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Tesis Doctoral

Physiological action of herbicides inhibiting amino acid biosynthesis and their sustainable mixtures in *Amaranthus palmeri* sensitive and resistant to glyphosate

Memoria presentada por D. Manuel Fernández Escalada para optar al grado de Doctor con Mención de Doctor Inernacional

Pamplona-Iruñea, junio de 2018

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AUTORIZACIÓN DE LAS DIRECTORAS DE TESIS

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

HACEN CONSTAR:

Que el trabajo titulado **"Physiological action of herbicides inhibiting amino acid biosynthesis and their sustainable mixtures in *Amaranthus palmeri* sensitive and resistant to glyphosate"** que presenta **D. Manuel Fernández Escalada** 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 del Departamento de Ciencias del Medio Natural 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-Iruñea, 23 de abril de 2018

Fdo. Ana Zabalza Aznárez

Fdo: Mercedes Royuela Hernando

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

Fernández-Escalada, M., Gil-Monreal, M., Zabalza, A., Royuela, M. Characterization of the *Amaranthus palmeri* phisiological response to glyphosate in susceptible and resistant populations. *J. Agric. Food Chem.* (2015). DOI: 10.1021/acs.jafc.5b04916. (Publication)

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Fernández-Escalada M., Zulet-González, A., Gil-Monreal, M., Zabalza, A., Royuela, M. Efecto del glifosato y de diferentes intermediarios metabólicos en la regulación de la ruta del siquimato. XVI congreso de la Sociedad Española de Malherbología (Pamplona-Iruña, 25th - 27th October, 2017). (Oral presentation

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La tesis Doctoral comprendida en este volumen es un compendio de trabajos publicados organizada en 3 capítulos. Los 2 primeros capítulos son artículos publicados en revistas científicas cuyo índice de impacto se encuentre incluido en la relación de revistas del *Journal of Citation Reports* y el tercer capítulo es un texto preparado en formato artículo. Además esta Tesis Doctoral consta de una introducción general, unos objetivos, unos materiales y métodos, una discusión general y unas conclusiones comunes a los 3 capítulos. Por último, la bibliografía es común a los 3 capítulos y se encuentra situada al final de este trabajo.

Publications included in this thesis:

-Publication 1: **Manuel Fernández-Escalada**, Miriam Gil-Monreal, Ana Zabalza, Mercedes Royuela

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Effects of EPSPS copy number variation (CNV) and glyphosate application on the aromatic and branched chain amino acid synthesis pathways in *Amaranthus palmeri.* 8:1–11 (2017) DOI: 10.3389/fpls.2017.01970.

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ABBREVIATIONS

AAA	Aromatic amino acid
AAAs	Aromatic amino acids
ABC	ATP-binding cassette transporter family
ACCase-inhibitors	Acetyl CoA carboxylase inhibitors
ADH	Alcohol dehydrogenase
ADT	arogenate dehydrogenase
ArDH	Arogenate dehydratase
AHAIR	Acetohydroxyacid isomerreductase
AHAS (ALS)	Acetohydroxyacid synthase
Ala	Alanine
ANOVA	Analysis of variance
AP	Alkaline phosphatase
Arg	Arginine
AS	Anthranilate synthase
Asn	Asparagine
Asp	Aspartic acid
BCAA	Branched-chain amino acid
BCAAs	Branched-chain amino acids
BCIP/NBT	5-bromo-4-chloro'-indolyphosphate/nitro-blue-tetrazolium chloride
cDNA	Complementary deoxyribonucleic acid
CM	Chorismate mutase
CNV	Copy number variation
CP	Crossing point
CS	Chorismate synthase
Ct value	Threshold cycle value
Cys	Cysteine
Da	Dalton
DAHPS	D-arabino-heptulosonate 7-phosphate synthase
DHAD	Dihydroxyacid dehydratase
DHQS	Dehydroquinate synthase
DNA	Deoxyribonucleic acid
DQSD	3-dehydroquinate dehydratase/shikimate dehydrogenase
DTT	Dithiothreitol
EC_{50}	Half maximal effective concentration
ECL	Enhanced chemiluminescence
EDTA	Ethylendiaminetetraacetic acid
EOBII	Emission of benzenoids II

