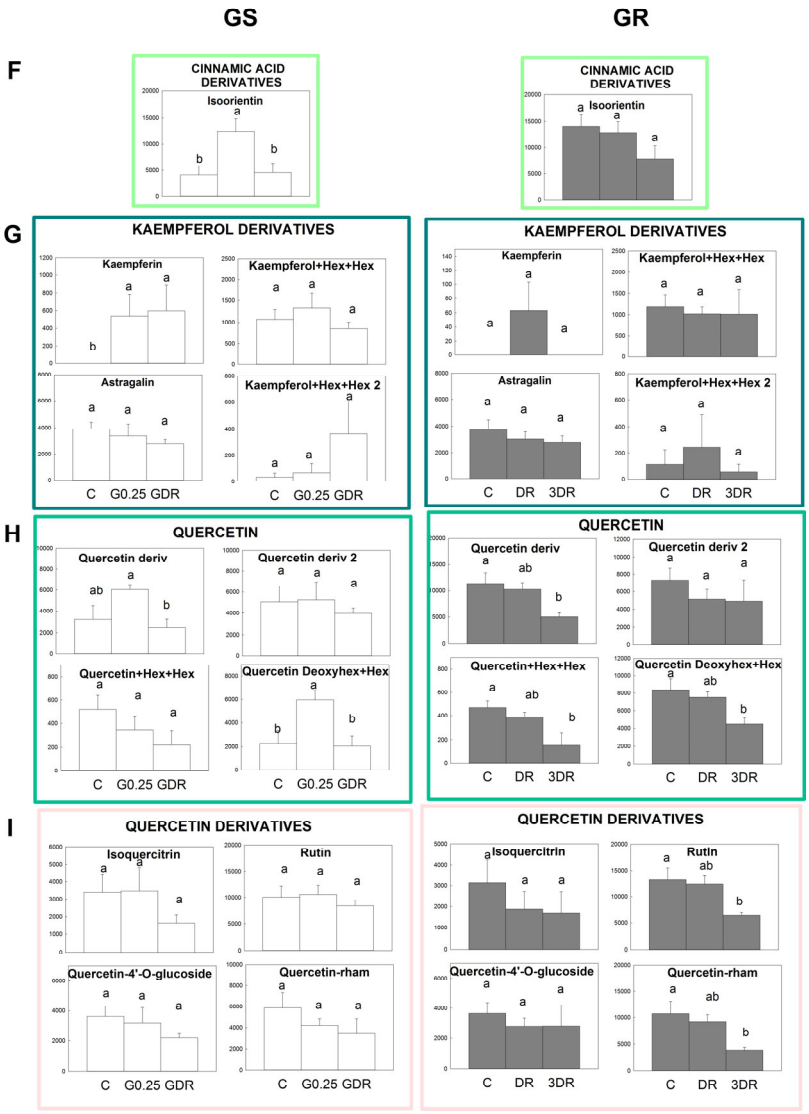

Figure 3.12. Flavonoids identified by LC-MS in glyphosate-sensitive (GS, white bars, left) and –resistant (GR, grey bars, right) *Amaranthus palmeri* plants expressed as normalized peak intensity. Cinnamic acid and derivatives (F; light green), kaempferol derivatives (G; dark green), quercetin (H; green) and quercetin derivatives (I; light pink). Plants were untreated (control) or treated with glyphosate in two different doses: 0.25 RD (recommended dose) or RD. Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tuckey).

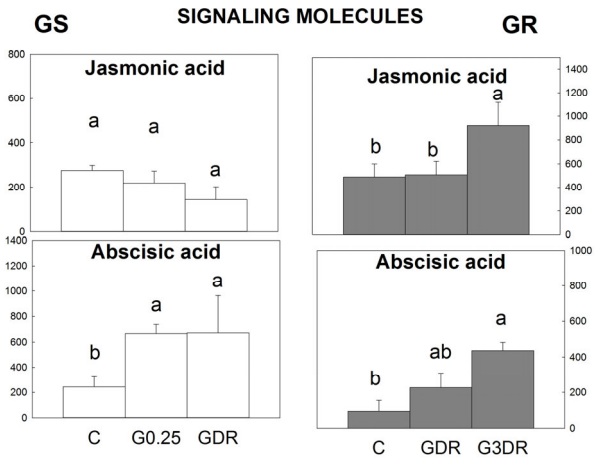

Figure 3.13. Jasmonic acid and abscisic acid, signaling molecules identified by LC-MS in glyphosate-sensitive (GS) and resistant (GR) *Amaranthus palmeri* plants expressed as normalized peak intensity. Plants were untreated (control) or treated with glyphosate in two different doses: 0.25 RD (recommended dose) or RD in GS and RD or 3 RD in GR. Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tuckey).

3.3.3. Metabolic profile of glyphosate-sensitive and resistant plants treated with quinate, a sublethal dose of glyphosate or their combination

In this section it is analyzed the comparison of the metabolome of GS or GR plants treated with a combination of quinate and a sub lethal dose of glyphosate with the individual treatments in order to ascertain any metabolic pattern that would explain the enhanced effect of the combined treatment in respect to glyphosate alone. In order to eliminate any possible enhancer effect of the surfactant that is applied with quinate, in this section the glyphosate applied alone was applied with the surfactant to ensure that the effects of the combined treatment were only elicited by the presence of quinate.

3.3.3.1. General overview and most significant metabolites detected by GC-MS

The dataset of metabolites positively identified by GC-MS was treated using PCA within each population to extract the parameters that are most important in assessing variation after quinate, glyphosate or the combination of both compounds (Figure 3.14).

The values were subjected to a PCA and converted to a set of two PCs that contributed 45.8% and 32.8% of the total variance, in GS and GR population respectively. The treatments were not clearly discriminated in any of the populations when the plot of component 1 and 2 were represented (Figure 3.14). In GS population, the component 1 would be related to the glyphosate effect, but apparently the component 2 would not explain differences (Figure 3.14.A). In GR population, the component 1 seems to be related to the glyphosate effect (Figure 3.14.B), and the component 2 with the presence of quinate, because both treatments would be located more separately from the others.

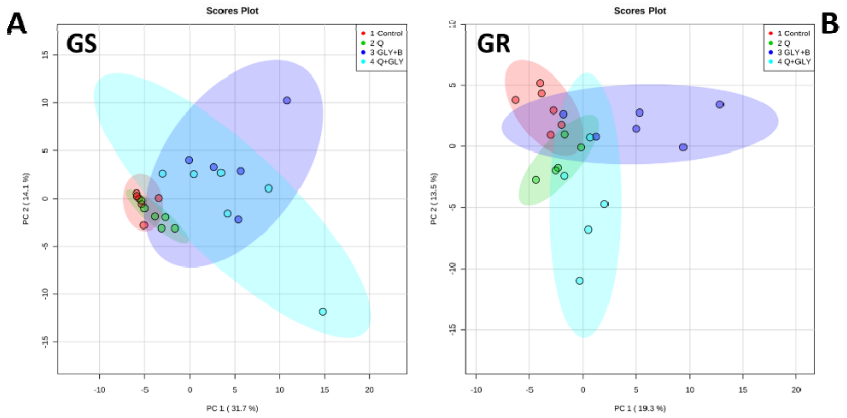

Figure 3.14. Principal component analysis (PCA) of metabolites detected by GC-MS in glyphosate-sensitive (GS; A; left) and glyphosate-resistant (GR; B; right) *Amaranthus palmeri* plants. . Plants were untreated (Control; red) or treated with quinate (Q; green), 0.25 the recommended dose (RD) of glyphosate in GS and RD in GR population with the surfactant (GLY+B; dark blue) and the combination of both compounds (Q+GLY; light blue).

The principal loadings of the componentes 1 and 2 were chosen and represented in the following figures, grouped as the previous section where the effect of glyphosate has been studied. On one hand, Figures 3.15 and 3.16 show metabolites of shikimate pathway or derivatives and AAs. On the other hand, carbohydrates, fatty acids, metabolites of the Krebs cycle and other are shown in Figures 3.17 and 3.18. In GS, the top loadings for component 1 were citrate/isocitrate, asparagine, S3P and stearic acid. For component 2, the 4 quinate derivatives identified, tyrosine and benzoate derivative. In GR population, some of the top loading for component 1 was shikimate, S3P, stearic acid and β-alanine. For component 2, quinate and their 4 derivatives and dehydroshikimate.

Quinate and quinate derivatives were accumulated when quinate (alone or combined) was supplied exogenously, as it was reported in Chapter 2 (Fig.2.4). In

general it was not detected any metabolic content change in any of the populations with two non-relevant exceptions: an increse in the content of sinapic acid derivative in GS population and fumarate in GR population (Figure 3.15, 16, 17 and 18).

Combined treatment did not provoke an exhacerbation of the glyphosate effect in the metabolic pool. As described in Chapter 2, only an enhancement of the content of quinate derivatives was observed (Figure 3.15.A and 3.16.A), suggesting that this acumulation could be related to the increase of the toxicity in GS population.

With each population, a heat map with the metabolites that were significantly different after ANOVA (Tuckey) was perfomed (Figure 3.19 and 3.20). Fourty metabolites in GS and 22 metabolites in GR were significantly differents. This result confirms a more significant effect of quinate and glyphosate on GS population than on GR population, where EPSPS overexpression would buffer the effect of the treatments. Data in each heat map were clustered and divided into 3 clusters according to metabolite pattern after the treatments. In each heat map, there were clearly detected differences between the treatments.

In GS population (Figure 3.19), a clear different metabolite profile was observed after each treatment. Quinate treatment did not cause any important perturbation to the plants, because the profile observed was similar to the observed in control plants. On the contraty, glyphosate alone and combined treatment provoked a very intense perturbation in the metabolome, showing an intense accumulation in clusters 2 and 3 in glyphosate treatment, and in the three clusters in the combined treatment. Thus, the combined treatment would be the most disturbing treatment on the metabolic profile, causing a general perturbation of the metabolome, result that would match with the lethality provoked by this treatment.

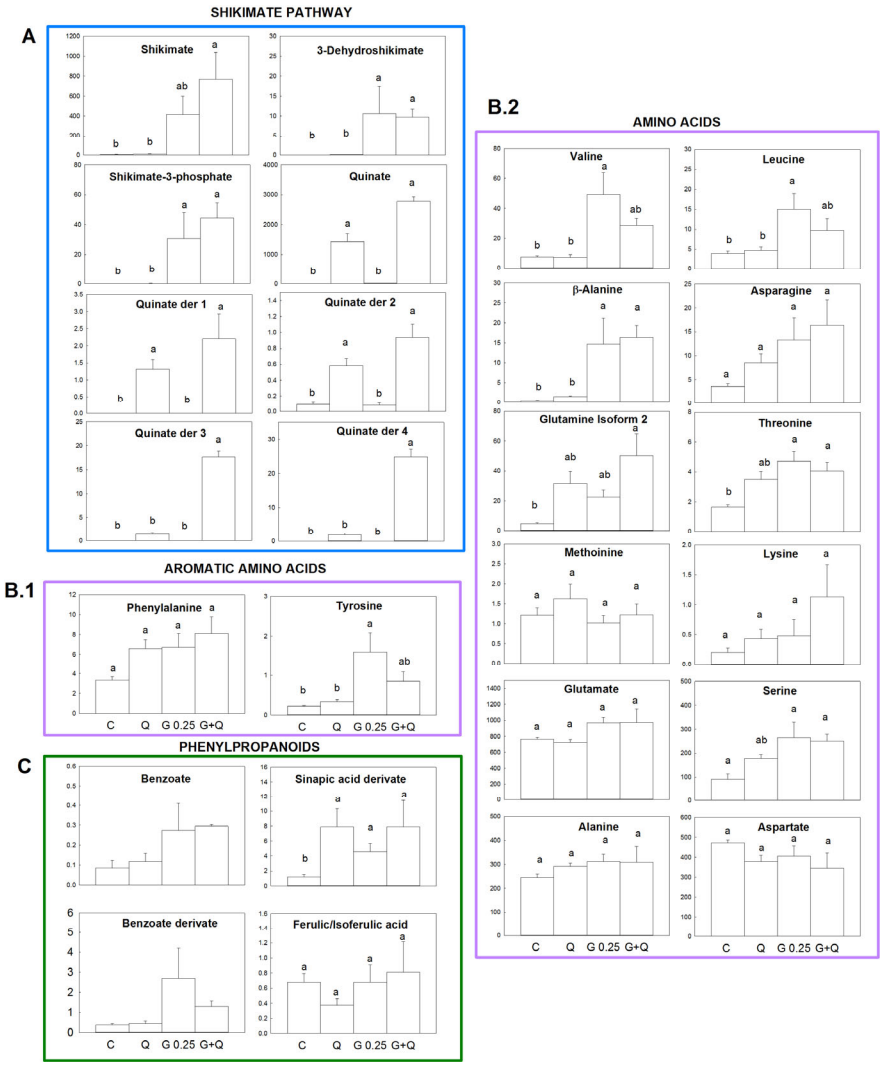

Figure 3.15. Principal metabolites detected by GC-MS in glyphosate-sensitive *Amaranthus palmeri* plants expressed as normalized peak intensity. Shikimate pathway (A; blue), aromatic amino acids (B.1, purple) and other amino acids (B.2; purple) and phenylpropanoids (C, green). Plants were untreated (control) or treated with treated with quinate (Q), 0.25 the recommended dose of glyphosate with the surfactant (GLY+B) and the combination of both compounds (Q+GLY). Different letters within each population indicate significant differences between treatments (*p*-value≤0.05, Tuckey).

The metabolites belonging to cluster 1 were more intensely accumulated after the combined treatment than after glyphosate alone in GS population. As expected, metabolites included in cluster 1, were quinate and the four derivatives detected. Glycine and xylitol were also included in this cluster. Moreover, metabolites that were included in cluster 2 and 3 were enhanced in those two

treatments very closely, suggesting that metabolites included in cluster 2 and 3 would be more related to the effect of the herbicide, like different AAs and carbohydrate metabolism.

The significant different metabolites were also clustered in the heatmap of the GR population (Figure 3.20). Cluster 1 was formed by metabolites detected more intensely in treatments where quinate was included, quinate applied alone and the combined treatment. Quinate and its derivatives were the metabolites included in this cluster. The combined treatment also provoked an increase in the content of the metabolites of cluster 3, which were metabolites related to the shikimate pathway and carbohydrate metabolism. However, in contrast to the results observed in GS population, it was not the most disturbing treatment in GR population. In this population, glyphosate alone provoked, in addition to the changes of cluster 3, an accumulation of the metabolites clustered in group 2, metabolites mostly no identified and some charbohydrates.

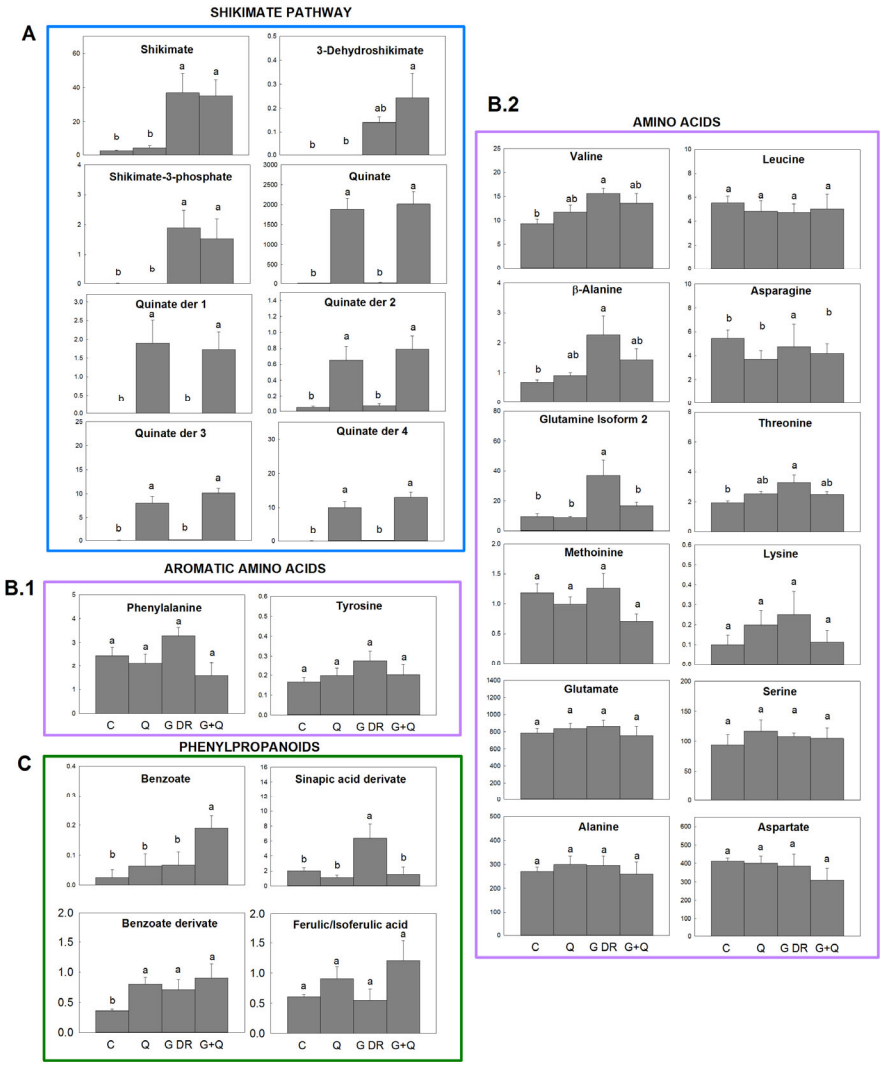

Figure 3.16. Principal metabolites detected by GC-MS in glyphosate-resistant *Amaranthus palmeri* plants expressed as normalized peak intensity. Shikimate pathway (A; blue), aromatic amino acids (B.1, purple) and other amino acids (B.2; purple) and phenylpropanoids (C, green). Plants were untreated (control) or treated with treated with quinate (Q), the recommended dose of glyphosate with the surfactant (GLY+B) and the combination of both compounds (Q+GLY). Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tuckey).

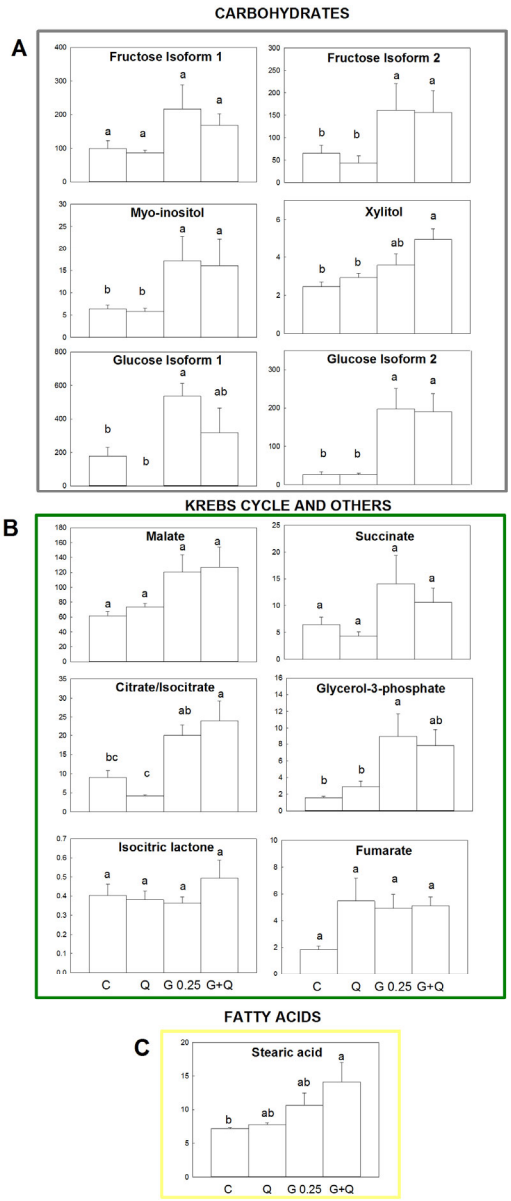

Figure 3.17. Principal metabolites detected by GC-MS in glyphosate-sensitive *Amaranthus palmeri* plants expressed as normalized peak intensity. Carbohydrates (A; grey), Krebs cycle and others (B; green) and fatty acids (C; yellow). Plants were untreated (control) or treated with treated with quinate (Q), 0.25 the recommended dose of glyphosate with the surfactant (GLY+B) and the combination of both compounds (Q+GLY). Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tuckey).

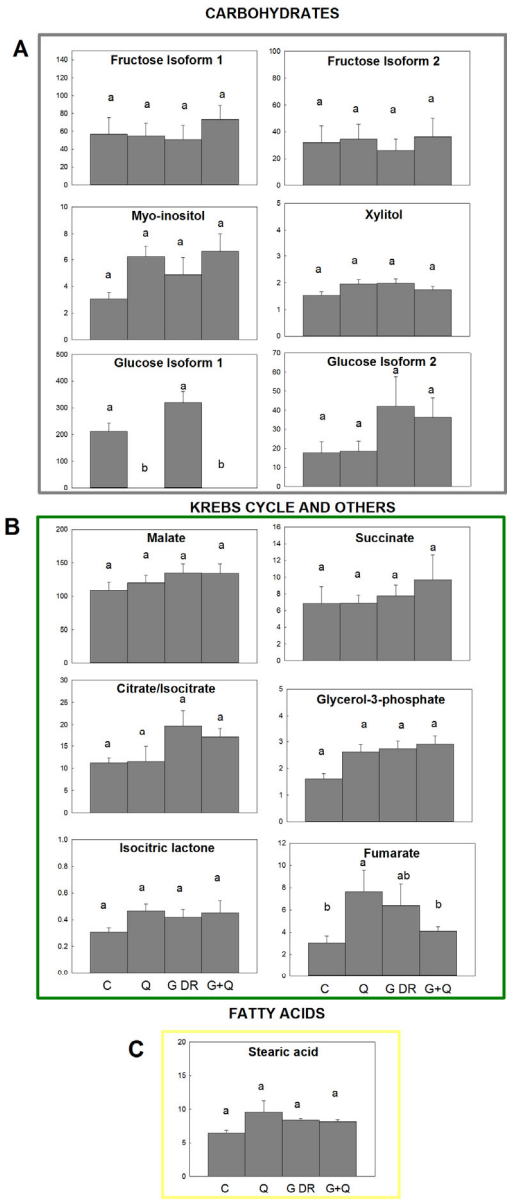

Figure 3.18. Principal metabolites detected by GC-MS in glyphosate-resistant *Amaranthus palmeri* plants expressed as normalized peak intensity. Carbohydrates (A; grey), Krebs cycle and others (B; green) and fatty acids (C; yellow). Plants were untreated (control) or treated with treated with quinate (Q), the recommended dose of glyphosate with the surfactant (GLY+B) and the combination of both compounds (Q+GLY). Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tuckey).