EPSPS	5-enolpyruvylshikimate 3-phosphate synthase
FITC	Fluorescein isothiocyanate
GABA	Gamma aminobutyiric acid
GD	General discussion
GI	General introduction
Gln	Glutamine
Glu	Glutamic acid
GM	Genetically modified
GOI	Gene of interest
GR	Glyphosate resistant
GS	Glyphosate sensitive
His	Histidine
HRAC	Herbicide Resistance Action Committee
HRP	Horseradish peroxidase
IAA	Indole-3-acetic acid
IC	Ion cromatograph
IgG	Immunoglobuline G
Ile	Isoleucine
LDH	Lactate dehydrogenase
Leu	Leucine
LSD test	Least significant difference test
M	Molar
MESG	2-amino, 6-meracptho, 7-methyl-purine riboside
Met	Methionine
MM	Materials and methods
MOPS	3-(N-morpholino) propanesulfonic acid
*m*RNA	Messenger ribonucleic acid
MTAB	Myristril trimethyl ammonium bromide
MYB	Myeloblastosis family
N	Normal
NADH	Nicotinamide adenine dinucleotide hydride
NCBI	National Center of Biotechnology Information
NST1	NAC secondary Wall thickening promoting factor 1
NST3	NAC secondary Wall thickening promoting factor 3
ODO1	Odorant1
ORCA3	Octadecanoid-derivative responsive Catharanthus AP2-domain
ORF	Open Reading frame
PAL	Phenylalanine ammonia lyase
PAR	Photosynthetically active radiation

PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex
PEP	Phosphoenolpyruvate
Phe	Phenylalanine
PMSF	Phenylmethylsulfonyl fluoride
PNP	Purine-nucleoside phosphorylase
PPO	Protoporphyrinogen oxidase
Pro	Proline
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
S3P	Shikimate-3-phosphate
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
Ser	Serine
SK	Shikimate kinase
SPSS	Statistical package for the social sciences
TA	Transaminase
TAIR	The Arabidopsis Information Resource
TAL	Tyrosine ammonia lyase
TAT	Tyrosine aminotransferase
TD	Threonine deaminase
Thr	Threonine
TPP (or ThDP)	Thiamine pyrophosphate
Trp	Tryptophan
TTBS	Twin tris buffer saline
Tyr	Tyrosine
Val	Valine

SUMMARY

Over the last century, the production of crops worldwide has substantially increased due to the improvement in crop varieties and in management techniques. One of the most powerful tools used to achieve this increase was herbicides, which eliminates the weeds that compete with the crops for natural resources. However, the sustainted use of herbicides has triggered the appearance of resistance in weeds, which is a global agricultural problem that has been exponentially increasing over the last 30 years.

Glyphosate is the most highly used herbicide worldwide due to its effectiveness and due to genetically modified crops that are resistant to this herbicide. The target site of glyphosate is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme in the biosynthetic pathway of the aromatic amino acids (AAAs). One of the worst weeds reported to have resistant populations to glyphosate is *Amaranthus palmeri*, whose resistance mechanism involves EPSPS gene amplification, which leads to an overexpression of the enzyme target of glyphosate.

The general objective of this work was to evaluate the physiological effects triggered by glyphosate that resulted in plant mortality in sensitive (GS) and resistant (GR) populations, focusing mainly on the consequences of glyphosate treatment in the AAA pathway and the branched-chain amino acid (BCAA) pathway. In addition, mixtures of glyphosate and the AHAS-inhibitor imazamox (an inhibitor of the BCAA pathway) were applied to obtain new insights into the regulation of both amino acid biosynthetic pathways and the possible cross-regulations and to confirm or reject the possibility of the employment of their mixture as an alternative to the application of glyphosate alone. To this end, the response to glyphosate of one glyphosate-sensitive (GS) and one glyphosate-resistant (GR) population of *A. palmeri* from North Carolina were compared at the molecular and biochemical levels.