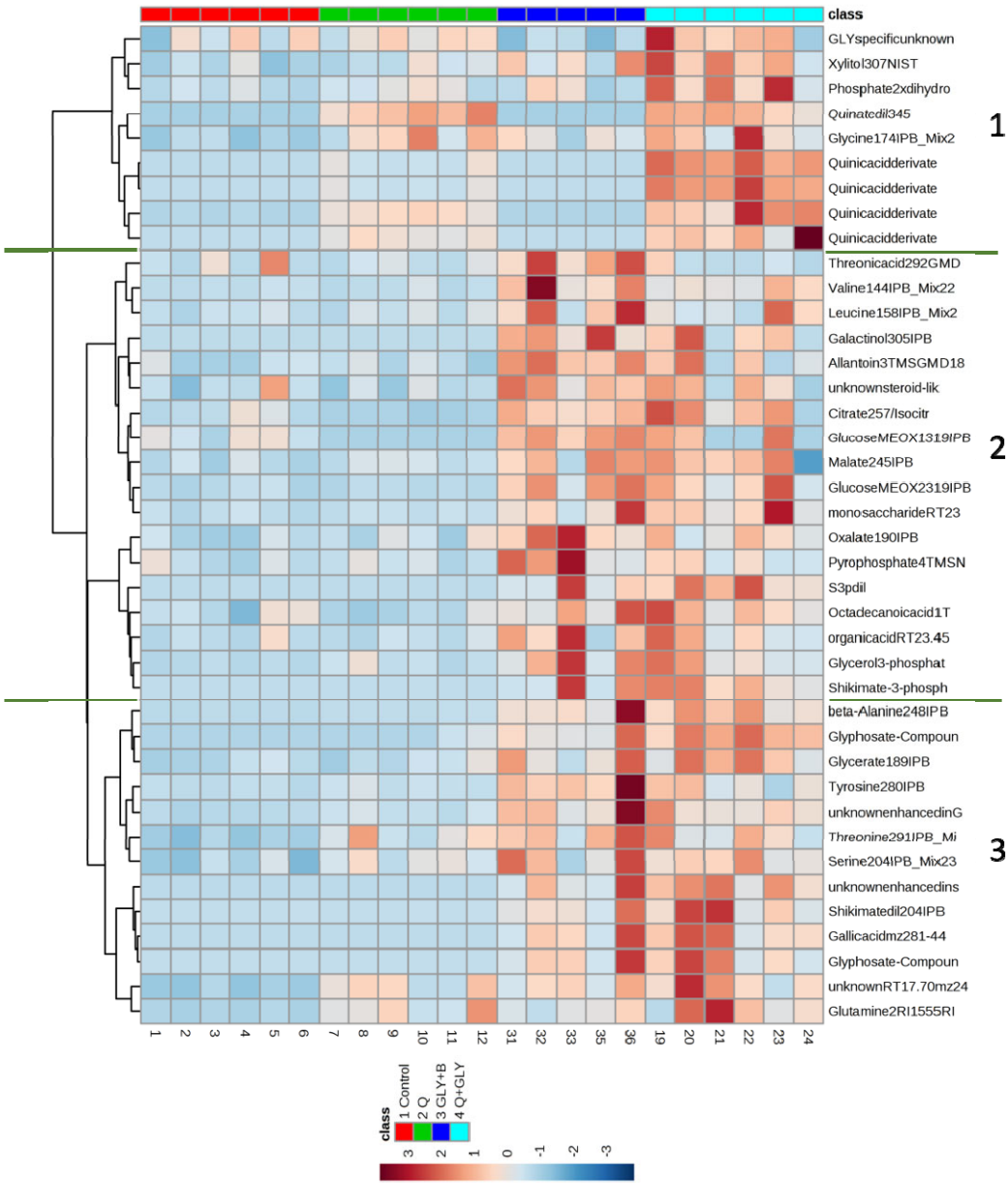

Figure 3.19. Heat map one-way hierarchical clustering of the ANOVA (Tuckey) significantly-different polar metabolites detected by GC-MS in glyphosate-sensitive (S) *Amaranthus palmeri* plants. Plants were untreated (Control) or treated with quinate (Q), 0.25 the recommended dose of glyphosate with the surfactant (GLY+B) and the combination of both compounds (Q+GLY). The algorithm for heat map clustering was based on the Euclidean distance measure for similarity.

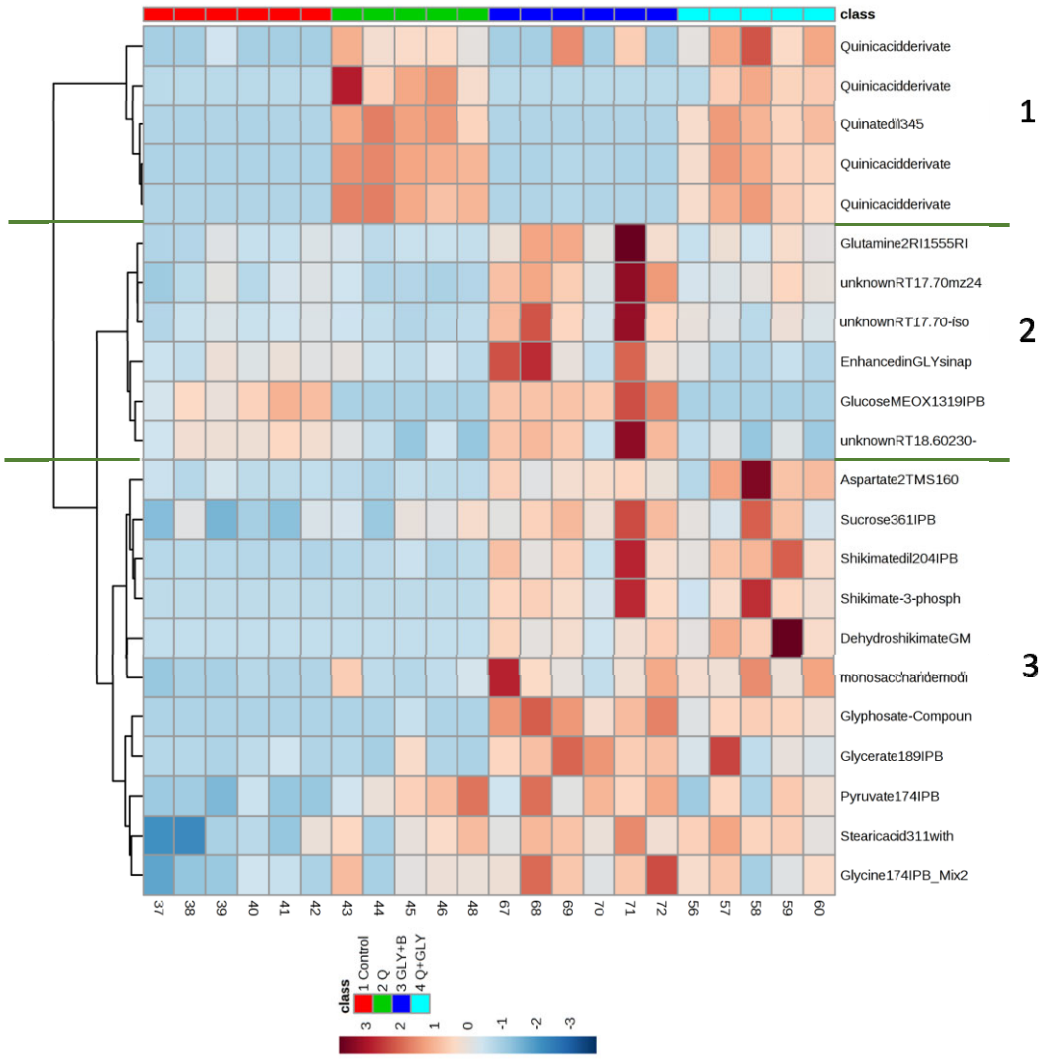

Figure 3.20. Heat map one-way hierarchical clustering of the ANOVA (Tuckey) significantly-different polar metabolites detected by GC-MS in glyphosate-resistant (R) *Amaranthus palmeri* plants. Plants were untreated (Control) or treated with quinate (Q), the recommended dose of glyphosate with the surfactant (GLY+B) and the combination of both compounds (Q+GLY). The algorithm for heat map clustering was based on the Euclidean distance measure for similarity.

3.3.3.2. Phenylpropanoid metabolites and metabolites of flavonoid bioynthetic pathways.

As it was evaluated with only glyphosate, metabolites related to the phenypropanoid and flavonoid biosynthetic pathways were evaluated after quinate, the low dose of glyphosate and the combined treatment. Thes secondary metabolites were obtained from the metabolites identified by LC-MS in the positive mode. The metabolites plotted, as in the previous section, were located and clasified within the scheme in Figure 3.21, where a simplified overview of the phenylpropanoid and flavonoid biosynthetic pathways is represented. Signaling molecules detected by LC-MS were represented in Figure 3.24.

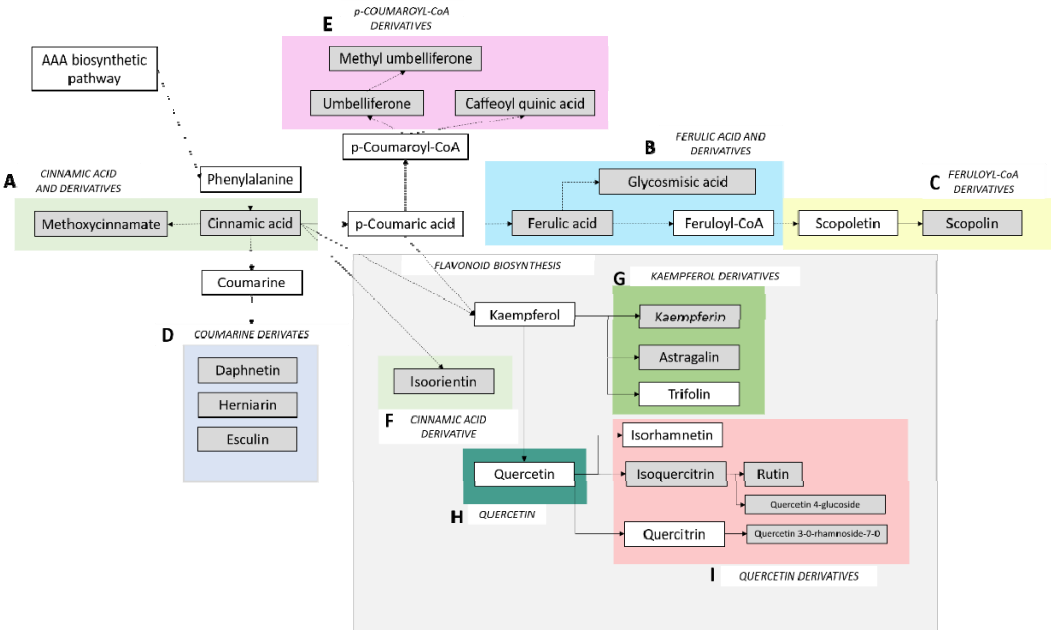

Figure 3.21. Simplification of the phenylpropanoid and flavonoid (squared in light grey) biosynthetic pathways. Metabolites in grey shows the metabolites identified by LC-MS; metabolites in white representes the metabolites no identified. One-step reactions are represented by arrows with continous line. Reactions containing more than one step are represented by arrows with discontinous line. The letters A-E and color code match will be ploted in Figures 3.22.1 and 3.22.2; letters F-I and color code match will be ploted in Figure 3.23.

Quinate applied alone did not provoke changes in the phenylpropanoid and flavonoid biosynthetic pathways in GS population (Figure 3.22.1 and 3.23). Despite that the shikimate pathway would be fueled by the entrance of quinate, the phenylpropanoid content were not affected 3 days after the application.

Zabalza et al. (2017) observed an increase in some phenylpropanoid content after quinate continuously applied through the nutrient solution, confirming that quinate was incorporated into the pathway affecting also the phenylpropanoid pathway. However, in this experiment, quinate application was sprayed to the leaves and this type of application would not be enough to detect changes in phenylpropanoids 2 days after. Indeed, previous studies with pea showed that the amount of three hydroxycinnamic acids (caffeic, ferulic, and p-coumaric acids) was not affected in the leaves after foliar application (Zulet et al. 2013b).

The combined treatment only exacerbated glyphosate effect in few of the metabolites identified. In GS population, only the phenylpropanoids cinnamic acid, scopolin and herniarin accumulation was higher in the combined treatment than the glyphosate alone. In the GR population, only the phenylpropanoid content of esculin was significant reduced after the combined treatment (Figure 3.22.2 and 3.23). Glyphosate increases PAL activity in plants, as has been reported previously (Marchiosi et al. 2009; Mobin et al. 2015; Zabalza et al. 2017). The increase in PAL activity after glyphosate treatment has been proposed to be related to the higher availability of Phe, substrate of the enzyme. Glyphosate treatment on sensitive A. palmeri plants increases the content of all free amino acids (Fernández-Escalada et al. 2016, Maroli et al.2018b), included Phe, due to an increase in the turnover of the existing proteins (Zulet et al., 2013a) The increase of PAL activity would lead to an increase of cinnamic acid, the product of the reaction. The tendency in increasing Phe content in the combined treatment in GS would match with the increase in cinnamic acid relative content.

The relative content of flavonoids was similar after glyphosate and after the combined treatment in both populations, evidencing that the overall flavonoid content is not easily affected by perturbations in the shikimate pathway. No striking changes in the GS population were detected in phenylpropanoid and flavonoid contents after the combined treatment, indicating that the lethality provoked by this treatment is not mediated by changes in these secondary metabolites.

Abscisic acid content was accumulated only in the combined treatment in GS population (Figure 3.24), suggesting a role of this hormone in the physiological response of this plants to the joint application of quinate and glyphosate.

As observed in the metabolites identified by CG-MS and LC-MS the exogenous supply of quinate did not affect the overall status of primary and secondary metabolites of GS or GR plants. Only an increase of quinate and its derivatives was detected in both populations. The applied dose of glyphosate affected more the content of primary metabolites than of secondary metabolites (mainly related

with shikimate pathways) and amino acids and the effect was more evident in GS than in GR, due to the different sensitivity of both populations to the herbicide.

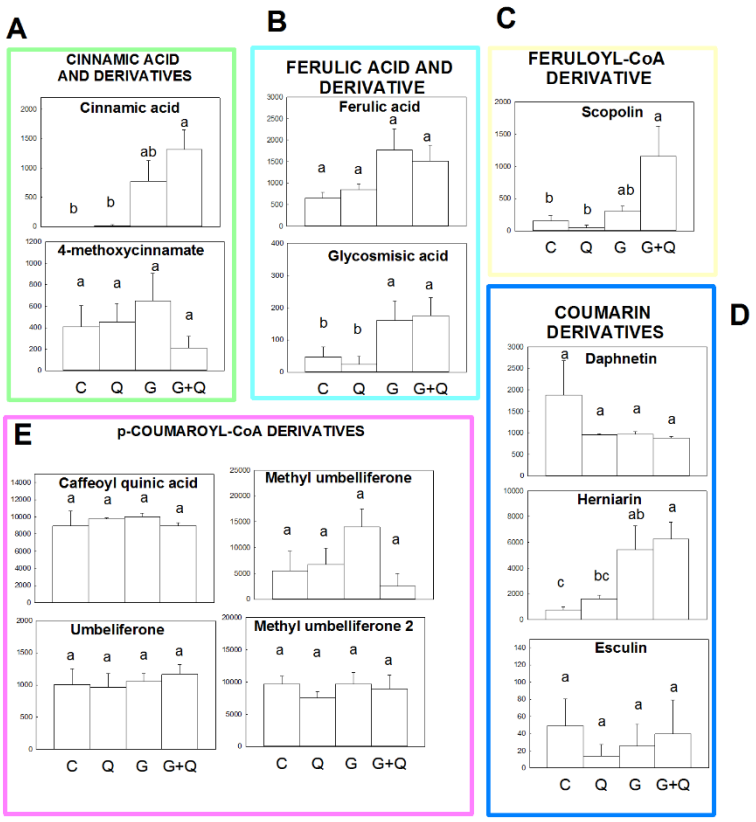

Figure 3.22.1. Phenylpropanoids identified by LC-MS in glyphosate-sensitive *Amaranthus palmeri* plants expressed as normalized peak intensity. Cinnamic acid and derivatives (A; green), ferulic acid and derivatives (B; light blue) and feruloyl-CoA derivative (C; yellow), coumarin derivatives (D; blue) and p-coumaroyl-CoA derivatives (E;pink). Plants were untreated (Control) or treated with quinate (Q), 0.25 the recommended dose of glyphosate with the surfactant (G) and the combination of both compounds (G+Q). Different letters within each population indicate significant differences between treatments (*p*-value≤0.05, Tuckey).

The comparison of the combined treatment (lethal) with glyphosate alone (sub lethal) allows to detect the changes in the metabolic profile that would be related to such increased efficacy. The comparison of the overall metabolic profile between glyphosate alone and combined shows few changes, at the level of the increase of the quinate derivatives (Figure 3.19). Indeed, the specific enhancement of these derivatives was proposed in Chapter 2 (Fig. 2.4) to explain the increased efficacy of the combined treatment. Results of this chapter support these previous results and, moreover, show that this change is the most

significant one detected after the combined and not after the individual treatment.

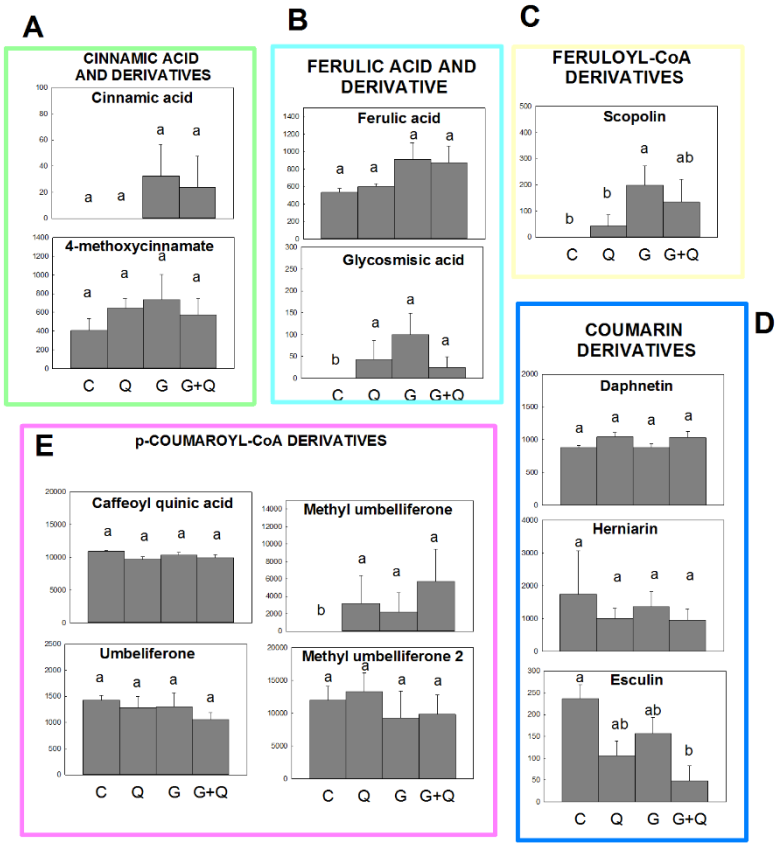

Figure 3.22.2. Phenylpropanoids identified by LC-MS in glyphosate-resistant *Amaranthus palmeri* plants expressed as normalized peak intensity. Cinnamic acid and derivatives (A; green), ferulic acid and derivatives (B; light blue) and feruloyl-CoA derivative (C; yellow), coumarin derivatives (D; blue) and p-coumaroyl-CoA derivatives (E;pink). Plants were untreated (Control) or treated with quinate (Q), the recommended dose of glyphosate with the surfactant (G) and the combination of both compounds (G+Q). Different letters within each population indicate significant differences between treatments (p-value≤0.05, Tuckey).

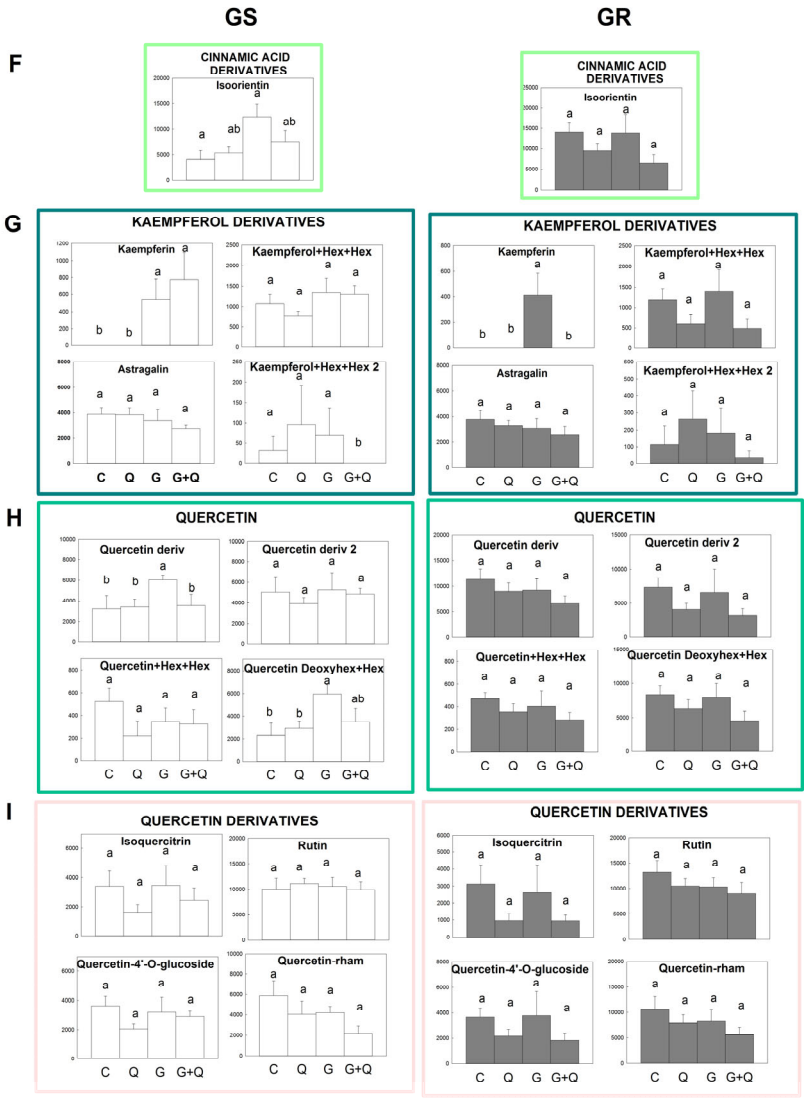

Figure 3.23. Flavonoids identified by LC-MS in glyphosate-sensitive (GS, left, white bars) and –resistant (GR, right, grey bars) *Amaranthus palmeri* plants expressed as normalized peak intensity. Cinnamic acid and derivatives (F; light green), kaempferol derivatives (G; dark green), quercetin (H; green) and quercetin derivatives (I; light pink). Plants were untreated (Control) or treated with quinate (Q), the recommended dose of glyphosate with the surfactant (G) and the combination of both compounds (G+Q). Different letters within each population indicate significant differences between treatments (*p*-value≤0.05, Tuckey)

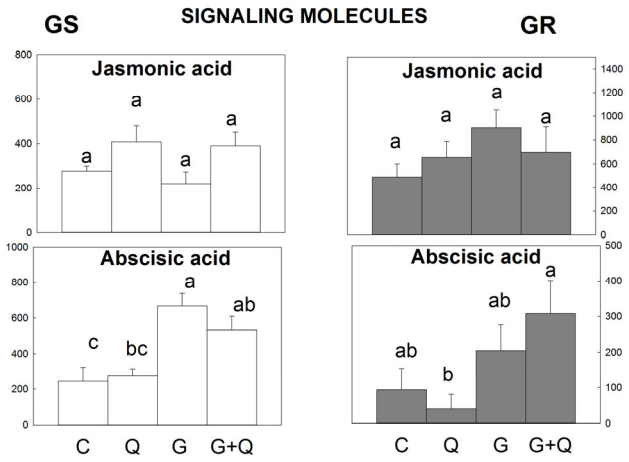

Figure 3.24. Jasmonic acid and abscisic acid, signaling molecules identified by LC-MS in glyphosate-sensitive (GS) and resistant (GR) *Amaranthus palmeri* plants expressed as normalized peak intensity. Plants were untreated (Control) or treated with quinate (Q), 0.25 the recommended dose (RD) of glyphosate in GS and RD in GR with the surfactant (G) and the combination of both compounds (G+Q). Different letters within each population indicate significant differences between treatments (*p*-value≤0.05, Tuckey).