In this work, a correlation between *EPSPS* overexpression, EPSPS protein content and EPSPS activity and the level of resistance of the *A. palmeri* GR population was confirmed. A high copy number variation (CNV) of EPSPS had no major pleiotropic effect on the studied parameters; the AAA and BCAA pathway expression, free amino acid profiles and carbohydrate contents were similar for both populations in the untreated plants. This suggests that the CNV

of *EPSPS* had no major pleiotropic effects on the physiology of the resistant plants.

The significant increase in shikimate content with increasing doses of glyphosate in the GS population (but not in the GR population) suggests that the damage produced in the sensitive population was higher. The lower general increase in total free and aromatic amino acid (AAA) content in the GR population than in the GS population confirmed that the GS population damage was higher.

The transcriptional response of the GS and GR populations to glyphosate treatment was studied, and a general induction of AAA pathway enzyme expression was identified. Very high increases in the *Anthranilate synthase* enzyme transcript levels were measured, while *Chorismate mutase* transcripts were not increased, suggesting a preferential flux of carbon towards the formation of tryptophan instead of tyrosine or phenylalanine after the regulatory point of chorismate formation. The absence of a response of BCAA gene expression to glyphosate treatment and the AAA gene expression after imazamox treatment suggests that no cross regulation exists between the AAA and BCAA pathways at the transcriptional level, despite their close relationship.

Finally, interactions between mixtures of two different doses of glyphosate and one of imazamox were tested on *A. palmeri*. A general antagonistic effect was detected in the main physiological markers (shikimate, amino acid and carbohydrate levels) because effects that were detected after the application of the mixtures were mostly lower than the effects seen when applying the individual compounds. This general physiological antagonism suggests that the doses cannot be lowered in the mixtures of glyphosate and imazamox that are to be applied in the field.

To summarize, this study describes new physiological insights into the characterization of the glyphosate-resistant weed population of North Carolina and unravels the mode of action of glyphosate on sensitive and resistant plants when applied alone or in combination with imazamox.

RESUMEN

Durante el siglo pasado, la producción agrícola se ha ido incrementando en todo el mundo gracias a la mejora en sus técnicas de manejo. Una de las herramientas más poderosas para alcanzar ese incremento en la producción fueron los herbicidas, los cuales eliminan las malas hierbas que compiten con los cultivos por los recursos naturales. Sin embargo, el uso inapropiado de los herbicidas ha desencadenado la aparición de resistencias en malas hierbas, un problema global para la agricultura que se ha ido incrementando durante los últimos 30 años.

El glifosato es el herbicida más usado en todo el mundo debido a su efectividad como herbicida total, y sobre todo desde la aparición de cultivos genéticamente modificados resistentes a este herbicida. La diana del glifosato es la enzima 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), en la ruta biosintética de los aminoácidos aromáticos (AAA). Una de las peores malas hierbas que se ha descrito como resistente al glifosato es *Amaranthus palmeri*, cuyo mecanismo de resistencia es la amplificación génica de la enzima *EPSPS*, lo que conlleva una sobreexpresión de ésta enzima diana del glifosato.

El objetivo general de este trabajo fue evaluar los efectos fisiológicos desencadenados por el glifosato que llevan a los individuos de las poblaciones sensible (GS) y resistente (GR) a la muerte, centrándolo fundamentalmente en las consecuencias del tratamiento con glifosato sobre la ruta de los AAA y la ruta de los aminoácidos ramificados (BCAA). Además, se aplicarion mezclas de glifosato y el inhibidor de la AHAS imazamox (inhibidor de la ruta de los BCAA) para profundizar en la regulación de ambas rutas biosintéticas de aminoácidos y su posible regulación cruzada, además de para confirmar o rechazar la posibilidad del empleo de las mezclas como una alternativa a la aplicación del glifosato en solitario. Para estas metas, la respuesta al glifosato de una población sensible (GS) y otra resistente (GR) de *A. palmeri* obtenidas en Carolina del Norte, fueron comparada a los niveles molecular y bioquímico.