3.4. CONCLUSIONS

- Untreated plants of GS and GR populations of *Amaranthus palmeri* plants showed no differences in the metabolite profile determined by GC- and LC-MS, showing that the overexpression of EPSPS does not lead to changes in metabolite content.

- In GS, there were detected dose-dependent changes in the metabolic pool of primary and shikimate pathway metabolism after glyphosate while in GR, only the highest glyphosate dose applied provoked changes, due to the less effect of the herbicide because the EPSPS overexpression. The changes observed were related to the physiological disturbances described previously for the herbicide: shikimate pathway and amino acid content.

- No general pattern of phenylpropanoids or signalling molecules could be outlined in the response to the herbicide, but abscisic acid and ferulic acid and its derivatives were affected similarly in both populations. The pattern of flavonoids after glyphosate was different between populations, observing a decrease in quercetine content in GR population after glyphosate treatment.

- The comparison of the metabolic profile after the lethal dose of glyphosate in GS plants with the other sublethal doses in both populations suggest that lethality is associated to changes in the metabolites of the shikimate pathway, carbohydrates and amino acids.

- The exogenous supply of quinate did not affect the overall status of primary and secondary metabolites of GS or GR plants, as detected by GC- and LC-MS. Only an increase of quinate and its derivatives was detected in both populations.

- The higher toxicity observed in the combined treatment was not related to a higher effect on the metabolome, as after glyphosate treatment similar metabolic changes were detected. There were only detected changes in the content of the quinate derivatives, differences that would be related to the increased toxicity of the combined treatment.

3.5. SUPPLEMENTAL TABLES

Supplemental Table 1. Metabolites identified and fragment ions (m/z) in *Amaranthus palmeri* GS and GR plants by GC-MS.

Metabolites	Fragment Ions (m/z)
2-Aminoadipate	260
2-Isopropylmalate	275
2-Keto-l-gluconic acid	292
2-Oxoglutarate	198
Adenine	264
Adenosine	236
Adenosine-5-monophosphate	169
Alanine	116
Allantoin	189, 259, 359
Arabinose	307
Arginine	157
Asparagine	231
Aspartate	160
Aspartate	232
Benzoate / Benzoic acid	179
Benzoate derivate	179, 245
beta-Alanine	248
Caffeic acid	219
Caffeic acid	396
cis-Aconitate	229
Citrate / Isocitrate	257
Dehydroshikimate	296, 402, 417 (386)
Ethanolamine	174
Ferulic acid / Isoferulic acid	338
Fructose 1	307
Fructose 2	307
Fructose-6-P	315
Fumarate	245
Galactinol	305
Gallic acid	281, 443 (458)
Glucose 1	319

Glucose 2	319
Glucose-6-P	387
Glutamate	246
Glutamine 1	RI 1488
Glutamine 2	RI 1555
Glutaric acid	261
Glycerate	189
Glycerol 3-phosphate	357
Glycine	174
Glyphosate-Compound RT 19.00 m/z	282
Glyphosate-Compound RT 22.40 m/z	212
Glyphosate-Compound RT 23.00 m/z	155
Hexadecanoic acid	313
Isocitric lactone	303
Isoleucine	158
Leucine	158
Lysine	156
Malate	245
Maleic acid	245
Methionine	176
monosaccharide RT 23.20	204
monosaccharide+modification	319
myo-Inositol	305
Myo-Inositol-1-phosphate	318
Octadecadienoic acid	337
Octadecanoic acid	341
Octadecenoic acid	339
organic acid RT 23.07 galactaric acid	333
organic acid RT 23.45 Glucaric acid	333
Oxalate	190
Phenylalanine	192
Phosphate + 2x dihydroxypropyl group	357-503-445-299
Phosphorylated saccharide RT 27.25	387
phosphorylated saccharide RT 29.75	387
Proline	142
Pyrophosphate 4TMS	451

Pyruvate	174
Quinate	345
Quinic acid derivative (glycosylated?) RT 31.40 mz	255-204-361 (345)
Quinic acid derivative (glycosylated?) RT 31.80 mz	255-204-361 (345)
Quinic acid derivative (glycosylated?) RT 32.10 mz	255-204-361 (255)
Quinic acid derivative (glycosylated?) RT 32.40 mz	255-204-361 (345)
Ribitol	217
Serine	204
Shikimate	204
Shikimate-3-phosphate	483
Sinapic acid derivate	227, 301, 329 (344)
Stearic acid	311, 453
Succinate	247
Sucrose	361
Threitol	217
Threonic acid	292
Threonine	291
Tyramine	174
Tyrosine	280
Uracil	255
Valine	144
Xylitol	307
Xylitol or similar monosaccharide / alcohol	307
unknown	*245, 308, 410, 424*
unknown	*224, 296, 386*
Unknown amin RT 15.65	*174, 156*
unknown amin RT 17.30	*174*
unknown phosphorylated compound RT 32.90	*169, 450, 384*
unknown RT 17.70	*275*
unknown RT 17.70	*244, 275*
unknown RT 18.60	*230, 257, 359, 374*
unknown RT 22.40	*424*
unknown RT 22.70	*155, 271, 299, 447*
unknown RT 25.30	*204, 210, 235*
Unknown RT 25.30	*204, 217, 235*
unknown RT 33.60	*307, 375, 219, 714*

unknown RT 34.90	*307, 375, 219, 714*
unknown RT 35.12	*307, 375, 219, 714*
unknown RT 35.46	*204, 217, 361, 597*
unknown steroid-like compound isomer 1 RT 28.40	*245, 360, 430, 520, 592*
unknown steroid-like compound isomer 2 RT 28.55	*245, 360, 430, 520, 592*

Supplemental Table 2. Metabolites identified in *Amaranthus palmeri* GS and GR plants by LC-MS. Confidence level of annotation (According to Matern et al., 2019) is as follows: 1, verified with mass, retention time, and CID spectrum of a commercial standard or a synthesized standard; 2, putatively annotated compounds based on CID spectrum interpretation, data base hits, and literature; 3, possible structure or metabolite family based on hits in data bases; 4, unknown.

			Quantifier ion		
Annotation	**Elemental composition**	**RT [min]**	**Type**	**m/z meas.**	**Ann. level**
Pipecolinic acid	$C_6H_{11}NO_2$	0.37	[M+H]+	130.09266	2
L-Histidine	$C_6H_9N_3O_2$	0.37	[M+H]+	156.0744	1
L-Proline	$C_5H_9NO_2$	0.4	[M+H]+	116.07113	1
Ribulose 1,5-bisphosphate	$C_4H_4O_7P_2$	0.42	[M+H]+	226.95123	1
Cytosine	$C_4H_5N_3O$	0.43	[M+H]+	112.05308	1
D-Ribose 5-phosphate	$C_5H_{11}O_8P$	0.43	[M+K]+	268.9814	1
L-Valine	$C_5H_{11}NO_2$	0.43	[2M+Na]+	257.14615	1
unknown ubiquitous metabolite	$C_4HO_2PS_4$	0.44	[M+H]+	240.86606	4
D-Xylitol	$C_5H_{11}NaO_5$	0.45	[M+Na]+	175.05737	1
Betaine	$C_5H_{11}NO_2$	0.47	[2M+H]+	235.16485	1
Trigonelline	C_7H_7NO2	0.47	[M+H]+	138.05513	1
Nicotinic acid	$C_6H_5NO_2$	0.48	[2M+H]+	247.07745	1
D-Mannitol	$C_6H_{14}O_6$	0.48	$[M+CO_2H_2+H]+$	229.09487	1
Betaine	$C_5H_{11}NO_2$	0.48	[M+H]+	118.08674	1
D-Sucrose	$C_{12}H_{22}O_{11}$	0.5	[M+Na]+	365.10311	1
Fructose/Glucose	$C_6H_{12}O_6$	0.5	[M+Na]+	203.0513	3
L-Asparagine	$C_4H_8N_2O3$	0.5	[2M+Na]+	287.10002	1
Adenine	$C_5H_5N_5$	0.5	[M+H]+	136.06184	1
Quinate	$C_7H_{12}O_6$	0.51	[M+Na]+	215.05107	1
L-Arginine	$C_6H_{14}N_4O_2$	0.51	$[M+CH_2O_2Na]+$	243.11056	1
L-Glutamine	$C_5H_{10}N_2O_3$	0.54	[M+H]+	147.07496	1
Glutamic acid	$C_5H_9NO_4$	0.55	[M+H]+	148.06083	1

Inosine 5'-monophosphate	$C_{10}H_{13}N_4O_8P$	0.57	[M+H]+	349.06017	1
L-Ureidosuccinic acid	$C_4H_7NO_4$	0.62	[M-CHNO+H]+	134.04472	1
Glutathione	$C_{10}H_{17}N_3O_6S$	0.64	[M]+	307.08285	1
Citric acid	$C_6H_8O_7$	0.64	[M+K]+	230.99227	1
2-maleylacetate	$C_6H_4O_5$	0.64	[M-2H]+	157.01305	3
Adenosine 5'-monophosphate	$C_{10}H_{14}N_5O_7P$	0.64	[M+H]+	348.06929	1
Nicotinic acid	$C_6H_5NO_2$	0.64	[M+H]+	124.03988	1
Methionine	$C_5H_{11}NO_2S$	0.64	[M+H]+	150.0567	1
Succinic acid	$C_4H_6O_4$	0.65	[M+H]+	119.03968	1
Aminoundecanoic acid	$C_{11}H_{23}NO_2$	0.66	[M+H]+	202.17957	3
Uridine	$C_9H_{12}N_2O_6$	0.66	[M+H]+	245.07566	1
Adenine	$C_5H_5N_5$	0.66	[M+H]+	136.06284	1
Nicotinamide	$C_6H_6N_2O$	0.67	[M+H]+	123.04904	1
L-Tyrosine	$C_9H_{11}NO_3$	0.68	[M-NH_2]+	165.05422	1
L-Tyrosine	$C_9H_{11}NO_3$	0.68	[M+H]+	182.08059	1
Adenosine	$C_{10}H_{13}N_5O_4$	0.69	[M+H]+	268.10259	1
Tyramine	$C_8H_{11}NO$	0.73	[M-NH_2]+	121.06483	1
Guanosine	$C_{10}H_{13}N_5O_5$	0.74	[M+H]+	284.09886	2
Tyramine	$C_8H_{11}NO$	0.74	[M+H]+	138.08954	1
Inosine	$C_{10}H_{12}N_4O_5$	0.76	[M+H]+	269.09141	1
Hypoxanthine	$C_5H_4N_4O$	0.76	[M+H]+	137.04988	1
Gamma-methyl L-glutamate	$C_6H_9NO_3$	0.92	[M-H_2O+H]+	144.06494	3
L-Phenylalanine	$C_9H_{11}NO_2$	1.21	[M+H]+	166.08536	1
Phenylalanine	$C_9H_{11}NO_2$	1.23	[M-CH_2O+H]+	120.08096	1
L-Phenylalanine	$C_9H_{11}NO_2$	1.3	[M+CH_2O_2Na]+	234.07395	1
Phenylalanylglutamate	$C_{14}H_{18}N_2O_5$	1.47	[M+H]+	295.13017	3
D-pantothenic acid	$C_9H_{17}NO_5$	1.59	[M+H]+	220.11212	1
HDMBOA, 2-Hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3(4H)-one	$C_{10}H_{11}NO_5$	1.64	[M+H]+	226.0706	3
Protocatechuic acid	$C_7H_6O_4$	1.79	[M+H]+	155.03549	1
L-Tryptophan	$C_{22}H_{24}N_4O_4$	2.33	[2M+H]+	409.1869	1
3,4-Dimethoxyphenethylamine	$C_{10}H_{15}NO_2$	2.34	[M+H]+	182.11745	1
Tryptophan, in-source fragment	$C_{11}H_{12}N_2O_2$	2.35	[M-CHO_2]+	159.09081	1
Tryptophan, in-source fragment	$C_{11}H_{12}N_2O_2$	2.36	[M-C_2H_4NO]+	146.06003	1

L-Tryptophan	$C_{11}H_{12}N_2O_2$	2.37	[M+H]+	205.09657	1
5-S-Methyl-5-thioadenosine	$C_{11}H_{15}N_5O_3S$	2.48	[M+H]+	298.09673	3
Gentisic acid	$C_7H_6O_4$	2.52	[M-H_2O+H]+	137.0248	1
Anthranilate	$C_7H_7NO_2$	2.52	[M+H]+	138.05398	3
Leucyl-Proline	$C_{11}H_20N_2O_3$	2.57	[M+H]+	229.15289	1
Esculin	$C_{15}H_{16}O_9$	2.71	[M+H]+	341.08723	1
3-Amino-4-hydroxybenzoic acid, 5-amino saliciylic acid	$C_7H_7NO_3$	2.77	[M+H]+	154.04987	3
2-Hydroxybenzoic acid, Salicylic acid	$C_7H_6O_3$	2.78	[M-H_2O+H]+	121.02945	1
Tryptamine	$C_{10}H_{12}N_2$	2.79	[M+H]+	161.10689	1
2,4 Dihydroxybenzoic acid	$C_7H_6O_4$	2.9	[M-H_2O+H]+	137.02384	2
β-Carbocine-3-carboxylic acid	$C_{12}H_{12}N_2O_2$	3	[M+H]+	217.09646	2
Hydroxycoumarin 1	$C_9H_6O_3$	3.09	[M+H]+	163.04201	3
Methyl 4-methoxycinnamate	$C_{11}H_{12}O_3$	3.15	[M+H]+	193.0832	3
ubiquitous metabolite	$C_{11}H_{28}N_2O_{12}$	3.17	[M+H]+	381.17402	4
Scopoletin hexose-pentose	$C_{21}H_{26}O_{13}$	3.19	[M+H]+	487.14409	3
Scopolin	$C_{16}H_{18}O_9$	3.2	[M+H]+	355.10117	1
L-Tyrosyl-L-leucine	$C_{15}H_{22}N_2O_4$	3.27	[M+H]+	295.17048	2
Quercetin-3-O-glc-1-3-rham-1-6-glucoside	$C_{33}H_{40}O_{21}$	3.33	[M+H]+	773.20921	3
Fraxin	$C_{16}H_{18}O_{10}$	3.42	[M-$C_6H_{10}O_5$+H]+	209.0463	1
Derivative of dicaffeoyl quinic acid	$C_{24}H_{18}O_{12}$	3.57	[M+H]+	499.087	3
Unknown Hexoside	$C_{19}H_{30}O_8$	3.64	[M+H]+	387.20108	3
7,8-Dihydroxycoumarin	$C_9H_6O_4$	3.67	[M+H]+	179.03724	1
9,10-Dihydrohydroxy-jasmonic acid sulfate	$C_{12}H_{20}O_7S$	3.68	[M+H]+	309.10273	2
Riboflavin	$C_{17}H_{20}N_4O_6$	3.71	[M+H]+	377.14348	1
β-d-glucosyl indole-3-carboxylate	$C_{15}H_{17}NO_7$	3.85	[M+H]+	324.10978	2
Quercetin + Hex + Hex + Fuc	$C_{33}H_{40}O_{21}$	3.92	[M+H]+	773.20551	3
Quercetin + Hex+Hex	$C_{27}H_{30}O_{17}$	3.96	[M+H]+	627.1529	3
Quercetin Deoxyhex Deoxyhex Hex	$C_{33}H_{40}O_{20}$	4.01	[M+H]+	757.21447	3
Saponarin	$C_{27}H_{30}O_{15}$	4.01	[M+H]+	595.16569	2
Quercetin Deoxyhex Hex	$C_{27}H_{30}O_{16}$	4.02	[M+H]+	611.15714	3
Isoorientin	$C_{21}H_{20}O_{11}$	4.02	[M+H]+	449.10799	3

1-O-Feruloyl-beta-D-glucose	$C_{16}H_{20}O_9$	4.06	[M+H]+	357.11916	3
N-Phenylacetyl-Aspartic acid	$C_{12}H_{13}NO_5$	4.07	[M+H]+	252.0869	2
Hydroxyjasmonic acid	$C_{12}H_{18}O_4$	4.11	[M+H]+	227.12685	3
Sinapyl alcohol	$C_{11}H_{12}O_3$	4.17	[M-H_2O+H]+	193.08493	1
Phthalide	$C_8H_6O_2$	4.18	[M+H]+	135.04479	1
Leucyl-Phenylalanine	$C_{15}H_{22}N_2O_3$	4.19	[M+H]+	279.16744	1
Carveol	$C_{10}H_{16}O$	4.2	[M+H]+	135.11612	3
Quercetin+hex+deoxyhex	$C_{27}H_{30}O_{16}$	4.23	[M+H]+	611.15799	3
fragment of Quercetin+hex+deoxyhex	$C_{21}H_{20}O_{12}$	4.24	[M+H]+	465.10365	3
fragment of Quercetin+hex+deoxyhex	$C_{15}H_{10}O_7$	4.24	[M+H]+	303.0499	3
Unknown, similar to Tyrosine	$C_9H_{11}NO_3$	4.26	[M+H]+	182.08109	4
Unknown, similar to Feruloyl glycerol	$C_{13}H_{16}O_6$	4.29	[M+H]+	269.10204	4
Methylumbelliferone	$C_{10}H_8O_3$	4.39	[M+H]+	177.05444	3
Feruloyl dehydrotyramine	$C_{18}H_{17}NO_4$	4.4	[M+H]+	312.12362	3
Scopoletin	$C_{10}H_8O_4$	4.41	[M+H]+	193.05111	1
Methylumbelliferone	$C_{10}H_8O_3$	4.44	[M+H]+	177.05434	3
Rutin, Quercetin 3-o-rutinoside	$C_{27}H_{30}O_{16}$	4.45	[M+H]+	611.15984	1
Rutin, in-source fragment 302	$C_{15}H_{10}O_7$	4.45	[M+H]+	303.0498	1
Rutin, in-source fragment 464	$C_{21}H_{20}O_{12}$	4.45	[M+H]+	465.10349	1
Gibberellic acid	$C_{19}H_{18}O_4$	4.49	[M-H_4O_2+H]+	311.12722	1
Leuko-DOPA-chrome	$C_9H_9NO_4$	4.56	[M+H]+	196.06113	3
Quercetin-3-o-glucoside, in-source fragment -Glc	$C_{15}H_{10}O_7$	4.59	[M+H]+	303.04987	1
Quercetin-3-glucoside, Isoquercitrin	$C_{21}H_{20}O_{12}$	4.59	[M+H]+	465.10251	1
Benzoic acid	$C_7H_6O_2$	4.64	[M+H]+	123.04509	1
N-(3-Hydroxypropyl)-phthalimide; 1H-Isoindole-1,3(2H)-dione	$C_{11}H_{11}NO_3$	4.76	[M+H]+	206.0799	1
Guaiacylglycerol β-coniferyl ether (G(8-O-4)G)	$C_{20}H_{24}O_7$	4.81	[M+H]+	377.16009	3
Kaempferol 3-O-beta-Glc-7-O-alpha-Rha	$C_{27}H_{30}O_{15}$	4.81	[M+H]+	595.1655	2
4-Hydroxy-3,5-dimethylbenzoic acid	$C_9H_{10}O_3$	4.85	[M+H]+	167.07077	2
Unknown, similar to	$C_9H_{11}NO_3$	4.86	[M+H]+	182.08115	4

Tyrosine					
5-Methoxyindole-3-acetic acid	$C_{11}H_{11}NO_3$	4.98	[M+H]+	206.07929	1
Unknown polysaccharide	$C_{12}H_{22}O_{11}$	5.31	[M+H]+	365.10049	4
Kaempferin	$C_{21}H_{20}O_{10}$	5.41	[M+H]+	433.1236	3
3,4-dimethoxycinnamic acid	$C_{11}H_{12}O_4$	5.6	[M+H]+	209.08191	1
Simulanol (S(8-5)G)	$C_{21}H_{24}O_7$	5.67	[M+H]+	389.16803	2
N-feruloyltyramine	$C_{18}H_{19}NO_4$	5.84	[M+H]+	314.13753	2
Glyphosate constituent 1	$C_{16}H_{33}NO_3$	5.89	[M+H]+	288.2528	4
Abscisic acid	$C_{15}H_{18}O_3$	5.9	[M+H]+	247.13147	1
Glycosmisic acid	$C_{20}H_{20}O_7$	5.91	[M+H]+	373.13667	2
p-Coumaric acid-O-dihydroxy-cinnamoyl methyl ester	$C_{19}H_{16}O_6$	5.92	[M+H]+	341.10174	3
Glyphosate constituent 2	$C_{16}H_{33}NO_3$	6.06	[M+H]+	288.25272	4
(E)-Cinnamic acid	$C_9H_8O_2$	6.09	[M+H]+	131.04966	1
Herniarin	$C_{10}H_8O_3$	6.17	[M+H]+	177.05415	1
Glyphosate constituent 3	$C_{16}H_{33}NO_3$	6.3	[M+H]+	288.25232	4
Amaranthus triterpenoid, 1258 (fragment 502)	$C_{30}H_{46}O_6$	6.64	[M+H]+	503.33567	3
Genistein	$C_{15}H_{10}O_5$	6.77	[M+H]+	271.06077	1
Amaranthus triterpenoid, 792	$C_{40}H_{56}O_{16}$	6.79	[M+H]+	793.36031	3
Amaranthus triterpenoid, 840 (fragment 502)	$C_{30}H_{46}O_6$	6.79	[M+H]+	503.33582	3
Jasmonic acid	$C_{12}H_{18}O_3$	6.82	[M+H]+	211.13316	1
Amaranthus triterpenoid, 840	$C_{41}H_{60}O_{18}$	6.82	[M+H]+	841.38418	3
Amaranthus triterpenoid, 1216 (fragment 810)	$C_{40}H_{58}O_{17}$	6.89	[M+H]+	811.38031	3
Amaranthus triterpenoid, 1216 (fragment 764)	$C_{39}H_{56}O_{15}$	6.89	[M+H]+	765.37209	3
Amaranthus triterpenoid, 1216 (fragment 810)	$C_{40}H_{58}O_{17}$	6.98	[M+H]+	811.37071	3
Amaranthus triterpenoid, 1216 (fragment 502)	$C_{30}H_{46}O_6$	6.98	[M+H]+	503.3365	3
Farnesal	$C_{15}H_{24}O$	7.16	[M+H]+	203.17828	2
Amaranthus triterpenoid, 794 (fragment 486)	$C_{30}H_{46}O_5$	7.26	[M+H]+	487.33998	3
Amaranthus triterpenoid, 794 (fragment 308)	$C_{10}H_{12}O_{11}$	7.27	[M+H]+	309.04793	3
Amaranthus triterpenoid, 1405 (fragment 808)	$C_{41}H_{60}O_{16}$	7.59	[M+H]+	809.39309	3