En este trabajo se ha confirmado la correlación entre la sobreexpresión génica, el contenido proteico y el nivel de actividad de la enzima EPSPS con el nivel de resistencia de la población GR de *A. palmeri*. Los altos niveles de variación en el número de copias génicas (CNV) de la enzima EPSPS no

tuvieron efectos importantes en los parámetros estudiados: la expresión de las rutas de biosíntesis de los AAA y de los BCAA, el contenido en aminoácidos libres y el contenido en carbohidratos fue similar para ambas poblaciones en condiciones de control. Esto sugiere que la CNV de la enzima *EPSPS* no tuvo un efecto pleiotrópico importante en la fisiología de las plantas resistentes.

La acumulación de siquimato producida tras el tratamiento con glifosato en la población GS (pero no en la GR) indicó que la afección producida en la población sensible fue mayor. Los incrementos en los niveles de aminoácidos totales libres y aromáticos, menores en la población GR que en la GS, confirmaron que el daño producido en la población sensible fue mayor.

Se estudió la respuesta transcripcional de las poblaciones GS y GR al tratamiento con glifosato, y se detectó una inducción general de la expresión de las enzimas de la ruta de los AAA. Se encontraron incrementos muy grandes en los niveles de tránscritos de la enzima *Antranilato sintasa* mientras que los niveles de tránscritos de la enzima *Corismato mutasa* no se incrementaron, lo que sugiere un flujo preferencial de carbono hacia la formación de triptófano en vez de tirosina o fenilalanina tras el punto regulatorio de la formación de corismato en presencia del herbicida. La ausencia de respuesta al tratamiento con glifosato en la expresión génica de la ruta biosintética de los BCAA, y de la ruta de los AAA al imazamox, sugirió que no existe regulación cruzada entre las rutas biosintéticas de los AAA y de los BCAA a nivel transcripcional, a pesar de su estrecha relación.

Por último, las interacciones entre las mezclas de dos dosis diferentes de glifosato y una de imazamox se estudiaron en *A. palmeri*. Se detectó un efecto antagonista general en los principales parámetros fisiológicos: siquimato, aminoácidos y niveles de carbohidratos, porque los efectos detectados con las mezclas fueron en su mayoría menores que la adición de los efectos individuales de cada herbicida. Este antagonismo fisiológico general sugiere que al aplicar la mezcla de glifosato e imazamox en campo, las dosis recomendadas no pueden ser disminuidas.

Resumiendo, esta Tesis describe nuevos aspectos fisiológicos en la caracterización de la población de Carolina del Norte resistente al glifosato y desentraña el modo de acción del glifosato en plantas sensibles y resistentes cuando éste es aplicado individualmente o combinado con imazamox.

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GENERAL INTRODUCTION

GI.1 OVERVIEW: HERBICIDE RESISTANCE

GI.1.1 Herbicides

In a world with a growing population and increasing demand for food, innovations in agronomy are not an option but a necessity. In this context, in the middle of the past century, the “green revolution” began, which substantially increased the production of crops all over the world thanks to improvements in crop management and adjusting fertilization, irrigation, rotation and weed control techniques, among many others. These investigations and new applications brought many advantages but also some disadvantages, such as water contamination, risks to health and the environment, loss of soils and ecosystems, and increased resistance in non-beneficial organisms, such as weeds.

Weeds increase the cost of crop production because they compete with the crops for available resources such as water, nutrients, light and space, and they can host dangerous pests for crops and make harvest difficult.

Several methods have been implemented to avoid the problems derived from weeds. Although there are other methods to control weeds, including agronomical and mechanical measures, chemical methods are the most extensively used, and among them, herbicides are the most important tools to control weeds (Edwards et al. 2014).