Amaranthus triterpenoid, 824 (fragment 780)	$C_{40}H_{60}O_{15}$	7.84	[M+H]+	781.39422	3
Biochanin A, 5,7-Dihydroxy-4'-methoxyisoflavone (internal standard)	$C_{16}H_{12}O_5$	8.79	[M+Na]+	307.05808	1
Biochanin A, 5,7-Dihydroxy-4'-methoxyisoflavone (internal standard)	$C_{16}H_{12}O_5$	8.79	[M+H]+	285.07614	1
Phytosphingosine	$C_{18}H_{37}NO_3$	9.32	[M+H]+	316.28323	3
Methyl octanoate	$C_9H_{18}O_2$	9.35	[M+H]+	159.13763	3
Dioscin	$C_{39}H_{63}O_{12}$	9.7	[M+H]+	724.44094	3
Lauric acid diethanolamide	$C_{16}H_{33}NO_3$	9.72	[M+H]+	288.25262	3
Glc-Glc-octadecatrienoyl-sn-glycerol (isomer 1)	$C_{33}H_{56}O_{14}$	9.8	[M+H]+	659.36079	3
Monolinolenin (isomer 1)	$C_{21}H_{36}O_4$	9.8	[M+H]+	353.26758	3
Glc-octadecatrienoyl-sn-glycerol (isomer 1)	$C_{27}H_{46}O_9$	10.03	[M+H]+	515.31914	3
Monolinolenin (isomer 2)	$C_{21}H_{36}O_4$	10.03	[M+H]+	353.26763	3
Glc-Glc-octadecatrienoyl-sn-glycerol (isomer 2)	$C_{33}H_{56}O_{14}$	10.04	[M+H]+	699.35227	3
Oxo-phytodienoic acid	$C_{18}H_{28}O_3$	10.24	[M+H]+	315.1929	2
Capric acid	$C_{10}H_{20}O_2$	10.32	[M+H]+	173.15204	1
Lysophosphatidylcholine (isomer 1)	$C_{26}H_{50}NO_7P$	10.62	[M+H]+	520.33946	3
Glc-octadecatrienoyl-sn-glycerol (isomer 2)	$C_{27}H_{46}O_9$	10.75	[M+H]+	497.31063	3
Linolenoylglycerol	$C_{21}H_{36}O_4$	10.75	[M+H]+	353.26762	3
Lysophosphatidylcholine (isomer 2)	$C_{26}H_{50}NO_7P$	10.84	[M+H]+	520.33978	3
Lysophosphatidylcholine (isomer 3)	$C_{24}H_{50}NO_7P$	11.03	[M+H]+	496.34017	3
Hydroxyoctadecadienoic acid (isomer 1)	$C_{18}H_{32}O_3$	11.33	[M+H]+	319.22361	3
Lysophosphatidylcholine (isomer 4)	$C_{26}H_{52}NO_7P$	11.44	[M+H]+	522.35566	3
1-Oleoyl-sn-glycero-3-phosphocholine	$C_{26}H_{52}NO_7P$	11.67	[M+H]+	522.35557	3
Hydroxyoctadecadienoic acid (isomer 2)	$C_{18}H_{30}O_3$	11.68	[M+H]+	277.21434	3
Lauric acid	$C_{12}H_{24}O_2$	12.09	[M+H]+	201.18435	1
Phthalic anhydride	$C_8H_4O_3$	12.2	[M+H]+	149.02348	3
1-Stearoyl-2-hydroxy-sn-glycero-3-phosphocholine	$C_{26}H_{54}NO_7P$	12.7	[M+H]+	524.37025	3

1-Stearoyl-sn-glycero-3-phosphate	$C_{21}H_{41}O_7P$	13.64	[M+H]+	437.2642	3
Linoloylglycerol	$C_{21}H_{38}O_4$	13.7	[M+H]+	377.26543	3
Oxylipin	$C_{36}H_{60}O_4$	13.72	[M+H]+	557.45365	3
linolenic acid (isomer1)	$C_{18}H_{30}O_2$	13.72	[M+H]+	279.23108	3
monoglyceride	$C_{20}H_{34}O_3$	13.89	[M+H]+	345.23948	3
linolenic acid (isomer 2)	$C_{18}H_{30}O_2$	13.89	[M+H]+	279.22526	3
13-apo-β-carotenone	$C_{18}H_{26}O$	13.97	[M+H]+	259.20489	3
Oleamide	$C_{18}H_{35}NO$	14.31	[M+H]+	282.27812	3
1-Palmitoylglycerol	$C_{19}H_{38}O_4$	14.36	[M+H]+	353.26486	3
17-Octadecynoic acid	$C_{18}H_{32}O_2$	14.58	[M+H]+	263.23577	3
1-Stearoyl-rac-glycerol	$C_{21}H_{42}O_4$	15.83	[M+H]+	359.31449	3
Diacylglycerine	$C_{39}H_{64}O_5$	17.82	[M+H]+	613.481	3

CHAPTER 4.

Regulation of the shikimate pathway by final products or intermediates

4.1. OBJECTIVE

The previous chapters have shown that the AAA biosynthetic pathway is disrupted and its regulation is affected after the inhibition of the EPSPS enzyme by the herbicide glyphosate, after applying quinate and after the application of the combination of both compounds. However, the regulatory mechanisms underlying response of the pathway remain unclear, and the specific role of the intermediates or final products has not been deeply studied.

The aim of this chapter was to **evaluate the role of aromatic amino acids and the intermediates in the regulation of shikimate pathway. It was performed by analyzing if aromatic amino acids could revert the effects of glyphosate on the pathway and if the supply of intermediates of the pathway could mimic the glyphosate effects, using *Amaranthus palmeri* sensitive (GS) and resistant (GR) plants to glyphosate.** To this end, leaf disks of GS and GR *Amaranthus palmeri* plants were incubated with glyphosate, intermediates and final products of the AAA biosynthetic pathway and it was assessed the relative expression level of the enzymes and protein content of the pathway.

This aim was approached by three specific objectives:

- To determine if the final products of the pathway, the AAAs, would have a role in the pathway regulation it was studied the effect of AAA exogenous application on the pathway performance and if they could revert the herbicide effect. Leaf disks were incubated with AAAs, glyphosate, and their combination and it was measured: shikimate content, protein content and transcript level of the shikimate pathway.

- To discern whether any metabolite of the pathway plays a key role in the shikimate pathway regulation it was studied the effect of their exogenous application on the pathway performance and if they could mimic the glyphosate effect on the pathway. Leaf disks were incubated with four different intermediates of the AAA biosynthetic pathway: shikimate, quinate, chorismate and anthranilate and their effect was monitored at the level of shikimate content, protein content and transcript level of the shikimate pathway.

- To evaluate the potential role of the metabolites of the shikimate pathway on other amino acid biosynthetic pathway that has been proposed to be cross-regulated with the AAA biosynthetic pathway. It

was determined the relative mRNA level of the genes of the BCAA pathway in leaf disks incubated with glyphosate, final products and intermediates of the AAA biosynthetic pathway.

4.2. EXPERIMENTAL APPROACH

4.2.1. Plant material and treatment application

Amaranthus palmeri plants of GS and GR populations were grown hydroponically under controlled conditions as described in Section MM.1.1. Before performing the experiment, the EPSPS relative genomic copy number (Table 4.1) was determined in 48 plants of GR population in order to select specifically the individuals with a similar relative genomic copies (between 60 and 100). After evaluating the results, 30 plants were selected with this characteristic trying to reduce the variability of the population and to uniform the response due to the EPSPS overexpression.

Table 4.1. Analytical determinations realized before performing the experiments 4.A and 4.B and their sections in this document.

Nucleic acid determination	
EPSPS relative genomic copy number	Section M.M.2.1.1.

Treatments (Table 4.2) were applied by incubating leaf disks during 24 h under continuous light at 24 °C. Solutions were prepared freshly and pH was adjusted to 7.0 with NaOH in all the treatments as described in section M.M.1.3.

Table 4.2. Treatments applied to glyphosate sensitive and glyphosate resistant *Amaranthus palmeri* leaf disks in Chapter 4. (a.e.=acid equivalent).

Identification	Treatment	Dose
C	Control	-
G	Glyphosate	1.75 g a.e.L^{-1}
S	Shikimate	20 mM
Q	Quinate	50 mM
Ch	Chorismate	1 mM
At	Anthranilate	1 mM
AAA	Aromatic amino acids	10 µM (each AAA)
AAA + G	Aromatic amino acids + Glyphosate	10 µM+ 1.75 g a.e.L^{-1}

4.2.2. Shikimate, enzyme content and transcript level determination

As described in section M.M.2.5, for shikimate content determination one disk was placed in each well of a 96-well microtiter plate containing 100 µL of treatment solutions. From the same leaf it was excised a disk for each treatment, in order to obtain one disk per treatment from the same plant. For enzyme content and transcript level determinations, 25 or 45 disks were placed, respectively, in a 6-well microplate containing 2.5 mL of each treatment. In each well, disks of different leaves were incubated, but the same proportion of original plants was maintained in all treatment tested. Disks were washed thoroughly before freezing. In both incubation options, each well was considered as a biological replicate.

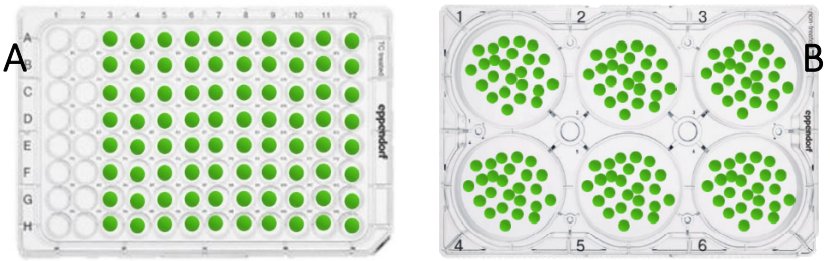

Figure 4.1. Leaf disk incubation system. Leaf disks were excised from glyphosate sensitive and glyphosate resistant plants of *Amaranthus palmeri* and incubated for 24 h. One disk per well was incubated for shikimate content determination (A) and 25 or 45 were incubated for enzyme content and transcript level determination, respectively (B).

Table 4.3. Analytical determinations realized before performing in Chapter 4 and their sections in this document.

Metabolite content	
Shikimate	Section M.M.2.5.
Enzyme content	
DAHPS	Section M.M.2.2.
EPSPS	Section M.M.2.2.
Relative gene expression	
Transcript level (AAA biosynthetic pathway)	Section M.M.2.1.2.

4.2.3. Statistical analysis

Analysis were performed using 10 biological replicates for shikimate content determination and 3 or 4 biological replicates for enzyme content or nucleic acid determination, respectively. Statistical analysis were different in each experiment performed, each corresponding to each specific objective. First, in the experiment of glyphosate and aromatic amino acids, differences between treatments for each population were evaluated performing one-way ANOVA with a multiple-comparison adjustment (Tuckey) at $p \leq 0.05$. Second, in the experiment using the intermediates the difference between each parameter of untreated disks and disks of each treatment was evaluated using Student's t test ($p \leq 0.05$). Third, for the evaluation of the effects on the expression level of BCAA pathway genes, differences between untreated disks and disks of each treatment Student's t tests ($p \leq 0.05$) were performed.

4.3. RESULTS AND DISCUSSION

4.3.1. Possible pathway regulation by aromatic amino acids. Could AAAs revert the changes induced in the pathway by glyphosate?

In this section, leaf disks were incubated with glyphosate, the three AAAs (Tyr, Phe and Trp), and the combination of the glyphosate and the AAAs for 24 h at 24°C. After incubation, shikimate content, DAHPS and EPSPS protein content and transcript level of the genes of the AAA biosynthetic pathway was measured.

The combined treatment AAA+G was studied trying to unravel whether the availability of the final products of the pathway could reverse the physiological effects of the herbicide on the shikimate pathway. The reversion of glyphosate effect with exogenous AAAs has been previously evaluated by various authors, but it has not been completely elucidated. It was suggested that Tyr and Phe are the amino acids with greater glyphosate reversing effect (Gresshoff 1979; Eason et al. 2000). Indeed, studies in *E. coli* suggested a synergistic interaction of these two amino acids in the reversion of the inhibitory effect of the herbicide (Gresshoff 1979). Nevertheless, these previous studies focused on reversion of effects on growth parameters, while this study deals specifically with the potential reversion of the effects of glyphosate on the shikimate pathway by AAAs.

4.3.1.1. Shikimate content

Shikimate content was determined in leaf disks of *Amaranthus palmeri* plants after 24 h incubation with treatment solutions (Figure 4.2). Shikimate content was very low and similar between untreated plants in both populations, while after glyphosate treatment, a significant increase in shikimate content was observed in both populations. This result validates this incubation system as an experimental approach to study the physiological effects of glyphosate, because it was detected a similar effect than when the herbicide is pulverized to the plants (as observed in the previous chapters of this study). Moreover, as detected before, the increase was more intense on GS population than in GR. In this experiment, in GS population, glyphosate treated plants showed an 11-fold increase in shikimate content compared to control plants, while in GR population it was 1.7 fold.

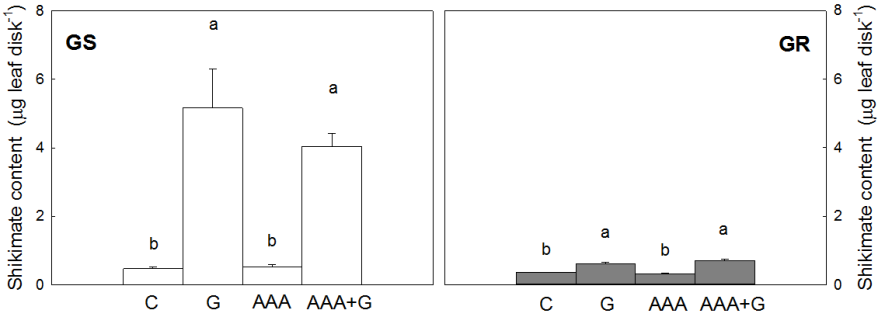

Figure 4.2. Shikimate content. Leaf disks of glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bar, right; GR) *Amaranthus palmeri* populations were incubated 24 h with water (C), aromatic amino acids (AAA), glyphosate (G) or the combination of aromatic amino acids and glyphosate (AAA+G) (Mean ± SE; n=10). Different letters indicate differences between treatments in each population (*p* value ≤ 0.05, Tukey).

AAAs applied alone did not modify the shikimate content in any of the populations, suggesting that the exogenous supply of the end products of the pathway does not modify the intermediate content of the pathway. Moreover, when AAAs were applied in combination with glyphosate (AAA+G treatment) the shikimate content detected was not significantly different to the one provoked by the herbicide applied alone, neither enhancing nor relieving the herbicide effect.

In both populations the accumulation of shikimate was similar in the two treatments with glyphosate, regardless of the presence of AAAs (Figure 4.2). This result suggests that shikimate accumulation would be directly related to the EPSPS inhibition and not to other physiological changes caused by the effect of the herbicide, such as a potential transitory modification on AAA content.

4.3.1.2. Enzyme content

DAHPS and EPSPS enzyme content was determined by inminoblotting (Figure 4.3). Glyphosate and AAA treatments did not provoke any significantly differences applied alone. The incubation with both compounds combined provoked different response depending on the population. In GS, it provoked an increase in DAHPS content (Figure 4.3.A), but did not modify the content of EPSPS enzyme (Figure 4.3.B). On the contrary, DAHPS content was not altered by this treatment but a significant increase in EPSPS content was observed in GR population (Figure 4.3.B).

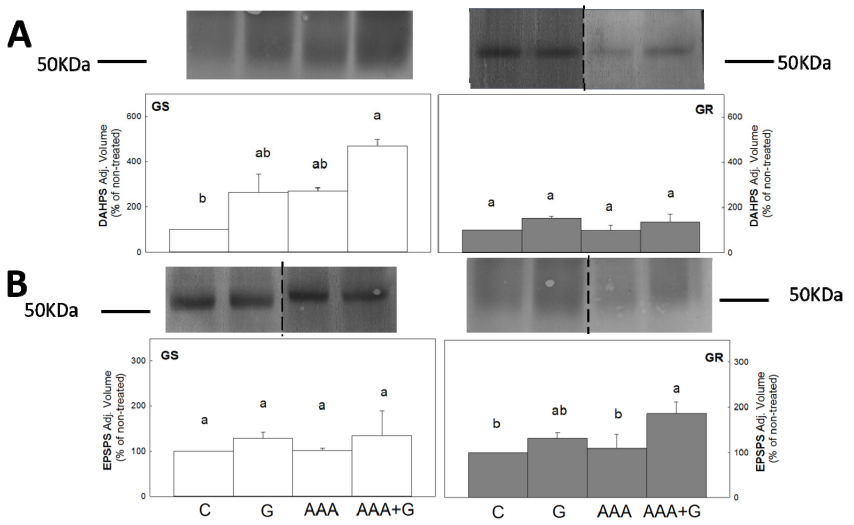

Figure 4.3. Enzymes of the aromatic amino acid biosynthetic pathway. (A) Normalization of the quantity of 3-deoxy-D-arabino-heptulosonate-7-phosphate-synthase (DAHPS). Top: Representative immunoblots for DAHPS are plotted, and lanes contained 40 µg of total soluble proteins. (B) Normalization of the quantity of 5-enolpyruvylshikimate 3-phosphate (EPSPS). Top: Representative immunoblots for EPSPS are plotted. Lanes contained 80 µg of protein for GS or 15 µg of protein for GR of total soluble proteins. In blots, each vertical dividing line indicates lane removal from the original blot. Leaf disks of glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bar, right; GR) *Amaranthus palmeri* populations were incubated 24 h with water (C), aromatic amino acids (AAA), glyphosate (G) or the combination of aromatic amino acids and glyphosate (AAA+G) (Mean ± SE; n=3). Different letters indicate differences between treatments in each population (p value ≤ 0.05, Tukey).

It was not detected increase of DAHPS or EPSPS enzyme content after incubation with glyphosate, contrary to previous reports (Pinto et al. 1988; Fernández-Escalada et al. 2017). In this experiment, the lack of increase in their content maybe related to the short incubation period or the low dose applied. In the previous experiments where an increase in DAHPS and EPSPS content was observed, time of treatment was 48 or 72h (Baerson et al. 2002; Fernández-Escalada et al. 2017).

In microbes, it has been widely described that the expression of DAHPS is regulated in response to cellular levels of AAAs. However, most of the studies performed in plants suggest that DAHPS would not be regulated by AAAs. Only few exceptions have reported a regulatory effect of the AAA levels on the activity of this enzyme *in vitro* in plants (Graziana and Boudet 1980; Suzich et al. 1985). Therefore, as it was expected, AAA treatment applied alone did not modify DAHPS content in this experiment. The application of AAAs and glyphosate together induced a significant increase of the DAHPS content in GS population (Figure 4.3), enhancing the non significant increase detected after glyphosate and

supporting that, in plants, the DAHPS content is not downregulated when AAA are externally supplied.

4.3.1.3. Transcript level

Relative transcript level of the genes of the AAA biosynthetic pathway was determined after incubation with the treatments. Transcript level of the pre-*EPSPS* genes: *DAHPS, DHQS, DQSD* and *SK* (Figure 4.4); post *EPSPS* genes: *EPSPS, CS* and *AS* (Figure 4.5); and post-chorismate genes *CM2, CM1-3, ADHα* and *ADH β* (Figure 4.6) was measured and expressed normalized to the *β-tubulin* expression level as housekeeping gene.

Glyphosate produced an increase in the relative expression level of the genes of the pre-chorismate part of the AAA biosynthetic pathway in GS population (Figures 4.4 and 4.5). However, in the post-chorismate pathway glyphosate treatment only provoked an increase in *ADHα* gene (Figure 4.6. C). In GR population, the response did not follow the same pattern, in which glyphosate treatment did not induce the relative expression level of the genes of the pathway, and even it was detected a decrease in the *ADHα* relative gene expression level (Figure 4.4.C).

The increase of relative transcript level of the genes of the AAA biosynthetic pathway detected after glyphosate in the GS population match with results previously reported in these populations (Fernández-Escalada et al. 2017) and in other species (Baerson et al. 2002; Garg et al. 2014); validating leaf disks incubation as a method of treatment to study the physiological effects of glyphosate and external supply of products and intermediates of the shikimate pathway.

The only effect detected after AAAs treatment was a decrease of *DQSD* relative mRNA level in GS plants (Figure 4.4.C) and no effects were observed in GR population. In plants, there is limited information about the effect of AAA levels on the expression of the shikimate pathway genes. Although the underlying molecular mechanisms are currently unknown, it has been suggested that reduced level of AAAs may act as a signal to induce the expression of the shikimate pathway and restore the carbon flux through the pathway in plants (Maeda and Dudareva 2012). In concordance with this hypothesis, it could have been expected a decrease in the relative expression level after the exogenous supply of AAAs. Nevertheless, this did not happen as no changes in the relative expression level were detected in GS or GR population after AAA supply. In the

same way as the results obtained in this study, the increase of Phe and Tyr in *Arabidopsis* transgenic plants had a very minor influence, if at all, on the transcriptome (Dubouzet et al. 2007; Tzin et al. 2009). Interestingly, the only exception was the decrease in the relative gene expression of the bifunctional enzyme *DQSD* gene in GS, suggesting a higher sensitivity of this enzyme to AAA level and that the AAA content would act as a signal in this gene regulation.

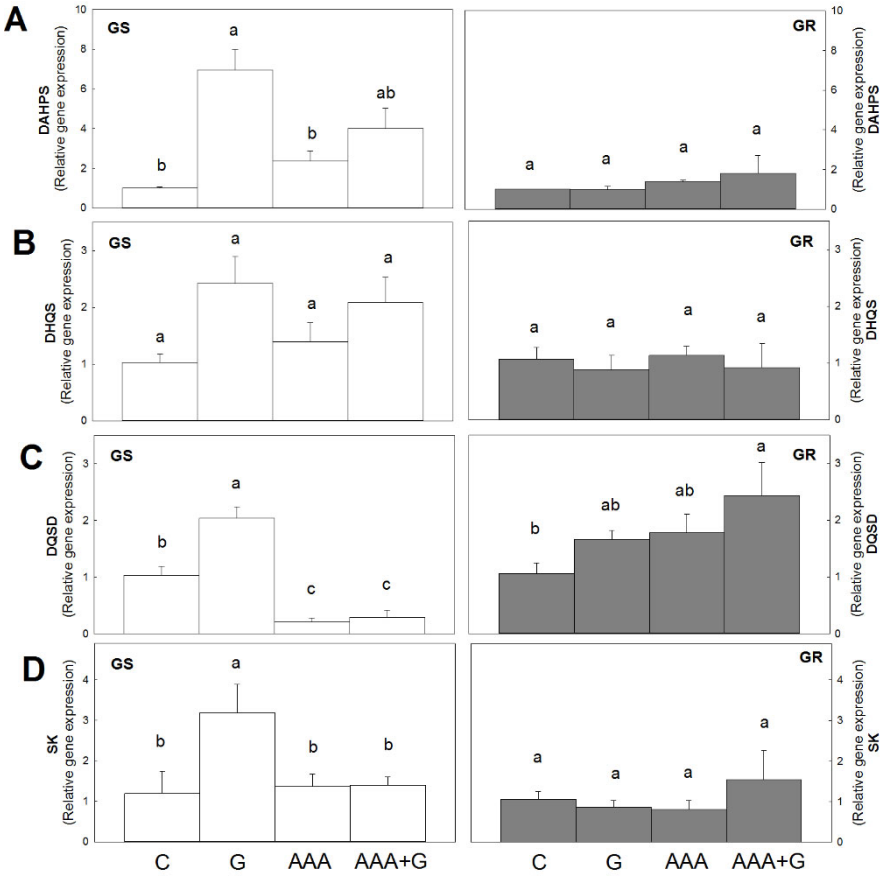

Figure 4.4. Transcript abundance of genes in the pre-EPSPS aromatic amino acid (AAA) biosynthetic pathway was measured in *Amaranthus palmeri* leaf disks. Glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations were incubated for 24 h with water (Control, C), aromatic amino acids (AAA), glyphosate (G) or the combination of aromatic amino acids and glyphosate (AAA+G). Relative transcript abundance was normalized using the normalization gene *beta tubulin* and each population to its own daily control of 3-Deoxy-d-arabino-heptulosonate-7-phosphate synthase (*DAHPS*; A), dehydroquinate synthase (*DHQS*; B), 3-dehydroquinate dehydratase/shikimate dehydrogenase (*DQSD*; C), shikimate kinase (*SK*; D) (Mean ± SE; n=4). Different letters indicate differences between treatments in each population (*p* value ≤ 0.05, Tukey).

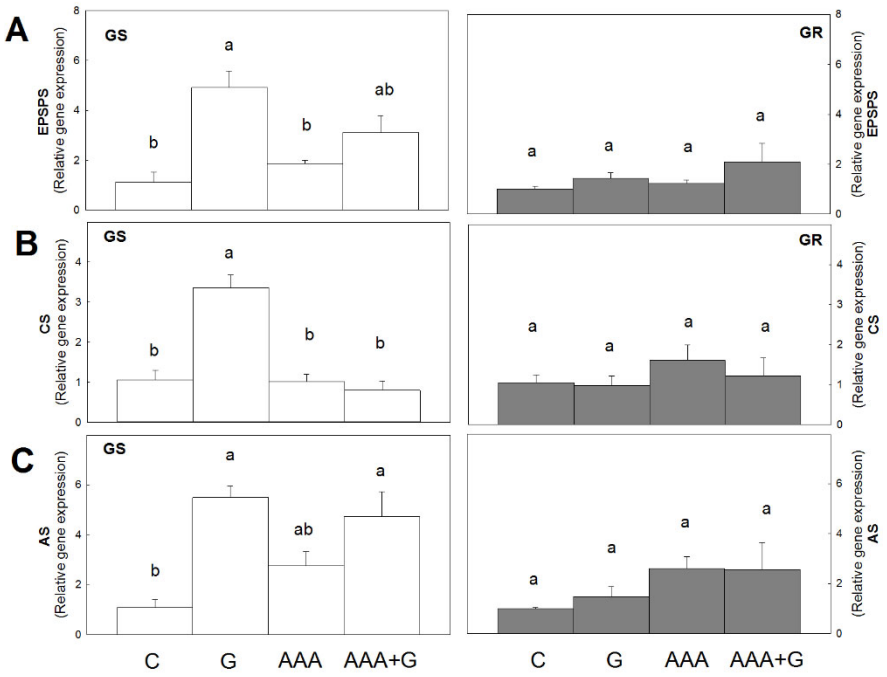

Figure 4.5. Transcript abundance of genes in the post-EPSPS aromatic amino acid (AAA) biosynthetic pathway was measured in *Amaranthus palmeri* leaf disks. Glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations were incubated for 24 h with water (Control, C), aromatic amino acids (AAA), glyphosate (G) or the combination of aromatic amino acids and glyphosate (AAA+G). Relative transcript abundance was normalized using the normalization gene beta tubulin and each population to its own daily control of 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*; A), chorismate synthase (*CS*; B), anthranilate synthase (*AS*; C) (Mean ± SE; n=4). Different letters indicate differences between treatments in each population (*p* value ≤ 0.05, Tukey).

When AAAs were applied in combination with glyphosate (AAA+G), a different response was observed in both populations. On one hand, in GR population no changes after the combined treatment were detected, with the exception of DQSD where a significant increase related to control vales was detected (Figure 4.4). On the other hand, the presence of AAAs in the combined treatment reversed the increase in transcript levels of the shikimate pathway (Figures 4.4 to 4.6) detected after the herbicide alone in GS population. In this treatment, the effect of glyphosate in the relative expression level of the genes *DQSD, SK, CS* or *ADHα* seemed to be abolished by the presence of AAAs. Other genes of the AAA biosynthetic pathway, for example *DAHPS* and *EPSPS*, showed a mixed effect of both compounds in relative gene expression. These results would match with authors that propose that the AAAs reverse the glyphosate effect (Gresshoff 1979; Killmer et al. 1981; Eason et al. 2000) and suggest that reduced level of AAAs may act as a signal to induce the expression of the shikimate pathway. It

seems that, in the GS population, the increase in gene expression of the pathway after glyphosate might be mediated by a transitory lack of the AAAs, as their exogenous supply can abolish the gene upregulation.

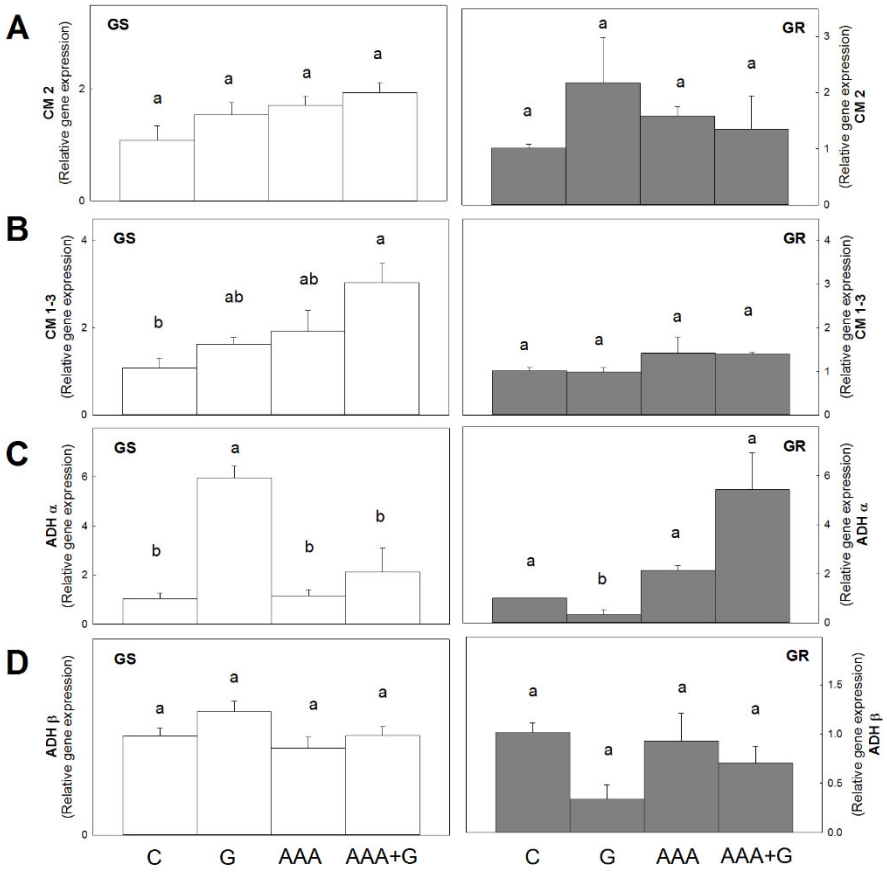

Figure 4.6. Transcript abundance of genes in the post-chorismate aromatic amino acid (AAA) biosynthetic pathway was measured in *Amaranthus palmeri* leaf disks. Glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations were incubated for 24 h with water (Control, C), aromatic amino acids (AAA), glyphosate (G) or the combination of aromatic amino acids and glyphosate (AAA+G). Relative transcript abundance was normalized using the normalization gene beta tubulin and each population to its own daily control of chorismate mutase isoform 2 (*CM2*; A), chorismate mutase isoforms 1 and 3 (*CM 1-3*; B), arogenate dehydrogenase isoform α (*ADHα*; C) and arogenate dehydrogenase isoform β (*ADHβ*; D) (Mean ± SE; n=4). Different letters indicate differences between treatments in each population (p value ≤ 0.05, Tukey).

In this study, the exogenous supply of AAAs did not induce any important change in the transcriptome of the shikimate pathway, nevertheless, when applied in combination with glyphosate the upregulation of gene expression detected after glyphosate was abolished. The AAA+G treatment did not show a very clear

pattern in the possible reversion of glyphosate effect, as it was different depending on the parameter evaluated: shikimate content or gene expression level. Summarizing, it can be proposed that the effect of glyphosate on relative expression level is mediated by a transitory lack of the final products, as detected in the GS population. On the contrary, it seems that shikimate accumulation is a dose-response direct effect of EPSPS inhibition detected in both populations and can not be abolished by an increase in the AAA availability.

4.3.2. Shikimate pathway regulation by intermediates of the pathway

After evaluating the role of the final products of the pathway in the response of the shikimate pathway to glyphosate, it was approached if the response was mediated by any specific intermediate. So, it was evaluated if the supply of any intermediate of the pathway could mimic the physiological effects detected in glyphosate-treated plants.

Leaf disks of plants of both populations were incubated with different intermediates related to the shikimate pathway: shikimate, quinate, chorismate and anthranilate. Shikimate, chorismate and anthranilate are specific intermediates of the pathway while quinate is a metabolite formed in a secondary branch of the shikimate pathway. All previous known parameters affected by glyphosate were measured: shikimate, DAHPS and EPSPS enzyme content and relative transcript level of the genes of the AAA biosynthetic pathway.

4.3.2.1. Shikimate content Shikimate content was determined (Figure 4.7) after the incubation with the intermediates and the pattern observed in both populations was similar. The incubation with shikimate increased shikimate content, confirming that the compound was absorbed (Figure 4.7). Quinate treatment also increased shikimate content after the incubation. In both compounds, the increase observed in GS was more intense than in GR population. Interestingly, anthranilate provoked a slight significant increase in shikimate content in both populations, similar to the increase detected after quinate incubation.

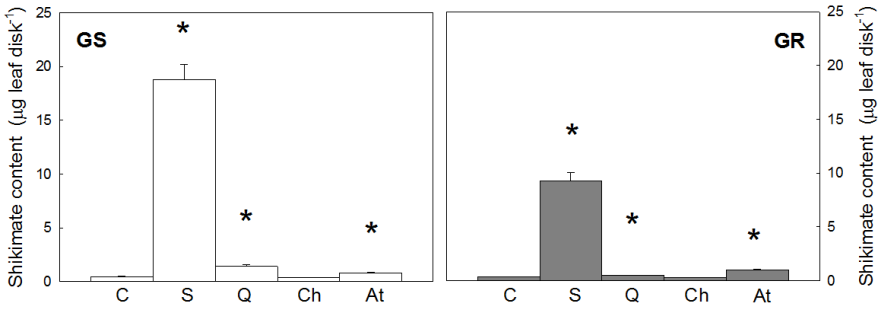

Figure 4.7. Shikimate content. Leaf disks of glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bar, right; GR) *Amaranthus palmeri* populations were incubated 24 h with water (C), shikimate (S), quinate (Q), chorismate (Ch) or anthranilate (At) (Mean ± SE; n=10). * Symbol indicates differences between control and treatment in each population (*p* value ≤ 0.05).

4.3.2.2. Enzyme content

DAHPS and EPSPS content were determined after disk incubation in both populations (Figure 4.8). The incubation with shikimate and quinate did not provoke any changes in the amount of these two enzymes in any population. Incubation with anthranilate provoked different response depending on the population and on the specific enzyme, because this intermediate provoked an increase in EPSPS content in GS (Figure 4.8.B) and a decrease in DAHPS expression in GR population (Figure 4.8.A). Interestingly, chorismate was the only intermediate that provoked a general significant increase of both enzymes in both populations.

4.3.2.3. Transcript level

Relative transcript level of the genes of the AAA biosynthetic pathway (*DAHPS, DHQS, DQSD, SK, EPSPS, CS, AS, CM2, CM1-3, ADHα* and *ADH β*) were determined

and expressed normalized to the *β-tubulin* expression level as housekeeping gene after incubation with the intermediates shikimate (Figure 4.9), quinate (Figure 4.10), chorismate (Figure 4.11) and anthranilate (Figure 4.12).

The incubation with shikimate provoked an upregulation of more than the half of the genes of the AAA pathway in GS population (Figure 4.9). Actually, in this population the relative mRNA level was more affected in the post EPSPS part of the shikimate pathway. Interestingly, this pattern was not observed in GR population, in which only *DQSD* and the isoform *ADHα* increased their relative mRNA level after shikimate.

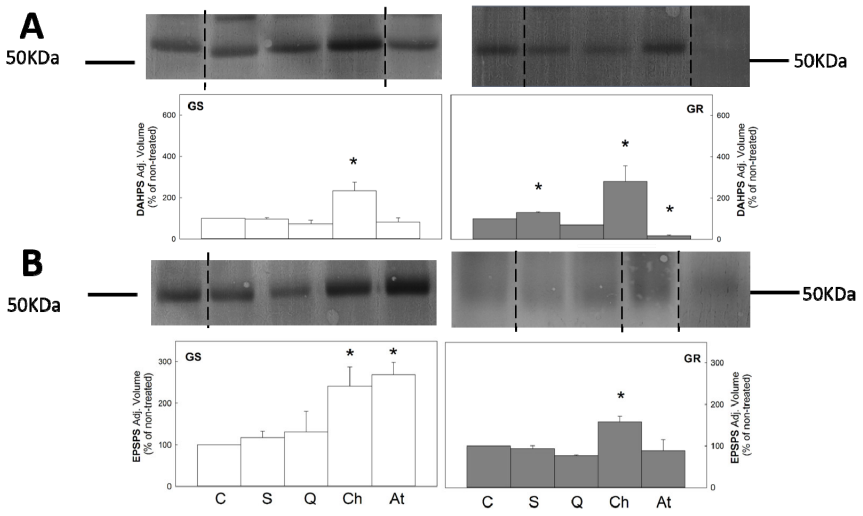

Figure 4.8. Enzymes of the aromatic amino acid biosynthetic pathway. (A) Normalization of the quantity of 3-deoxy-D-arabino-heptulosonate-7-phosphate-synthase (DAHPS). Top: Representative Immunoblots for DAHPS are plotted, and lanes contained 40 µg of total soluble proteins. (B) Normalization of the quantity of 5-enolpyruvylshikimate 3-phosphate (EPSPS). Top: Representative Immunoblots for EPSPS are plotted. Lanes contained 80 µg of protein for GS or 15 µg of protein for GR of total soluble proteins. In blots, each vertical dividing line indicates lane removal from the original blot. Leaf disks of glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bar, right; GR) *Amaranthus palmeri* populations were incubated 24 h with water (C), shikimate (S), quinate (Q), chorismate (Ch) or anthranilate (At) (Mean ± SE; n=3). * Symbol indicates differences between control and treatment in each population (p value ≤ 0.05).

In GS population, quinate treatment (Figure 4.10) also provoked induction of the relative expression level in more than the half (six out of 11) of the genes of the pathway, although the level of increase was milder than the detected after shikimate. In GR population, four genes of the pathway were upregulated after quinate incubation. One of them was *DQSD*, which showed the opposite behavior in GS population, where it was decreased.

The incubation with chorismate (Figure 4.11) did not produce any overall changes in the transcript level in any of the two populations. In GS, only upregulation was observed in *DHQS*, *CS* and *AS* genes, being the most striking increase in *AS* gene, the enzyme that uses chorismate as a substrate. In GR population no changes in relative gene expression were observed.

As chorismate, anthranilate did not provoke any overall or significant effect on gene expression level of the shikimate pathway (Figure 4.12). Interestingly, only a reduction in the relative transcript level of the plastid isoform of *CM (CM1-3*) and *ADHα* were observed in GS. In GR population, the upregulation of *SK* gene was the only significant increase detected.

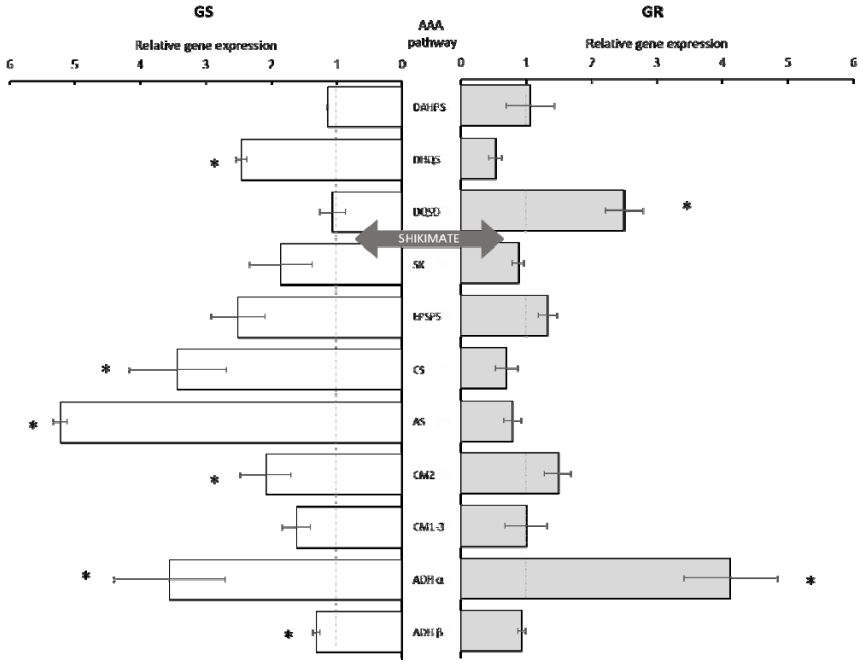

Figure 4.9. Transcript abundance of genes in the EPSPS aromatic amino acid (AAA) biosynthetic pathway was measured in *Amaranthus palmeri* leaf disks. Glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations were incubated for 24 h with **shikimate**. Relative transcript abundance was normalized using the normalization gene beta tubulin and each population to its own daily control of 3-Deoxy-d-arabino-heptulosonate-7-phosphate synthase (*DAHPS*), dehydroquinate synthase (*DHQS*), 3-dehydroquinate dehydratase/shikimate dehydrogenase (*DQSD*), shikimate kinase (*SK*), 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*), chorismate synthase (*CS*), anthranilate synthase (*AS*), chorismate mutase isoform 2 (*CM2*), chorismate mutase isoforms 1 and 3 (*CM1-3*), arogenate dehydrogenase isoform α (*ADHα*) and arogenate dehydrogenase isoform β (*ADHβ*) (Mean ± SE; n=4). * Symbol indicates differences between control and treatment in each population (*p* value ≤ 0.05).

The shikimate pathway uses carbon from primary metabolism to form the essential AAAs, not only as components of protein synthesis but also serve as precursors for a wide range of secondary metabolites with multiple biological functions in plants. Due to its importance for plant biology, the synthesis of AAA is a tightly regulated process controlled at different levels, and not completely elucidated. In this experiment, it has been approached the transcriptional regulation of the shikimate pathway genes by four metabolites of the same pathway. Nevertheless, it has to be pointed out that regulation of the pathway can take place at posttranscriptional or allosteric regulation level.

The incubation with **shikimate** provoked different response in both populations: Shikimate was accumulated in the leaves when was applied exogenously, although shikimate accumulation in GS was around 2 fold higher than in GR, suggesting that the overexpression of EPSPS in GR would be related to a capability of metabolizing further the compound. Interestingly, it was also detected shikimate accumulation after quinate incubation, which was not observed in the previous chapters of this study, when quinate was exogenously applied to the leaves of the whole plant. This can be explained by the methodology used in each experiment, as quinate applied onto the leaves of a whole plant would be metabolized and/or translocated, while quinate application to a leaf disk can not be further translocated and has to be metabolized by the own disk, as a carbon input of the shikimate pathway and increasing the shikimate content.

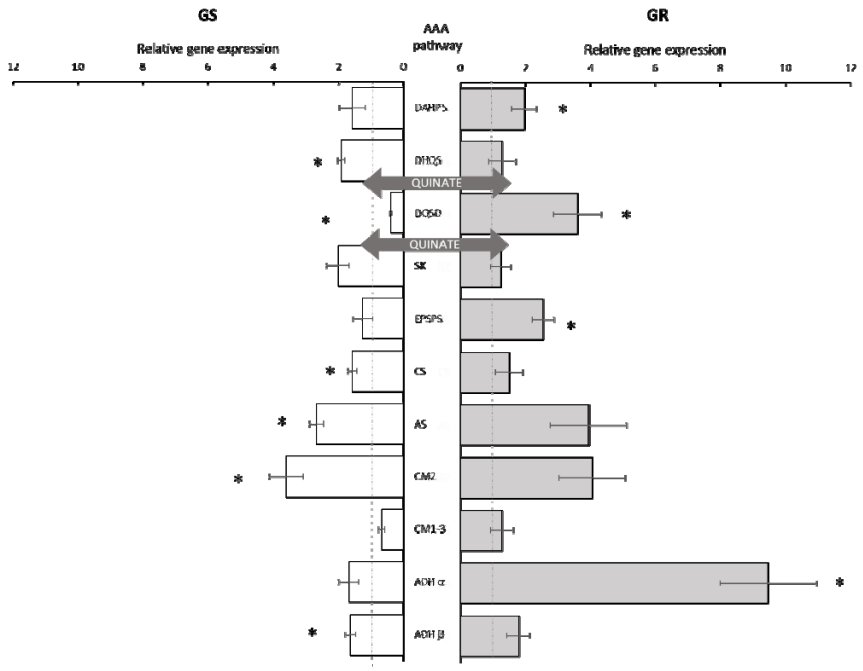

Figure 4.10. Transcript abundance of genes in the EPSPS aromatic amino acid (AAA) biosynthetic pathway was measured in *Amaranthus palmeri* leaf disks. Glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations were incubated for 24 h with **quinate**. . Relative transcript abundance was normalized using the normalization gene beta tubulin and each population to its own daily control of 3-Deoxy-d-arabino-heptulosonate-7-phosphate synthase (*DAHPS*), dehydroquinate synthase (*DHQS*), 3-dehydroquinate dehydratase/shikimate dehydrogenase (*DQSD*), shikimate kinase (*SK*), 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*), chorismate synthase (*CS*), anthranilate synthase (*AS*), chorismate mutase isoform 2 (*CM2*), chorismate mutase isoforms 1 and 3 (*CM1-3*), arogenate dehydrogenase isoform α (*ADHα*) and arogenate dehydrogenase isoform β (*ADHβ*) (Mean ± SE; n=4). * Symbol indicates differences between control and treatment in each population (*p* value ≤ 0.05).