Herbicides are small molecules (molecular mass of 700 Da or less) that inhibit specific molecular target sites within plant biochemical pathways and/or physiological processes (Dayan et al. 2010), which usually trigger a large number of negative effects in the plants that lead to death. In addition, the affinity of these compounds for their respective biological targets makes them useful tools to study plant biochemical and physiological processes (Dayan et al. 2010).

At this point, two concepts that will be used in this work must be clarified: the mechanism of action and the mode of action of an herbicide. The mechanism of action is the target site or biochemical process that is specifically blocked by the herbicide, and the mode of action could be defined as the subsequent physiological processes that lead them to the death of the plant.

Herbicides can be classified in a variety of ways according to their chemical structure group, selectivity, application characteristics or mechanism of action. Classification based on the mechanism of action is one of the most useful and includes at least 25 groups that encompass all major plant functions: photosynthesis; fatty acid, amino acid, protein and pigment synthesis; the hormone system; and the cell cycle and cell wall formation. The most widespread herbicide site of action classification system is the HRAC classification (Herbicide Resistance Action Committee). This system differentiates groups of actions named with letters of the alphabet and describes not only the chemical family belonging to a specific site of action but all compounds, via their common names, included in each family. Délye et al. (2013) summarized this classification system taking into account the cellular distribution of the sites of action (Fig. GI.1).

In this work, herbicides of family B and family G were used. Both of them affect the amino acid synthesis of the plant (Fig. GI.1). Family G affects the aromatic amino acid biosynthesis pathway (AAA pathway), with the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) being the target of herbicides belonging to this family (Steinrücken and Amrhein 1980). The unique herbicide of this family is glyphosate, which has been commercialized and is the most highly used herbicide worldwide. Glyphosate is a broad-spectrum herbicide that controls a large number of weeds. It is a total, systemic herbicide that can be used in pre- or post-emergence (Shaner 2000; Duke and Powles 2008). The use of glyphosate is allowed in crops in Spain (Regulation (EC) Nº 1107/2009).

Family B herbicides affect the branched-chain amino acid biosynthesis pathway (BCAA pathway). This family of herbicides inhibits the acetohydroxyacid synthase (AHAS) enzyme blocking BCAA pathway, which leads to plant death. AHAS-inhibitors are selective herbicides with the capability to control many weed species at low rates and with low risk to mammalian health, characteristics that have led them to be intensively used worldwide in most of the important crops (Powles and Yu 2010).

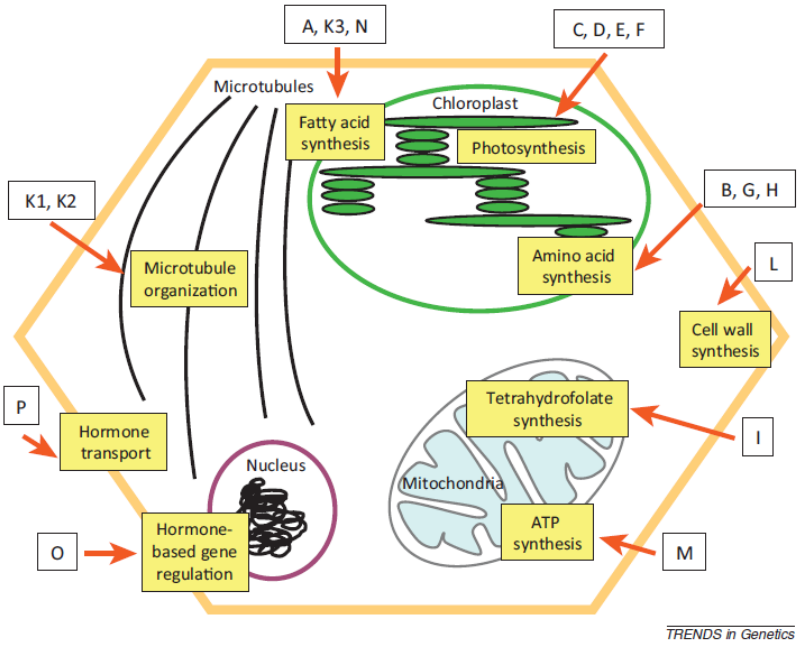

Figure GI.1. Cellular targets of herbicide action and herbicide classification by mechanism of action according to the Herbicide Resistance Action Committee (HRAC). Each capital letter in box is a family of herbicides that has the same mechanism of action. Herbicides target only a few proteins or processes among the tremendous range present in plants. (Délye et al. 2013).