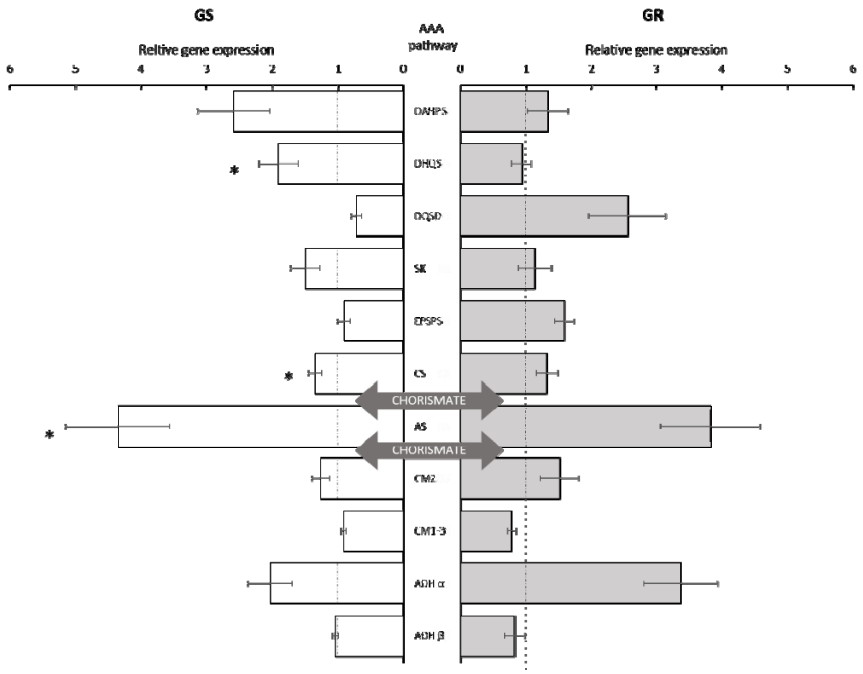

Figure 4.11. Transcript abundance of genes in the EPSPS aromatic amino acid (AAA) biosynthetic pathway was measured in *Amaranthus palmeri* leaf disks. Glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations were incubated for 24 h with **chorismate**. . Relative transcript abundance was normalized using the normalization gene beta tubulin and each population to its own daily control of 3-Deoxy-d-arabino-heptulosonate-7-phosphate synthase (*DAHPS*), dehydroquinate synthase (*DHQS*), 3-dehydroquinate dehydratase/shikimate dehydrogenase (*DQSD*), shikimate kinase (*SK*), 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*), chorismate synthase (*CS*), anthranilate synthase (*AS*), chorismate mutase isoform 2 (*CM2*), chorismate mutase isoforms 1 and 3 (*CM1-3*), arogenate dehydrogenase isoform α (*ADHα*) and arogenate dehydrogenase isoform β (*ADHβ*) (Mean ± SE; n=4). * Symbol indicates differences between control and treatment in each population (*p* value ≤ 0.05).

Upregulation of 6 genes of the shikimate pathway (mainly located in the post-EPSPS part) was detected after shikimate and quinate incubation in GS, which was quite similar to the response observed after glyphosate treatment. The increase in shikimate content would be responsible of the increase in the relative expression level of the genes of the AAA biosynthetic pathway. Shikimate content is highly accumulated after glyphosate application due to the EPSPS inhibition. So, it can be proposed that shikimate accumulation has a role in the increase in the relative expression level of the genes of the pathway when the herbicide is applied. Moreover, this increase in the relative transcript level was mainly observed in GS population, and not in GR, which can be related to the higher shikimate accumulation detected in GS than in GR after shikimate incubation.

Moreover, effect of quinate on the gene upregulation would be mediated by shikimate accumulation too, as shikimate was accumulated after quinate treatment in both populations and relative expression was significantly higher after quinate in 6 and 4 genes of GS and GR populations, respectively.

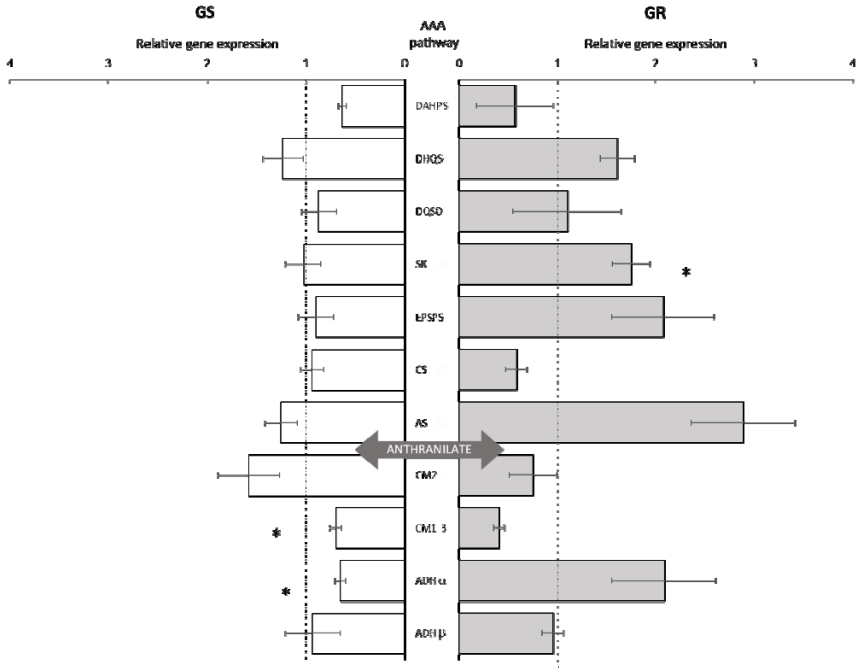

Figure 4.12. Transcript abundance of genes in the EPSPS aromatic amino acid (AAA) biosynthetic pathway was measured in *Amaranthus palmeri* leaf disks. Glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations were incubated for 24 h with **anthranilate**. . Relative transcript abundance was normalized using the normalization gene beta tubulin and each population to its own daily control of 3-Deoxy-d-arabino-heptulosonate-7-phosphate synthase (*DAHPS*), dehydroquinate synthase (*DHQS*), 3-dehydroquinate dehydratase/shikimate dehydrogenase (*DQSD*), shikimate kinase (*SK*), 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*), chorismate synthase (*CS*), anthranilate synthase (*AS*), chorismate mutase isoform 2 (*CM2*), chorismate mutase isoforms 1 and 3 (*CM1-3*), arogenate dehydrogenase isoform α (*ADHα*) and arogenate dehydrogenase isoform β (*ADHβ*) (Mean ± SE; n=4). * Symbol indicates differences between control and treatment in each population (*p* value ≤ 0.05).

The incubation with **quinate** provoked an increase in the relative transcript level of the shikimate pathway. These results do not match with the results obtained in Chapters 1 and 2, where quinate treatment provoked a decrease in the relative transcript level. However, the different doses, timing and incubation system should be taken into account when interpreting the observed differences. In this experiment, the dose applied to the disks was lower than the applied in the previous chapters. Maybe if the dose applied were low as in this experiment, the flux in the pathway would not be high enough to provoke a reduction on the

relative gene expression. On the other hand, the harvest in this experiment was realized 24 h after the incubation. The most intense reduction of the relative transcript level observed in Chapter 1 after quinate treatment was observed 48 h after the application (Figures 1.5 to 1.7), so 24 h could be not time long enough to observe a general reduction on the relative mRNA level of the genes of the AAA biosynthetic pathway.

The incorporation of the quinate on the shikimate pathway can be through two different pathways: on the one hand, through the reversible quinate dehydrogenase enzyme to 3-dehydroquinate and, on the other hand, through the quinate hydrolyase enzyme to shikimate (Figure GI.7). This means that the carbon flux would be more intense on the pathway just before or just after DQSD complex. The reduction on the relative gene expression in the *DQSD* complex gene in GS population after quinate would be related to the main incorporation of quinate to the pathway after DQSD. The increase of the flux just in that point would act as a signal to reduce the relative gene expression of the enzyme in order to regulate the pathway. However, the response of *DQSD* in GR population after quinate was the contrary: it seems that quinate would be incorporated into the pathway as 3-dehydroquinate, inducing the expression of the enzyme metabolizing it, although the reasons remain to be elucidated. Interestingly, the increase in the relative transcript level of one of the isoforms of *CM* and *ADH* genes in GS and *ADH* in GR after quinate would confirm that, despite the low dose applied, quinate would direct the flux to the synthesis of Tyr and Phe, as observed in Chapter 1 (Figure 1.4).

Chorismate treatment caused a striking upregulation of *AS* gene in both populations, although it was only significant in GS population (Figure 4.11). Curiously, chorismate is the common substrate of the enzymes *AS* and *CM*, as the first step in the bifurcation of the pathway, but only the relative transcript level of the *AS* gene was increased while no effect on *CM* transcript levels was detected. It seems that, as happens with glyphosate (Fernández-Escalada et al. 2017), the flux would be redirected towards the Trp biosynthetic pathway when chorismate is added exogenously. On the other hand, chorismate increased the content of the enzymes DAHPS and EPSPS (Figure 4.8). However, the increase in these two enzymes does not match with the results observed in the relative transcription level of the genes (Figure 4.11), in which no changes were detected. It seems that the increase in the content of both enzymes would be due to post-transcriptional regulation process elicited by the presence of chorismate.

Anthranilate was the intermediate that affected less the expression level of the pathway. Interestingly, it was observed a decrease in the relative transcript abundance of *CM1-3* and *ADHα* after incubation with anthranilate in GS population. It was observed a different response on the two isoenzymes of *CM*: the plastidics 1 and 3 isoenzymes but not the cytosolic isoform 2 would be downregulated by anthranilate. In the same way, the two isoenzymes of *ADH* showed also different response, and the *ADHα* expression was the only downregulated by anthranilate. Anthranilate incubation also provoked a significant increase in shikimate content (as shikimate or quinate incubation) but no general changes in relative gene expression were observed. This pattern suggests that it has to exist another factor or signal in addition to shikimate accumulation in the induction of the relative gene expression of the AAA biosynthetic pathway after quinate or shikimate. Anthranilate incubation provoked a decrease in the DAHPS enzyme content in GR population, (Figure 4.8.B), which should be mediated by post-transcriptional regulation as no effects of anthranilate in *DAHPS* gene expression were detected.

How the carbon flow into the shikimate pathway is regulated in plants remains ambiguous. Siehl (1997) suggested that the inhibition of DAHPS activity by arogenate, a metabolite of the post-chorismate part of the pathway, was the key regulation process for the shikimate pathway. In the case of glyphosate exposure, this important regulatory pathway cannot occur since chorismate and all its by-products are not synthesized, resulting in an increase in the flux through this pathway and the accumulation of S3P (Gomes et al. 2014) and shikimate. If this DAHPS feedback control by arogenate was correct, it could be expected downregulation in DAHPS expression and protein content after the external supply of chorismate, a metabolite in the pathway downstream arogenate. Nevertheless, contrary to expected, chorismate incubation induced DAHPS protein content and EPSPS protein content in both populations. No changes in the gene expression level of both enzymes were detected in any of the populations after chorismate, indicating that the upregulatory mechanism was posttranscriptional. Anyway, results indicate that DAHPS or EPSPS expression is not feedback inhibited by chorismate.

In plants, the regulation of AAA biosynthetic pathway has been described to be complicated. The principal reason is the subcellular localization of the enzymes, which provokes that the regulation occurs mostly at gene expression level. However, AAA biosynthesis is also subject to complex post-transcriptional and allosteric regulations (Mir et al. 2015). The results obtained in this experiment suggest that any perturbation on the pathway would provoke changes in the

relative transcript level of the genes in order to reestablish the flux in the pathway and confirm a complex regulation of the shikimate pathway where regulation mechanisms interact at different levels and behave different in each population.

Interestingly, some of the responses were similar in both populations and some others were more intense in GS population than in GR population. On one hand, the effect of the intermediate chorismate on the content of DAHPS and EPSPS enzymes was similar in both populations. On the other hand, shikimate treatment induced a higher upregulation of the shikimate pathway genes in GS population than in the GR population and anthranilate only downregulated the relative expression of the genes of the Tyr and Phe branch in GS population. Overall, results suggest that the overexpression of the EPSPS of the GR population would have an effect on the plasticity of the pathway, as GS population seemed to be the more susceptible to the regulation.

Shikimate was the metabolite that provoked a deregulation more intense in the pathway, and when shikimate content was accumulated by shikimate or quinate treatment, the relative transcript level of most of the genes of the AAA biosynthetic pathway seemed to increase. As previously suggested, the rapid accumulation of shikimate would be provoked by a significant loss of feedback control of the AAA biosynthetic pathway (Marchiosi et al. 2009).

The possibility of unravel whether the response of the plants to glyphosate treatment would be mediated by any intermediate of the pathway or not was approached in this experiment. The results obtained evidence that no intermediate fully mimicked the effect of the herbicide. Nevertheless, although the toxic effect of the herbicide would be due to a combination of different factors, shikimate incubation elicited, as glyphosate, upregulation of most of the shikimate pathway genes. Indeed, effect was higher in GS population than in GR population and it was related to the level of shikimate accumulated, resembling the different sensitivity of both populations to glyphosate.

4.3.3. Regulation of the expression of the BCAA biosynthetic pathway by glyphosate and intermediates of the AAAs pathway

The different amino acids are synthesized by a number of distinct metabolic networks, which are expected to possess regulatory cross interactions between them for proper coordination of their interactive functions, such as incorporation

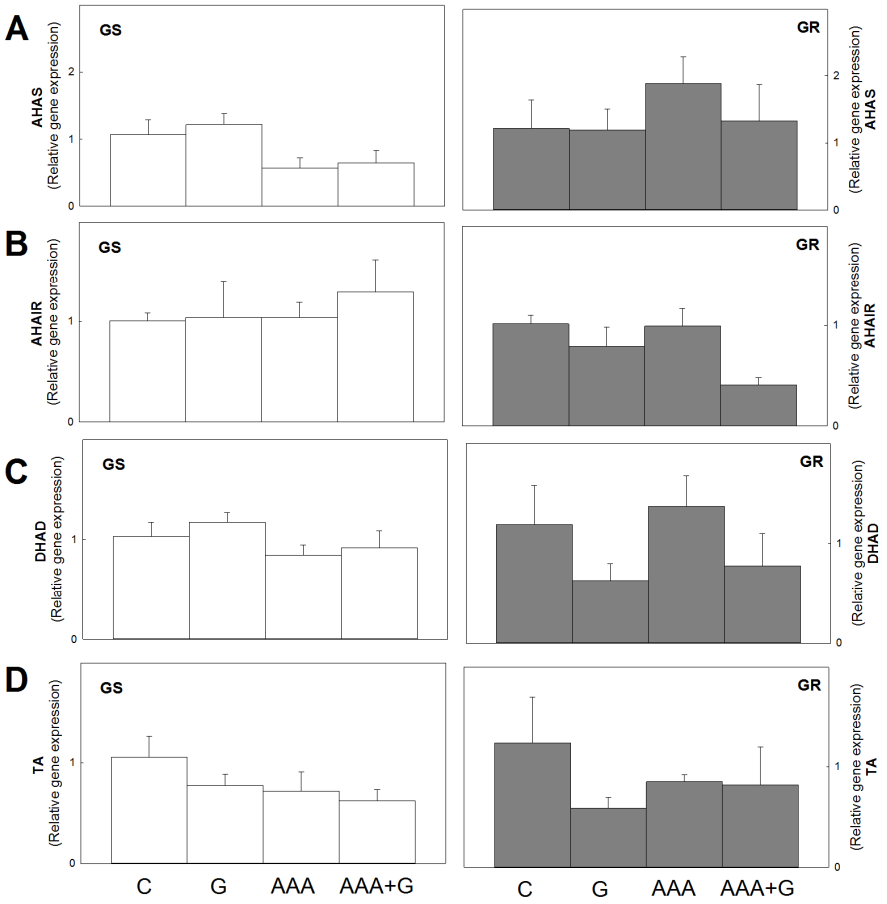

Figure 4.13. Transcript abundance of genes in the branched chain amino acid (BCAA) biosynthetic pathway was measured in *Amaranthus palmeri* leaf disks. Glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations were incubated for 24 h with water (Control, C), aromatic amino acids (AAA), glyphosate (G) or the combination of aromatic amino acids and glyphosate (AAA+G). Relative transcript abundance was normalized using the normalization gene beta tubulin and each population to its own daily control of acetohydroxy acid synthase (*AHAS*; A), acetohydroxyacid isomer-reductase (*AHAIR*; B), dihydroxyacid dehydratase (*DHAD*; C) and transaminase (*TA*; D) (Mean ± SE; n=4). * Symbol indicates differences between control and treatment in each population (*p* value ≤ 0.05).

into proteins(Less et al. 2010). Yet, individual amino acid metabolic networks are also expected to cross interact and have been proposed by previous studies (Guyer et al. 1995; Noctor et al. 2002; Pratelli and Pilot 2014). In particular, several authors have proposed cross-regulation between AAA and BCAA pathways (Noctor et al. 2002). Previous studies with plants of the same populations of *A. palmeri* treated with glyphosate provoked an increase in BCAA content (Fernández-Escalada et al. 2016), suggesting a possible effect of the herbicide on the BCAA biosynthetic pathway.

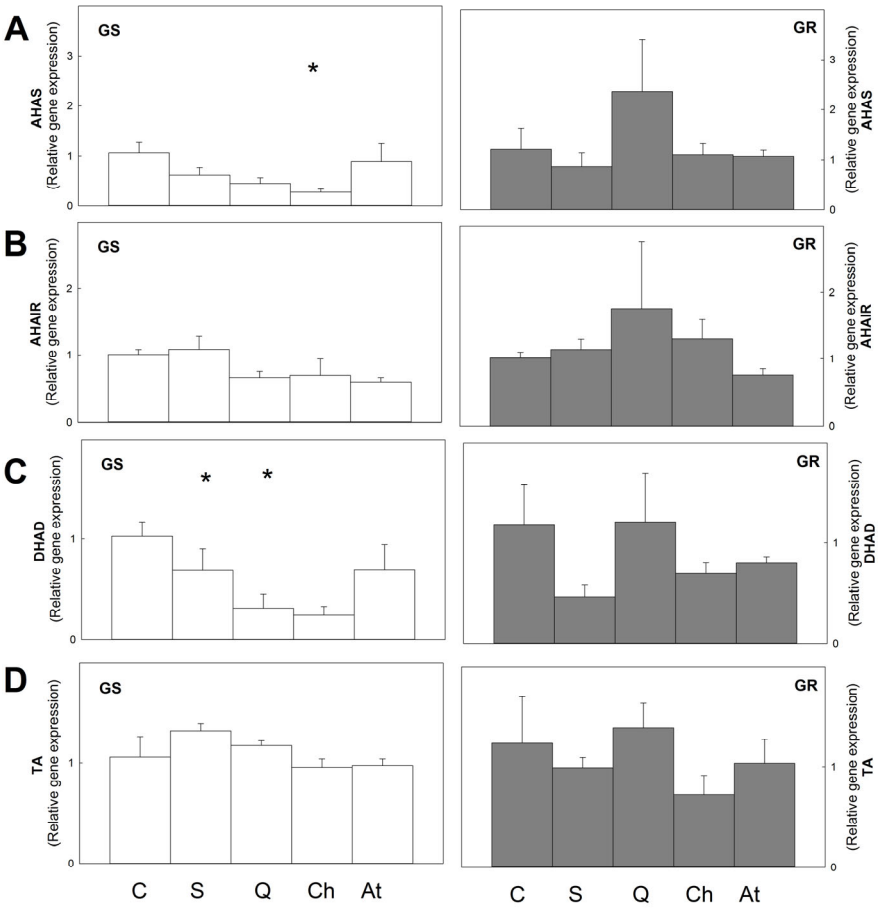

Figure 4.14 Transcript abundance of genes in the branched chain amino acid (BCAA) biosynthetic pathway was measured in *Amaranthus palmeri* leaf disks. Glyphosate-sensitive (white bars, left; GS) and glyphosate-resistant (gray bars, right; GR) populations were incubated for 24 h with water (C), shikimate (S), quinate (Q), chorismate (Ch) or anthranilate (At). Relative transcript abundance was normalized using the normalization gene beta tubulin and each population to its own daily control of acetohydroxy acid synthase (*AHAS*; A), acetohydroxyacid isomer-reductase (*AHAIR*; B), dihydroxyacid dehydratase (*DHAD*; C) and transaminase (*TA*; D) (Mean ± SE; n=4). * Symbol indicates differences between control and treatment in each population (p value ≤ 0.05).

Relative transcript level of the genes of the BCAA biosynthetic pathway (*AHAS, AHAIR, DHAD* and *TA*) was measured and also expressed normalized to *the β-tubulin* expression level as housekeeping gene after incubation with glyphosate, AAA, the combination of the two treatments (Figure 4.13) and the intermediates shikimate, quinate, chorismate or anthranilate (Figure 4.14).

Glyphosate, AAAs or their combination did not elicit any significant change in the expression level of the BCAA biosynthetic pathway (Figure 4.13). In the same way, after leaf disk incubation with the intermediates of the shikimate pathway, no general pattern or general effect of any intermediate was detected. Only quinate (Figure 4.14. C) and chorismate (Figure 4.14 A and C) provoked a decrease in the relative expression level of *DHAD* and *AHAS* and *DHAD* genes, respectively.

As previously reported (Fernández-Escalada et al. 2017), glyphosate did not provoke any significant change in the transcriptional levels of the genes of the BCAA pathway. Moreover, the results obtained in this study suggest that neither the inhibition by the herbicide nor the supply of the final products of the biosynthetic pathway would affect the BCAA pathway.

It was also reported the lack of effect of an herbicide inhibiting BCAA biosynthesis (imazamox) on the transcript levels of the genes of the AAA pathway after 3 days of treatment with the recommended dose (Fernández-Escalada et al. 2019). Taken together our results and that results, it can be suggested after 3 days of treatment there is no cross regulation between the AAA and BCAA biosynthetic pathways at transcriptional level.

4.4. CONCLUSIONS

- Leaf disk incubation is a valid methodology to unravel the shikimate pathway regulation because the previously known effects of glyphosate, shikimate accumulation and upregulation of the shikimate pathway genes were detected.

- The exogenous supply of AAAs did not induce any important change in the transcriptome of the shikimate pathway. Nevertheless, when applied in combination with glyphosate the upregulation of gene expression detected after glyphosate was abolished, suggesting that the effect of the glyphosate would be mediated by a lack of the final products of the pathway. AAAs did not attenuate the effect of glyphosate on shikimate accumulation, suggesting that shikimate accumulation is directly elicited by EPSPS inhibition and independent of the AAA level.

- The effect of glyphosate on shikimate pathway was not fully mimicked by shikimate, quinate, chorismate nor anthranilate. Each intermediate induced a different pattern on shikimate pathway expression and protein content, evidencing that shikimate pathway possess a complex regulation at different levels, transcriptional and posttranscriptional.

- The incubation with shikimate or quinate induced shikimate accumulation and induced the relative transcript level of most of the genes of the shikimate pathway, suggesting that transcription induction after glyphosate would be mediated, at least in part, by shikimate accumulation. Chorismate upregulated the expression of the chorismate-consuming enzyme AS, while anthranilate was the intermediate which less modify the shikimate pathway.

- DAHPS content is not feedback inhibited by the intermediate or product availability of the shikimate pathway as it was not possible to detect any decrease in DAHPS protein content after AAAs or intermediate supply. On the contrary, DAHPS content was increased by chorismate in both populations and by AAAs in GS population.

- In general the effects detected after the application of the intermediates were more severe in the GS than in the GR population, suggesting that the tight regulatory mechanisms that operate in the GS population are disrupted or buffered when the overexpression of the EPSPS is present,

as in the GR population, although mechanisms underlying this behavior remain to be elucidated.

- The expression of the genes of the BCAA biosynthetic pathway was independent of the AAA intermediate or glyphosate applications, confirming the lack of cross-regulation between AAA and BCAA biosynthetic pathways at transcript level at this time point.

GENERAL OVERVIEW

Glyphosate is a total herbicide whose site of action is the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme of the aromatic amino acid biosynthetic pathway, the shikimate pathway. Even the site of action is very well known, the toxic effect of herbicide has not been completely elucidated. It goes beyond the interaction with the target site and provokes a physiological roadblock that leads to plant death.

Some of the processes described as part of the glyphosate mode of action are: accumulation of the metabolites upstream the EPSPS and free amino acids, an increase of the transcripts of the genes of the inhibited pathway and accumulation of quinate. Quinate is a secondary metabolite formed in a lateral branch of the shikimate pathway. This quinate accumulation has been proposed as a key factor on the toxic response to the herbicide because some phytotoxic effects were detected when quinate was applied alone trying to mimic glyphosate. It was raised the possibility of using quinate as a glyphosate enhancer leading to reducing the glyphosate doses in the control of *Amaranthus palmeri.*

The shikimate pathway is directly affected by glyphosate and quinate, although their effects are contrary, glyphosate inhibiting the pathway and quinate enhancing the carbon flux. It can be hypothesized that they may interact in the pathway and enhance the toxicity of the herbicide, making the study of the application of both compounds together very interesting. Besides the management point of view, the specific physiological study of the pattern of shikimate after either compounds or their combination would provide new insights in the regulation of the pathway.

The intensive use of glyphosate has resulted in the evolution of resistance to this herbicide. *A. palmeri* is a troublesome weed that has become a major glyphosate-resistant weed and whose main mechanism of resistance is EPSPS gene amplification. The availability of a biotype with overexpression of the EPSPS enzyme provided an opportunity to analyze how overexpression of EPSPS affects the regulation of the shikimate pathway after glyphosate and/or quinate treatments.

The **main objective** of this work was to get new insights in the regulation of the shikimate pathway after glyphosate treatment by the use of the secondary metabolite quinate as an enhancer of the herbicide glyphosate in *A. palmeri* populations sensitive and resistant to glyphosate.

First of all, it had to be elucidated if it was possible to enhance the toxicity of glyphosate with quinate, what would make possible a better control of *A.*

palmeri. This was approached in Chapter 1 by studying whether the toxicity and physiological effects of glyphosate were affected by its combined application with quinate. First, it was determined the effect of the exogenous quinate application alone. It was observed that, in both populations, quinate was absorbed by the plant, and the carbon flux led to Phe and Tyr accumulation (Figure 1.4). However, only in GS population quinate supply provoked a decrease of the transcripts of the genes of the shikimate pathway (Figures 1.5, 1.6 and 1.7). It can be proposed that the downregulation of expression level of the shikimate pathway was only evident when the overexpression of EPSPS is not present. Second, quinate was then applied in combination with a sub lethal dose of glyphosate, in order to determine the possible enhancer effect of the mixture. It was observed that the most effective combination was when quinate was applied 24 h after glyphosate treatment (Figure 1.8), more than simultaneously or 24 h before. So, this combination was used in all experiments performed afterwards.

Applying quinate after glyphosate become lethal a sub lethal glyphosate dose in the GS population, laying the framework for the application of the both compounds to improve the efficacy of the herbicide and to reduce the doses in the control of the sensitive population (Figure 1.9). However, the effect in GR population was not lethal (Figure 1.10).

Moreover, when the previously reported herbicide markers (shikimate content and amino acid profile) were evaluated, it was confirmed that the combined treatment provoked changes in amino acid profile, exacerbating the glyphosate effect in GS population (Figures 1.13, 1.14 and 1.15). Applying exogenous quinate once the pathway is already blocked by the herbicide, would disturb the pathway more intensely than other combinations, due to an increase of the carbon flux trough the pathway when it is already inhibited.

Nevertheless, the sub lethal doses of glyphosate used in the first chapter were too low to induce any changes in the herbicide physiological markers in GR, so in the following chapters, the sub lethal glyphosate dose applied in GR was raised from 0.5 the recommended dose (RD) to 1 RD.

The objective of Chapter 2 was to unravel the pattern of the shikimate pathway after applying the combined treatment, trying to explain the increase of the efficacy detected in GS population, and was approached at metabolic and transcriptional level. An additional glyphosate dose was applied in both populations, in order to compare the physiological changes of a dose that would provoke the same visual toxicity than the combined treatment. This higher dose was 1RD for GS and 3RD for GR populations and was lethal in GS populations. The

combined treatment, where quinate was applied one day after the treatment with the sub lethal glyphosate dose, provoked the same visual effect in the plants than the higher glyphosate dose applied (Figure 2.2).

The content of the metabolites of the AAA biosynthetic pathway was determined by GC-MS. It was observed a dose-dependent increase of the metabolites upstream the EPSPS (Figure 2.5) and gallic acid (Figure 2.6) in both populations, increase that was not exacerbated in the combined treatment. The induced increase of 4 quinate derivatives content was enhanced after the combined treatment in GS population (Figure 2.4), suggesting that this accumulation could be related to the increase of the toxicity of this treatment. The EPSPS inhibition by the herbicide before the application of quinate would minimize the incorporation of the quinate through the pathway, leading to a metabolization of the compound to a lateral branch of the pathway. Of the detected phenylpropanoids, salicylic acid was the only one that was affected after the treatments, being accumulated in both populations after sub lethal doses of the herbicide (Figure 2.8). This accumulation could be related to possible changes in the oxidative status after glyphosate treatment, previously reported in other species.

The pattern of the shikimate pathway was also evaluated at the level of relative transcript level of the genes of the pathway. As previously reported, glyphosate provoked an upregulation of the genes of the pre-chorismate part of the pathway in both populations, effect that was observed even after the sub-lethal dose in GS (Figures 2.9 and 2.10), while the genes located in the post-chorismate part of the pathway were not affected after the herbicide treatment (Figure 2.11). The exacerbation of the phytotoxicity of the combined treatment was not related to any remarkable change in the relative gene expression, where a mixed pattern between the decrease provoked by quinate and the increase induced by glyphosate was observed. The results obtained in this chapter suggest that the enhancement of the toxicity observed after the combined treatment in GS population would be related mainly to the increase in the quinate derivative content, and not to changes at transcriptional level. These results raised the question if other changes in the metabolomic profile of the plants treated with the combined treatment could contribute to unravel the cause of the toxicity increase.

All effects detected in shikimate pathway by glyphosate and/or quinate were less intense in GR than in GS population, suggesting that EPSPS overexpression would confer the capability of minimize not only glyphosate effect by affecting directly

the EPSPS, but also the effect of quinate treatment of potentially increase the carbon flux through the pathway.

The metabolic characterization of sensitive and resistant populations of *A. palmeri* after applying quinate, glyphosate or their combination was approached in Chapter 3 by performing non-targeted metabolomic profiling by GC-MS and LC-MS. The comparison of the metabolite profile of untreated plants of both populations showed that there were no significant differences between populations, suggesting that the overexpression of the EPSPS in GR population would not affect the metabolite pool, and that under control conditions, the increase in EPSPS protein would not increase the carbon flux through the pathway (Figures 3.2 and 3.3).

To compare of the metabolite profile of treated plants of both populations, two approaches were developed, on one hand to evaluate a possible dose-response of metabolites after glyphosate treatment and, on the other, to compare the combination of quinate and glyphosate with the individual treatments. In each approach, a Principal Component Analysis (PCA) was performed within each population using the metabolites positively identified by GC-MS or LC-MS.

In the first approach, to evaluate a possible dose-response of metabolites after glyphosate treatment, it was studied the effect of the two doses of glyphosate (low and high) in the metabolic profile of both populations. A dose-dependent response of some primary metabolites was provoked by the herbicide in GS (Figure 3.5 and 3.6), observing the effect not only in metabolites related to the shikimate pathway, but also in carbohydrates, amino acids and fatty acids. In GR population the effect provoked was lower than in GS, due to the less effect of the herbicide because of the EPSPS overexpression (Figures 3.7 and 3.8). A heat map clearly showed that (Figure 3.9) the lethal phenotype detected in GS population after the high dose of the herbicide was related to an intense accumulation of the metabolites related to the shikimate pathway and other amino acids. A similar metabolome was also detected after the sub lethal treatments 0.5 RD in GS and 3 RD in GR. The secondary metabolism was studied by analyzing the metabolites related to the phenylpropanoid and flavonoid pathways from the metabolites detected and identified by LC-MS in the positive mode. No general pattern of phenylpropanoids or signaling molecules could be outlined in the response to the herbicide, and no striking changes were detected. Abscisic acid and ferulic acid and its derivatives were affected similarly in both populations (Figures 3.11 and 3.12). On the contrary, the pattern of flavonoids after glyphosate was different between populations, observing a decrease in quercetin content only in GR

population after glyphosate treatment. These results suggest that the lethality of the RD of glyphosate in GS plants is mediated by changes in metabolites of the shikimate pathway, carbohydrates and amino acids, while no changes in secondary metabolism seem to be involved.

In the second approach, the comparison of the metabolic profile of the combination of quinate and glyphosate with the individual treatments was performed. It showed that the increase of the toxicity of the combined treatment in the GS population was not related to any striking effect in the metabolome as similar metabolic changes were detected after glyphosate or the combined treatment. Among all primary and secondary metabolites evaluated, the quinate derivatives were the only compounds that were accumulated differently after the combined treatment. This result was described also in chapter 2, but the full metabolomic study performed in Chapter 3 stablishes that it was the only main change detected in the metabolic profile, suggesting its role in the increased toxicity of the combined treatment.

After that, the regulatory mechanisms underlying the response of the shikimate pathway remained unclear, and the specific role of the intermediates or final products had not been deeply studied. For that, the objective of Chapter 4 was to evaluate the role of aromatic amino acids and the intermediates in the regulation of shikimate pathway. This was performed by a different methodology of the previous chapters, by incubating leaf discs with the compounds for 24 h to analyze if aromatic amino acids could revert the effects of glyphosate on the pathway, and if the supply of intermediates of the pathway could mimic the glyphosate effects.

The reversion of glyphosate effects by aromatic amino acids supply was different depending on the evaluated parameter. While the upregulation of gene expression detected after glyphosate was neutralized in combination with the aromatic amino acids (Figure 4.4, 4.5 and 4.6), AAAs did not attenuate the effect of glyphosate on shikimate accumulation (Figure 4.2). These results suggests that the shikimate accumulation is directly elicited by EPSPS inhibition and independent of the AAA level while the upregulation of expression level of the pathway by glyphosate would be mediated by a lack of the final products of the pathway.

The effect of glyphosate on shikimate pathway was not fully mimicked by shikimate, quinate, chorismate nor anthranilate (Figures 4.9, 4.10, 4.11 and 4.12). Each intermediate induced a different pattern on shikimate pathway expression and protein content, evidencing that shikimate pathway possess a complex

regulation at different levels, transcriptional and posttranscriptional. However, shikimate was the metabolite that induced the relative transcript level of most of the genes of the shikimate pathway, suggesting that the transcription induction detected after glyphosate treatment would be mediated, at least in part, by shikimate accumulation.

In general the effects detected after the application of the intermediates were more severe in the GS than in the GR population, suggesting that the tight regulatory mechanisms that operate in the GS population are disrupted or buffered when the overexpression of the EPSPS is present, as in the GR population, although mechanisms underlying this behavior remain to be elucidated.

In summary, in this study the application of quinate and glyphosate together has been approached from two interesting and interdependent points of view. From a management point of view, 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.* From a physiological point of view, the comparison of the combined treatment with the individual treatment at transcriptomic and metabolomics level has provided new insights in the regulation of the shikimate pathway.

GENERAL BIBLIOGRAPHY

Ahsan N, Lee D-G, Lee K-W, et al (2008) Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. Plant Physiol Biochem 46:1062–1070.

Alcántara-de la Cruz R, Rojano-Delgado AM, Giménez MJ, et al (2016) First resistance mechanisms characterization in glyphosate-resistant *Leptochloa virgata*. Front Plant Sci 7:1742.

Aldesuquy HS (2015) Shikimic acid and salicylic acid induced protection on growth vigor, seedy yield and biochemical aspects of yielded seeds of *Vicia faba* plants infected by *Botrytis fabae*. J Plant Pathol Microbiol 06:1000304.

Arlt K, Brandt S, Kehr J (2001) Amino acid analysis in five pooled single plant cell samples using capillary electrophoresis coupled to laser-induced fluorescence detection. J Chromatogr A 926:319–325.

Armendáriz O, Gil-Monreal M, Zulet A, et al (2016) Both foliar and residual applications of herbicides that inhibit amino acid biosynthesis induce alternative respiration and aerobic fermentation in pea roots. Plant Biol 18:382–390.

Baerson SR, Rodriguez DJ, Biest NA., et al (2002) Investigating the mechanism of glyphosate resistance in rigid ryegrass (*Lolium ridigum*). Weed Sci 50:721–730.

Barroso AAM, de S Costa MG, Neto NJ, et al (2018) Protein identification before and after glyphosate exposure in *Lolium multiflorum* genotypes. Pest Manag Sci 74:1125–1133.

Baylis A (2000) Why glyphosate is a global herbicide: strengths, weaknesses and prospects. Pest Manag Sci 56:299–308

Becerril JM, Duke SO, Lydon J (1989) Glyphosate effects on shikimate pathway products in leaves and flowers of velvetleaf. Phytochemistry 28:695–699.

Bender J, Fink GR (1998) A Myb homologue, ATR1, activates tryptophan gene expression in *Arabidopsis*. Proc Natl Acad Sci 95:5655–5660.

Benesova M, Bode R (1992) Chorismate mutase isoforms from seeds and seedlings of *Papaver somniferum*. Phytochemistry 31:2983–2987

Bentley R, Haslam E (1990) The shikimate pathway — a metabolic tree with many branches. Crit Rev Biochem Mol Biol 25:307–384.

Binder S (2010) Branched-chain amino acid metabolism in *Arabidopsis thaliana.* Arab B e0137.

Binder S, Knill T, Schuster J (2007) Branched-chain amino acid metabolism in higher plants. Physiol Plant 129:68–78

Bohlmann J, Lins T, Martin W, Eilert U (1996) Anthranilate synthase from *Ruta graveolens*. Duplicated AS alpha genes encode tryptophan-sensitive and tryptophan-insensitive isoenzymes specific to amino acid and alkaloid biosynthesis. Plant Physiol 111:507–14.

Böttcher C, Centeno D, Freitag J, et al (2008a) Teaching (and learning from) metabolomics: The 2006 PlantMetaNet ETNA Metabolomics Research School. Physiol Plant 132:136–149.

Böttcher C, Von Roepenack-Lahaye E, Schmidt J, et al (2008b) Metabolome analysis of biosynthetic mutants reveals a diversity of metabolic changes and allows identification of a large number of new compounds in *Arabidopsis*. Plant Physiol 147:2107–2120.

Boudet A (1973) Quinic and shikimic acids in woody angiosperms. Phytochemistry 12:363–370

Boudet AM (2007) Evolution and current status of research in phenolic compounds. Phytochemistry 68:2722–2735.

Boudet AM (2012) Polyphenols: From plant adaptation to useful chemical resources. In: Cheynier V, Sarni-Manchado P, Quideau S (eds) Recent Advances in Polyphenol Research, First edition. Wiley-Backwell, Chichester, West Sussex, UK, pp 41–70

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Brunetti C, Sebastiani F, Tattini M (2019) Review: ABA, flavonols, and the evolvability of land plants. Plant Sci 280:448–454.

Brunharo C, Caio A, Patteron E, et al (2016) Confirmation and mechanism of glyphosate resistance in tall windmill grass (*Chloris elata*) from Brazil. Pest Manag Sci 72:1758–1764

Brunk DG, Rhodes D (1988) Amino acid metabolism of *Lemna minor* L . III. Responses to aminooyacetate. Plant Physiol 447–453.

Buchanan BB, Gruissem W, Jones R (2015) Biochemistry & Molecular Biology of Plants. John Wiley & Sons

Castrillón-Arbeláez P, Délano Frier J (2016) Secondary metabolism in *Amaranthus* spp.— a genomic approach to understand its diversity and responsiveness to stress in marginally studied crops with high agronomic potential. In: Shanker A, Shanker C (eds) Abiotic and Biotic Stress in Plants - Recent Advances and Future Perspectives. pp 186–211

Chandi A, Milla-Lewis SR, Giacomini D, et al (2012) Inheritance of evolved glyphosate resistance in a North Carolina Palmer amaranth (*Amaranthus palmeri*) biotype. Int J Agron 2012:Article ID 176108.

Chaudhari S, Jordan D, York A, et al (2017) Biology and management of glyphosate-resistant and glyphosate-susceptible Palmer amaranth (*Amaranthus palmeri*) phenotypes from segregating population. Weed Sci 65:755–768

Chen J, Huang H, Zhang C, et al (2015a) Mutations and amplification of EPSPS gene confer resistance to glyphosate in goosegrass (*Eleusine indica*). Planta 242:859–868.

Chen J, Huang Z, Zhang C, et al (2015b) Molecular basis of resistance to imazethapyr in redroot pigweed (*Amaranthus retroflexus* L.) populations from China. Pestic Biochem Physiol 124:43–47.

Cobb A, Reade J (2010) Herbicides and Plant Physiology. Wiley-Blackwell, Iowa, EEUU

Cousens RD, Fournier-Level A (2018) Herbicide resistance costs: what are we actually measuring and why? Pest Manag Sci 74:1539–1546.

Cromartie TH, Polge ND (2000) An improved assay for shikimic acid and its use as monitor for the activity of sulfosate. In: Weed Science Society of America Proceedings. pp 4, 121

Culpepper AS, Grey TL, Vencill WK (2006) Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) confirmed in Georgia. Weed Sci 54:620–626

Culpepper AS, Whitaker JR, MacRae AW, York AC (2008) Distribution of glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) in Georgia and North Carolina during 2005 and 2006. J Cotton Sci 12:306–310

Dayan FE, Duke SO, Grossmann K (2010) Herbicides as probes in plant biology. Weed Sci 58:340–350.

De Carvalho LC, Alves PL, González-Torralva F, et al (2012) Pool of resistance mechanisms to glyphosate in *Digitaria insularis*. J Agric Food Chem 60:615–622

de María N, Becerril JM, García-Plazaola JI, et al (2006) New insights on glyphosate mode of action in nodular metabolism: Role of shikimate accumulation. J Agric Food Chem 54:2621–2628.

Délye C (2012) Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: A major challenge for weed science in the forthcoming decade. Pest Manag Sci 69:176–187.

Délye C, Jasieniuk M, Le Corre V (2013) Deciphering the evolution of herbicide resistance in weeds. Trends Genet 29:649–658.

Dev A, Tapas S, Pratap S, Kumar P (2012) Structure and function of enzymes of shikimate pathway. Curr Bioinform 7:374–391

Dewey S, Appleby A (1983) A comparison between glyphosate and assimilate translocation patterns in Tall Morningglory (*Ipomoea purpurea*). Weed Sci 31:308–314

Dill GM (2005) Glyphosate-resistant crops: History, status and future. Pest Manag Sci 61:219–224

Dubouzet JG, Ishihara A, Matsuda F, et al (2007) Integrated metabolomic and transcriptomic analyses of high-tryptophan rice expressing a mutant anthranilate synthase alpha subunit. J Exp Bot 58:3309–3321.

Duke SO (1990) Overview of herbicide mechanisms of action. Environ Health Perspect 87:263–271

Duke SO (2011) Glyphosate degradation in glyphosate-resistant and -susceptible crops and weeds. J Agric Food Chem 59:5835–41.

Duke SO (2012) Why have no new herbicide modes of action appeared in recent years? Pest Manag Sci 68:505–512.

Duke SO (2018a) The history and current status of glyphosate. Pest Manag Sci 74:1027–1034.

Duke SO (2018b) Glyphosate: The world's most successful herbicide under intense scientific scrutiny. Pest Manag Sci 74:1025–1026.

Duke SO, Hoagland RE, Elmore CD (1979) Effects of glyphosate on metabolism of phenolic-compounds. Phenylalanine ammonia-lyase activity, free amino-acids, and soluble hydroxyphenolic compounds in axes of light-grown soybeans. Physiol Plant 46:307–317

Duke SO, Powles SB (2008) Glyphosate: a once-in-a-century herbicide. Pest Manag Sci 64:319–325

Duke SO, Powles SB, Sammons RD (2018) Glyphosate – How it became a once in a hundred year Herbicide and its future. Outlooks Pest Manag 29:247–251.

Durner J, Knorzer OC, Boger P (1993) Ketol-acid reductoisomerase from barley (*Hordeum vulgare*) - purification, properties, and specific-inhibition. Plant Physiol 103:903–910

Dyer WE (2018) Stress-induced evolution of herbicide resistance and related pleiotropic effects. Pest Manag Sci 74:1759–1768.

Dyer WE, Henstrad JM, Handa AK, Herrmann KM (1989) Wounding induces the first enzyme of the shikimate pathway in Solanaceae. Proc Natl Acad Sci 86:7370–7373

Eason JR, Johnston JW, De Vré L (2000) Reversal of glyphosate inhibition of *Sandersonia aurantiaca* flower senescence with aromatic amino acids. Postharvest Biol Technol 18:81–84.

Eberhard J, Ehrler TT, Epple P, et al (1996) Cytosolic and plastidic chorismate mutase isoenzymes from *Arabidopsis thaliana*: molecular characterization and enzymatic properties. Plant J 10:815–21

Edwards R, Hannah M (2014) Focus on weed control. Plant Physiol 166:1087–1089.

Fernández-Escalada, Gil-Monreal M, Zabalza A, Royuela M (2016) Characterization of the *Amaranthus palmeri* physiological response to glyphosate in susceptible and resistant populations. J Agric Food Chem 64:95–106.

Fernández-Escalada, Zulet-González A, Gil-Monreal M, et al (2017) Effects of EPSPS copy number variation (CNV) and glyphosate application on the aromatic and branched chain amino acid synthesis pathways in *Amaranthus palmeri.* Front Plant Sci 8:1970.