Since their discovery in the 1980s, new compounds belonging to this family of herbicides have been developed, and there are currently five different chemical classes of herbicides that inhibit AHAS: sulfonylureas, sulfonylaminocarbonyltriazolinones, imidazolinones, triazolopyrimidines and pyrimidinyl-oxy-benzoates (Powles and Yu 2010). Concerning imidazolinones, six herbicide active ingredients have been registered: imazamox, imazethapyr, imazapyr, imazamethabenz-methyl, imazaquin and imazapic, and they can be applied as a single active ingredient, as a combination of two ingredients, or in combination with other herbicides (Tan et al. 2006; Délye et al. 2016). Although six active ingredients have been registered, only the use of imazamox is allowed on crops in Spain (Regulation (EC) Nº 1107/2009). Imazamox is a selective herbicide that can be used on different crops such as *Medicago sativa*, *Pisum sativum*, *Vicia faba*, *Phaseolus vulgaris*, *Glycine max*, and *Papaver somniferum* and the Clearfield® crops of *Oryza sativa*, *Helianthus annuus* and *Zea mays*.

The BCAA and AAA biosynthetic pathways take place in the chloroplast and mainly take part in the building of proteins. Moreover, these pathways are involved in the synthesis of secondary metabolites related to a broad range of functions in the plant (Noctor et al. 2002; Sato et al. 2006), including defence functions against stress factors such as herbicides.

GI.1.2 Resistance, a global problem

Herbicide resistance is the ability of a weed biotype to survive an herbicide application, where under normal circumstances, that herbicide applied at the recommended rate would kill the weed. The resistance of weed biotypes to herbicides is a consequence of naturally occurring mutations and evolutionary processes. Individuals within a species that are best adapted and not susceptible to a particular practice, such as the application of a specific herbicide, are selected for and will increase in the population over time. Mitigating or slowing the evolution of herbicide resistance relies on reducing the selection pressure for resistance through the application of diverse weed management practices (Duke 2018).

Today, some integrated management techniques that take into account all the interactions in a crop environment are being proposed. However, the need for fast solutions to overcome the lack of efficient tools for the control of resistant weeds makes long-term techniques difficult to apply and makes faster solutions more attractive, even though they do not often solve the whole problem. Although chemical management has been very effective for weed control, this practice brings a variety of issues, including the potential risk for the development of resistant weeds (Fig. GI.2) that eventually results in the herbicides being inefficient tools for weed control; thus, making weed control much more expensive and difficult.

Long-term repeated application of the same herbicide, or other herbicides with the same site of action, accelerates the selection of resistant weeds to that type of herbicide (Neve et al. 2014). Thus, the classification of herbicides concerning their mechanism of action must be taken into account (Fig. GI.1). In this sense, the HRAC classification system is very useful because it classifies herbicides into families according to their mechanism of action, which is visually represented and summarized in Délye et al. 2013 (Fig. GI.1).

This classification groups herbicides that have the same affected processes in the weed; therefore, in case of the appearance of resistance when an herbicide is used, selection of an herbicide from another family would be the first alternative to improve weed control.