Fernández-Escalada M, Zulet-González A, Gil-Monreal M, Royuela M (2019) Physiological performance of glyphosate and imazamox mixtures on *Amaranthus palmeri* sensitive and resistant to glyphosate. Sci Rep 9:18225.

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

Franz J, Mao M, Sirkorski J (1997) Glyphosate: a unique global herbicide. ASC Monograph 189.

Funke T, Han H, Healy-Fried ML, et al (2006) Molecular basis for the herbicide resistance of Roundup Ready crops. Proc Natl Acad Sci 103:13010–13015.

Gaines TA, Zhang W, Wang D, et al (2010) Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. Proc Natl Acad Sci 107:1029–1034.

Gaines TA, Shaner DL, Ward SM, et al (2011) Mechanism of resistance of evolved glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*). J Agric Food Chem 59:5886–9.

Gaines TA, Wright A a, Molin WT, et al (2013) Identification of genetic elements associated with EPSPs gene amplification. PLoS One 8:e65819.

Gaines TA, Patterson EL, Neve P (2019) Molecular mechanisms of adaptive evolution revealed by global selection for glyphosate resistance. New Phytol 223:1770-1775.

Galili G, Amir R, Fernie AR (2016) The regulation of essential amino acid synthesis and accumulation in plants. Annu Rev Plant Biol 67:153–178.

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

Ge X, D'Avignon D, Ackerman J, Sammons R (2012) Vacuolar glyphosate-sequestration correlates with glyphosate resistance in ryegrass (*Lolium* spp.) from Australia, South America and Europe: a 31P NMR investigation. Pest Manag Sci 60:1245–1250

Giacomini D, Westra P, Ward SM (2014) Impact of genetic background in fitness cost studies: An example from glyphosate-resistant Palmer amaranth. Weed Sci 62:29–37.

Gigolashvili T, Berger B, Mock HP, et al (2007) The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. Plant J 50:886–901.

Goers SK, Jensen RA (1984) The differential allosteric regulation of two chorismate-mutase isoenzymes of *Nicotiana silvestris*. Planta 162:117–124

Gomes MP, Smedbol E, Chalifour A, et al (2014) Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid : an overview. J Exp Bot 65:4691–4703.

Gorzolka K, Lissel M, Kessler N, et al (2012) Metabolite fingerprinting of barley whole seeds, endosperms, and embryos during industrial malting. J Biotechnol 159:177–187.

Graziana A, Boudet AM (1980) 3-Deoxy-D-arabino heptulosonate 7-phosphate synthase from *Zea mays*: General properties and regulation by tryptophan. Plant Cell Physiol 21:193–802

Gresshoff PM (1979) Growth inhibition by glyphosate and reversal of its action by phenylalanine and tyrosine. Aust J Plant Physiol 6:177–85

Guo J, Carrington Y, Alber A, Ehlting J (2014) Molecular characterization of quinate and shikimate metabolism in *Populus trichocarpa*. J Biol Chem 289:23846–23858.

Guyer D, Patton D, Ward E (1995) Evidence for cross-pathway regulation of metabolic gene expression in plants. Proc Natl Acad Sci 92:4997–5000

Haslam E (1993) Shikimic acid. Metabolism and Metabolites. Wiley, John, Chichester, West Sussex, UK

Hayat Q, Hayat S, Irfan M, Ahmad A (2010) Effect of exogenous salicylic acid under changing environment: A review. Environ Exp Bot 68:14–25.

Healy-Fried ML, Funke T, Priestman MA, et al (2007) Structural basis of glyphosate tolerance resulting from mutations of Pro101 in *Escherichia coli* 5-enolpyruvylshikimate-3-phosphate synthase. J Biol Chem 282:32949–32955.

Heap I The international survey of herbicide resistant weeds. Online. Internet. June 2019. Available in www.weedscience.org

Heap I, Duke SO (2018) Overview of glyphosate-resistant weeds worldwide. Pest Manag Sci 74:1040–1049.

Hernandez A, Garcia-Plazaola JI, Becerril JM (1999) Glyphosate effects on phenolic metabolism of nodulated soybean (*Glycine max* L. Merr.). J Agric Food Chem 47:2920–2925.

Hey SJ, Byrne E, Halford NG (2010) The interface between metabolic and stress signalling. Ann Bot 105:197–203.

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Calif Agric Exp Stn Circ 347:1–32.

Horak MJ, Loughin TM (2000) Growth analysis of four *Amaranthus* species. Weed Sci 48:347–355

Ishimoto M, Rahman SM, Hanafy MS, et al (2010) Evaluation of amino acid content and nutritional quality of transgenic soybean seeds with high-level tryptophan accumulation. Mol Breed 25:313–326.

Kaya A, Yigit E (2014) The physiological and biochemical effects of salicylic acid on sunflowers (*Helianthus annuus*) exposed to flurochloridone. Ecotoxicol Environ Saf 106:232–238.

Keith B, Dong X, Ausubel FM, Fink GR (1991) Differential induction of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase genes in *Arabidopsis thaliana* by wounding and pathogenic attack. Proc Natl Acad Sci 88:8821–8825

Killmer J, Widholm J, Slife F (1981) Reversal of glyphosate inhibition of carrot cell culture growth by glycolytic intermediates and organic and amino acids. Plant Physiol 68:1299–1302

Koger CH, Shaner DL, Henry WB, et al (2005) Assessment of two nondestructive assays for detecting glyphosate resistance in horseweed (*Conyza canadensis*). Weed Sci 53:559–566.

Koo D-H, Molin WT, Saski CA, et al (2018) Extrachromosomal circular DNA-based amplification and transmission of herbicide resistance in crop weed *Amaranthus palmeri*. Proc Natl Acad Sci 115:3332–3337.

Küpper A, Borgato EA, Patterson EL, et al (2017) Multiple resistance to glyphosate and acetolactate synthase inhibitors in Palmer amaranth (*Amaranthus palmeri*) identified in Brazil. Weed Sci 65:317–326.

Kuroki G, Conn EE (1988) Increased chorismate mutase levels as a response to wounding in *Solanum tuberosum* L. tubers. Plant Physiol 86:895–898.

Labhilili M, Joudrier P, Gautier MF (1995) Characterization of cDNAs encoding Triticum durum dehydrins and their expression patterns in cultivars that differ in drought tolerance. Plant Sci 112:219–230.

Less H, Angelovici R, Tzin V, Galili G (2010) Principal transcriptional regulation and genome-wide system interactions of the Asp-family and aromatic amino acid networks of amino acid metabolism in plants. Amino Acids 39:1023–1028.

Leuschner C, Schultz G (1991a) Uptake of shikimate pathway intermediates by intact chloroplasts. Phytochemistry 30:2203–2207

Leuschner C, Schultz G (1991b) Non-light-dependent shikimate pathway in plastids from pea roots. Bot Acta 104:240–244

Leuschner C, Herrmann KM, Schultz G (1995) The metabolism of quinate in pea roots - Purification and partial characterization of a quinate hydrolyase. Plant Physiol 108:319–325

Li J, Peng Q, Han H, et al (2018) Glyphosate resistance in *Tridax procumbens* via a novel EPSPS Thr-102-Ser substitution. J Agric Food Chem 66:7880–7888.

Liu Y, Zhang Y, Liu Y, et al (2015) Metabolic effects of glyphosate on transgenic maize expressing a G2-EPSPS gene from *Pseudomonas fluorescens.* Plant Biochem Biotechnol 24:233–241.

Liu X, Ma D, Zhang Z, et al (2019) Plant lipid remodeling in response to abiotic stresses. Environ Exp Bot 165:174–184.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression Data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25:402–408.

Lopez-Nieves S, Yang Y, Timoneda A, et al (2017) Relaxation of tyrosine pathway regulation underlies the evolution of betalain pigmentation in Caryophyllales. New Phytol 217:896–908

Lorentz L, Gaines TA, Nissen SJ, et al (2014) Characterization of glyphosate resistance in *Amaranthus tuberculatus* populations. J Agric Food Chem 62:8134–8142.

Lydon J, Duke SO (1988) Glyphosate induction of elevated levels of hydroxybenzoic acids in higher-plants. J Agric Food Chem 36:813–818

Ma R, Kaundun SS, Tranel PJ, et al (2013) Distinct detoxification mechanisms confer resistance to mesotrione and atrazine in a population of waterhemp. Plant Physiol 163:363–377.

Maeda H, Shasany AK, Schnepp J, et al (2010) RNAi suppression of Arogenate Dehydratase1 reveals that phenylalanine is synthesized predominantly via the arogenate pathway in petunia petals. Plant Cell 22:832–849.

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

Malone JM, Morran S, Shirley N, et al (2016) EPSPS gene amplification in glyphosate-resistant *Bromus diandrus*. Pest Manag Sci 72:81–88.

Mao C, Xie H, Chen S, et al (2016) Multiple mechanism confers natural tolerance of three lilyturf species to glyphosate. Planta 243:321–335.

Marchiosi R, Lucio Ferrarese MDL, Bonini EA, et al (2009) Glyphosate-induced metabolic changes in susceptible and glyphosate-resistant soybean (*Glycine max* L.) roots. Pestic Biochem Physiol 93:28–33.

Maroli AS, Nandula VK, Dayan FE, et al (2015) 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 63:9199–9209.

Maroli AS, Gaines TA, Foley ME, et al (2018a) Omics in weed science: a perspective from genomics, transcriptomics, and metabolomics approaches. Weed Sci 66:681–695.

Maroli AS, Nandula VK, Duke SO, et al (2018b) Comparative metabolomic analyses of *Ipomoea lacunosa* biotypes with contrasting glyphosate tolerance captures herbicide-induced differential perturbations in cellular physiology. J Agric Food Chem 66:2027–2039.

Matern A, Böttcher C, Eschen-Lippold L, et al (2019) A substrate of the ABC transporter PEN3 stimulates bacterial flagellin (flg22)-induced callose deposition in *Arabidopsis thaliana*. J Biol Chem 294 (17): 6857-6870

Matsukawa T, Ishihara A, Iwamura H (2002) Induction of anthranilate synthase activity by elicitors in oats. Zeitschrift fur Naturforsch - Sect C J Biosci 57:121–128

Michitte P, De Prado R, Espinoza N, et al (2007) Mechanisms of resistance to glyphosate in a ryegrass (*Lolium multiflorum*) biotype from Chile. Weed Sci 55:435–440

Mir R, Jallu S, Singh TP (2015) The shikimate pathway: Review of amino acid sequence, function and three-dimensional structures of the enzymes. Crit Rev Microbiol 41:172–189.

Miteva LP-E, Ivanov S V, Alexieva VS (2010) Alterations in glutathione pool and some related enzymes in leaves and roots of pea plants treated with the herbicide glyphosate. Russ J Plant Physiol 57:131–136.

Mobin M, Wu C-H, Tewari RK, Paek K-Y (2015) Studies on the glyphosate-induced amino acid starvation and addition of precursors on caffeic acid accumulation and profiles in adventitious roots of *Echinacea purpurea* (L.) Moench. Plant Cell, Tissue Organ Cult 120:291–301.

Mohapatra S, Minocha R, Long S, Minocha SC (2010) Transgenic manipulation of a single polyamine in poplar cells affects the accumulation of all amino acids. Amino Acids 38:1117–29.

Mohler C (2001) Weed life history: identifying vulnerabilities. In: Liebman M, Mohler C, Staver C (eds) Ecological Management of Agricultural Weeds. Cambridge Uviersity Press, Cambridge, Great Britain, pp 40–98

Mortensen DA, Egan JF, Maxwell BD, et al (2012) Navigating a critical juncture for sustainable weed management. Bioscience 62:75–84.

Murphy B, Tranel P (2019) Target-site mutations conferring herbicide resistance. Plants 8:382.

Nandula VK, Wright AA, Bond JA, et al (2014) 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 70:1902–1909.

Neve P, Vila-Aiub M, Roux F (2009) Evolutionary-thinking in agricultural weed management. New Phytol 184:783–793

Neve P, Busi R, Renton M, Vila-Aiub M (2014) Expanding the eco-evolutionary context of herbicide resistance research. Pest Manag Sci 70:1385–1393

Ngo TD, Malone JM, Boutsalis P, et al (2018) EPSPS gene amplification conferring resistance to glyphosate in windmill grass (*Chloris truncata*) in Australia. Pest Manag Sci 74:1101–1108.

Noctor G, Novitskaya L, Lea PJ, Foyer CH (2002) Co-ordination of leaf minor amino acid contents in crop species: Significance and interpretation. J Exp Bot 53:939–945

Orcaray L, Igal M, Marino D, et al (2010) The possible role of quinate in the mode of action of glyphosate and acetolactate synthase inhibitors. Pest Manag Sci 66:262–269.

Orcaray L, Igal M, Zabalza A, Royuela M (2011) Role of exogenously supplied ferulic and p-coumaric acids in mimicking the mode of action of acetolactate synthase inhibiting herbicides. J Agric Food Chem 59:10162–10168.

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

Osipov VI, Aleksandrova LP (1982) Spatial-organization of quinic and shikimic acid biosynthesis in autotrophic cells of *Pinus sylvestris* needles. Sov Plant Physiol 29:217–222

Ossipov V, Bonner C, Ossipova S, Jensen R (2000) Broad-specificity quinate (shikimate) dehydrogenasefrom *Pinus taeda* needles. Plant Physiol Biochem 38:923–928.

Palma-Bautista C, Torra J, Garcia MJ, et al (2019) Reduced absorption and impaired translocation endows glyphosate resistance in *Amaranthus palmeri* harvested in glyphosate-resistant soybean from Argentina. J Agric Food Chem 67:1052–1060.

Pan D, Li QX, Lin Z, et al (2017) Interactions between salicylic acid and antioxidant enzymes tilting the balance of H_2O_2 from photorespiration in non-target crops under halosulfuron-methyl stress. Pestic Biochem Physiol 143:214–223.

Pan L, Yu Q, Han H, et al (2019) Aldo-keto reductase metabolizes glyphosate and confers glyphosate resistance in *Echinochloa colona.* Plant Physiol 181:1519-1534.

Perotti VE, Larran AS, Palmieri VE, et al (2019) A novel triple amino acid substitution in the EPSPS found in a high-level glyphosate-resistant *Amaranthus hybridus* population from Argentina. Pest Manag Sci 75:1242–1251.

Peters B, Strek HJ (2018) Herbicide discovery in light of rapidly spreading resistance and ever-increasing regulatory hurdles. Pest Manag Sci 74:2211–2215.

Pinto JEBP, Dyer WE, Weller SC, Herrmann KM (1988) Glyphosate induces 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase in potato (*Solanum tuberosum* L) cells grown in suspension-culture. Plant Physiol 87:891–893.

Powles SB (2008) Review evolved glyphosate-resistant weeds around the world: lessons to be learnt. Pest Manag Sci 64:360–365.

Powles SB (2010) Gene amplification delivers glyphosate-resistant weed evolution. Proc Natl Acad Sci 107:955–956.

Powles SB, Preston C (1995) Herbicide cross resistance and multiple resistance in plants. Monograph 2. Basel, Switzerland. Herbicide Resistance Action Comitee.

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

Pratelli R, Pilot G (2014) Regulation of amino acid metabolic enzymes and transporters in plants. J Exp Bot 65:5535–5556.

Reinink M, Borstlap AC (1982) 3-deoxy-d-arabino-heptulosonate 7-phosphate synthase from pea leaves: Inhibition by L-tyrosine. Plant Sci Lett 26:167–171.

Roma-Burgos N, Heap I, Rouse C, Lawton-Rauh A (2019) Evolution of Herbicide-Resistant Weeds. In: Korres N, Burgos NR, Duke SO (eds) Weed Control. Sustainability, Hazards and Risks in Cropping System Worldwide. CRC Press, Boca Raton, Florida, 92–132

Romero RM, Roberts MF, Phillipson JD (1995) Anthranilate synthase in microorganisms and plants. Phytochemistry 39:263–276.

Rubin JL, Jensen RA (1985) Differentially regulated isozymes of 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase from seedlings of *Vigna-radiata* [L] Wilczek. Plant Physiol 79:711–718

Salas RA, Dayan FE, Pan Z, et al (2012) EPSPS gene amplification in glyphosate-resistant Italian ryegrass (*Lolium perenne* ssp. multiflorum) from Arkansas. Pest Manag Sci 68:1223–1230.

Sammons RD, Gaines T A. (2014) Glyphosate resistance: State of knowledge. Pest Manag Sci 70:1367–1377.

Schmid J, Amrhein J (1999) The shikimate pathway. In: Singh B (ed) Plant Amino Acids: Biochemistry and Biotechnology. Marcel Dekker, New York, pp 147–169

Schönbrunn E, Eschenburg S, Shuttleworth WA, et al (2001) Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. Proc Natl Acad Sci 98:1376–80.

Siehl DL (1997) Inhibitors of EPSP sinthase, glutamine synthetase and histidine synthesis. In: Roe RM, Burton JD, Kuhr RJ (eds) Hebicide Activity: Toxicology, Biochemistry and Molecular Biology. IOS press, Amsterdam, pp 37–67

Siehl D, Conn E (1988) Kinetic and regulatory properties of arogenate dehydratase in seedlings of *Sorghum bicolor* (L.) Moench. Arch Biochem Biophys 260:822–29

Singh BK (1999) Biosynthesis of valine, leucine and isoleucine. In: Singh BK (ed) Plant Amino Acids: Biochemistry and Biotechnology. Marcel Dekker, New York, pp 227–247

Singh M, Widholm JM (1974) Measurement of the five enzymes which convert chorismate to tryptophan in wheat plants. Physiol Plant 32:240–246

Spitzer-Rimon B, Marhevka E, Barkai O, et al (2010) EOBII, a gene encoding a flower-specific regulator of phenylpropanoid volatiles' biosynthesis in Petunia. Plant Cell 22:1961–1976.

Steinrücken HC, Amrhein N (1980) The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochem Biophys Res Commun 94:1207–1212

Strobel NE, Kuc JA (1995) Chemical and biological inducers of systemic resistance to pathogens protect cucumber and tobacco plants from damage caused by paraquat and cupric chloride. Phytopathology 85:1306–1310

Suzich JA, Dean JFD, Herrmann KM (1985) 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase from carrot root (*Daucus-carota*) is a hysteretic enzyme. Plant Physiol 79:765–770

Taiz L, Zeiger E (2010) Plant Physiology, Fifth edition. Sinauer Associates, Inc., Sunderland, MA, USA

Takatsuji H, Mori M, Benfey PN, et al (1992) Characterization of a zinc finger DNA-binding protein expressed specifically in Petunia petals and seedlings. EMBO J 11:241–249

Tohge T, Watanabe M, Hoefgen R, Fernie AR (2013a) Shikimate and phenylalanine biosynthesis in the green lineage. Front Plant Sci 4:62.

Tohge T, Watanabe M, Hoefgen R, Fernie AR (2013b) The evolution of phenylpropanoid metabolism in the green lineage. Crit Rev Biochem Mol Biol 48:123–52.

Trenkamp S, Eckes P, Busch M, Fernie AR (2009) 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 5:277–291.

Tzin V, Malitsky S, Aharoni A, Galili G (2009) Expression of a bacterial bi-functional chorismate mutase/prephenate dehydratase modulates primary and secondary metabolism associated with aromatic amino acids in Arabidopsis. Plant J 60:156–167.

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

Tzin V, Galili G (2010b) The biosynthetic pathways for shikimate and aromatic amino acids in *Arabidopsis thaliana*. In: The Arabidopsis Book. p e0132

Ulanov A, Lygin A, Duncan D, et al (2009) Metabolic effects of glyphosate change the capacity of maize culture to regenerate plants. J Plant Physiol 166:978–87.

Van Der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 295:295–297.

Verdonk JC (2005) ODORANT1 Regulates fragrance biosynthesis in petunia flowers. Plant Cell 17:1612–1624.

Vila-Aiub MM, Balbi MC, Distéfano AJ, et al (2012) Glyphosate resistance in perennial *Sorghum halepense* (Johnsongrass), endowed by reduced glyphosate translocation and leaf uptake. Pest Manag Sci 68:430–436.

Vila-Aiub MM, Goh SS, Gaines TA, et al (2014) No fitness cost of glyphosate resistance endowed by massive EPSPS gene amplification in *Amaranthus palmeri.* Planta 239:793–801.

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

Vivancos PD, Driscoll SP, Bulman CA, et al (2011) 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 157:256–268.

Weinstein L, Porter C, Laurencot J (1959) Quinic acid as a precursor in aromatic biosynthesis in the rose. Contrib from Boyce Thompson Inst 20:121–134

Weinstein L, Porter C, Laurencot J (1961) Role of quinic acid in higher plants. Contrib from Boyce Thompson Inst 21:201–214

Westwood JH, Charudattan R, Duke SO, et al (2018) Weed management in 2050: Perspectives on the future of weed science. Weed Sci 66:275–285.

Wiersma AT, Gaines TA, Preston C, et al (2015) Gene amplification of 5-enol-pyruvylshikimate-3-phosphate synthase in glyphosate-resistant *Kochia scoparia*. Planta 241:463–474.

Wittembach VA, Teaney PW, Hanna WS, et al (1994) Herbicidal activity of an isopropylmalate dehydrogenase inhibitor. Plant Physiol 106:321–328

Wittenbach V, Abell LM (1999) Inhibition of valine, leucine and isoleucine biosynthesis. In: Singh BK (ed) Plant Amino Acids: Biochemistry and Biotechnology. Marcel Dekker, New York, pp 385–416

Yoshida S, Tazaki K, Minamikawa T (1975) Occurrence of shikimic and quinic acids in angiosperms. Phytochemistry 14:195–197

Yuan CI, Chaing MY, Chen YM (2002) Triple mechanisms of glyphosate-resistance in a naturally occurring glyphosate-resistant plant *Dicliptera chinensis.* Plant Sci 163:543–554.

Zabalza A, Zulet A, Gil-Monreal M, et al (2013) Branched-chain amino acid biosynthesis inhibitors: Herbicide efficacy is associated with an induced carbon-nitrogen imbalance. J Plant Physiol 170:814–21.

Zabalza A, Orcaray L, Fernández-Escalada M, et al (2017) The pattern of shikimate pathway and phenylpropanoids after inhibition by glyphosate or quinate feeding in pea roots. Pestic Biochem Physiol 141:96–102.

Zinellu A, Sotgia S, Posadino AM, et al (2005) Highly sensitive simultaneous detection of cultured cellular thiols by laser induced fluorescence-capillary electrophoresis. Electrophoresis 26:1063–1070.

Ziska L, Dukes J (2011) Weed Biology and Climate Change. Blackwell Publishing Ltd., Iowa, EEUU

Zulet A (2009) Evaluación del efecto fitotóxico de la aplicación foliar de quinato. Trabajo fin de Máster. Univsersidad Pública de Navarra

Zulet A, Gil-Monreal M, Villamor JG, et al (2013a) Proteolytic pathways induced by herbicides that inhibit amino acid biosynthesis. PLoS One 8:e73847.

Zulet A, Zabalza A, Royuela M (2013b) Phytotoxic and metabolic effects of exogenous quinate on *Pisum sativum* L. J Plant Growth Regul 32:779–788.

Zulet A, Gil-Monreal M, Zabalza A, et al (2015) Fermentation and alternative oxidase contribute to the action of amino acid biosynthesis-inhibiting herbicides. J Plant Physiol 175:102–112.