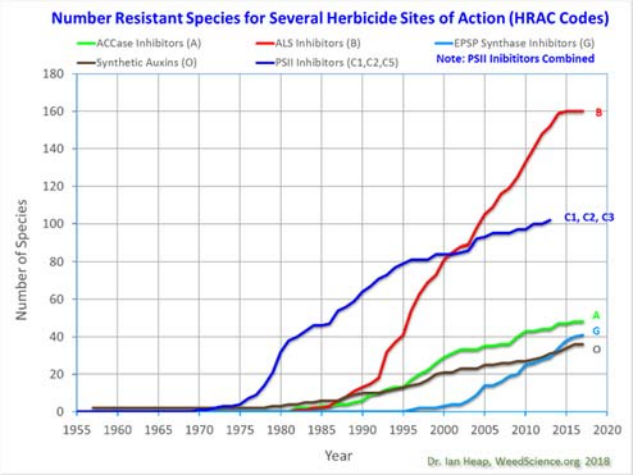

Figure GI.2. Evolution of the number of species that have at least one population resistant to herbicides, grouped for several mechanisms of action with Herbicide Resistance Action Committee (HRAC) codes (each different capital letter indicate a different mechanism of action) (Heap 2018).

In the last 40 years, the increase of resistance is a general feature among all chemical families (Fig. GI.2). In 1980, less than 50 different weed species showed at least one population with resistance to herbicides, while in 2018 this number has increased to close to 500 weeds (Heap 2018) (Fig. GI.2). In addition, another problem is that almost 100 weed species have populations that are resistant to multiple sites of action (Heap 2018), further complicating their management (Tetard-Jones et al. 2018). Multiple resistance is defined as the expression (within individuals or populations) of more than one resistance mechanism (Powles and Preston 1995). Multiple-resistant plants may possess from two to several distinct resistance mechanisms and may exhibit resistance to only a few or several herbicides (Powles and Preston 1995).

The selection pressure that chemicals place on weeds is the perfect example of evolutionary mechanisms and the “artificial” selection of some favourable mutations or developed abilities, which in this case allows weeds to be able to resist herbicides (Duke 2018). The global issue of herbicide resistance for weed management is a serious challenge for global food security (Délye et al. 2013, Tétard-Jones et al. 2018).

GI.1.3 Mechanisms of resistance to herbicides

After application, herbicide molecules penetrate into the plant and are translocated to the location of the target protein. The herbicide molecules accumulate at the location of the target proteins and bind to the target proteins, thereby disrupting biosynthetic pathways or vital cell structures and/or generating cytotoxic molecules (Fig. GI.3) (Délye et al. 2013). Several resistance mechanisms that interfere with the action for the herbicide at different steps have evolved in weeds (Délye et al. 2013).

Resistance can be classified into two main groups depending on the strategy used by the plant to survive the herbicide: non-target site and target site mechanisms (Powles and Preston 1995, Tétard-Jones et al. 2018).

- ✓ **Non-target site mechanisms**

Evolved non-target site herbicide resistance can be due to one or a combination of different mechanisms that make it difficult for an herbicide to reach its target or limits the amount of herbicide that reaches the target protein to a nonlethal dose. This type of mechanism, which is unspecific, includes decreased herbicide penetration into the plant, decreased rates of herbicide translocation, increased rates of herbicide sequestration/metabolism (Powles and Yu 2010), and protection against herbicide effects (Délye et al. 2013). Non-target site herbicide resistances are proposed to be irrespective of the mechanism of action of the herbicides applied (Sabbadin et al. 2017) and can be explained by following the path of the herbicide from its application to its final effect (Fig. GI.3).

Non-target site mechanisms of resistance include reduced penetration (Powles and Yu 2010) and translocation (Shaner 2009) (Fig. GI.3, letters A and B). A reduction in herbicide penetration has been reported in plants that are resistant to all major herbicides (Délye 2012), among them are glyphosate (Vila-Aiub et al. 2012) and AHAS-inhibitors (White et al. 2002). Differences in the physical and chemical properties of the cuticle of resistant plants cause a reduction in the retention of the herbicide solution or penetration through the cuticle (Délye 2012). This type of mechanism is not expected to confer high levels of resistance (Délye 2012).

If the herbicide reaches the target site, an enhanced metabolism can degrade the active herbicide molecules before it can affect its target (Fig. GI.3, letter C). Herbicide degradation is a complex process that involves the coordinated action of several types of enzymes (Délye 2012): cytochrome P450s, glutathione-S-transferases, glycosyl-transferases, esterases, ABC transporters and hydrolases (Yuan et al. 2007; Powles and Yu 2010; Délye et al. 2013; Brazier-Hicks et al. 2018). These proteins act in four consecutive steps. First, the herbicide molecules are transformed into more hydrophilic metabolites, usually via oxidation. They are then conjugated to a plant acceptor molecule (normally thiols or sugars) and the metabolites are transported to a vacuole and/or the cell wall where, finally, further degradation may occur (Yuan et al. 2007).

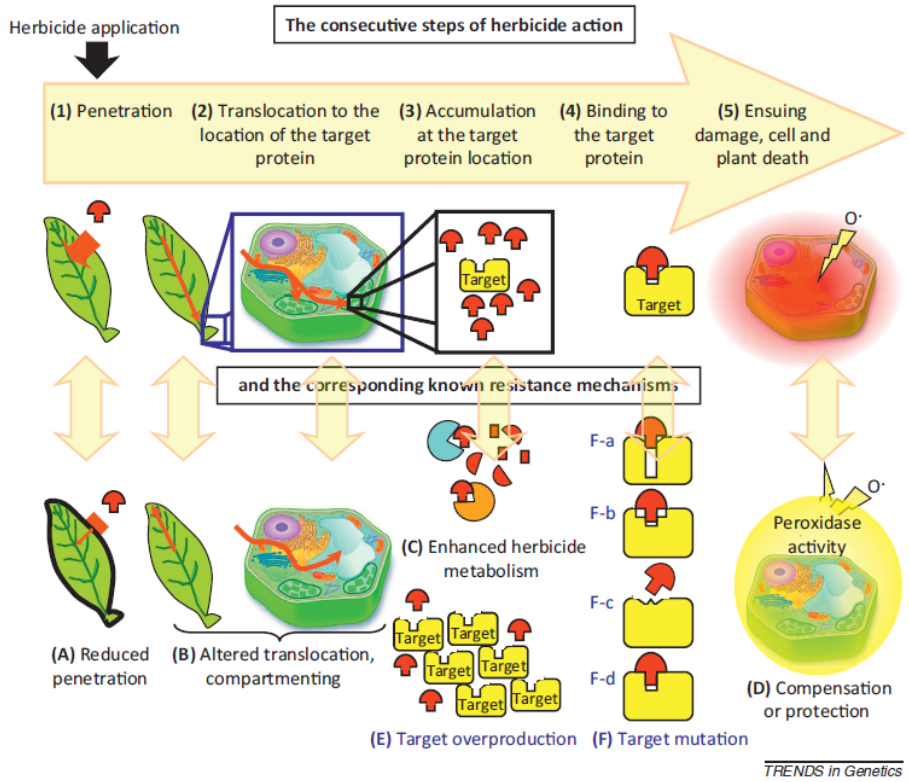

Figure GI.3 Resume of target site and non-target site mechanisms of resistance with the consecutive steps of herbicide action as a guide. Capital letters in black (A, B and D) indicate non-target site mechanism of resistance and capital letters in blue (E and F) indicate target site mechanism of resistance. Numbers 1 to 5 indicate the consecutive steps of herbicide action. (Taken from Délye et al. 2013).

If damage is produced by the herbicide, some plants can neutralize the effects of toxic molecules generated by the herbicide-target site interaction (Délye et al. 2013) (Fig. GI.3, letter D). An increase in the expression of oxidases and peroxidases protects the cells against oxidative damage, thereby giving the resistant plant more time to degrade the herbicide (Délye 2012).

The most negative aspect of non-target site resistance is an associated cross-resistance, which is unpredictable and is not specific to the mechanism of action of the herbicides (Edwards et al. 2014). Resistance that has been evolved by a given herbicide can confer cross-resistance to other herbicides with other mechanisms of action (Délye et al. 2013).

- ✓ **Target site mechanisms**

Evolved target-site resistance can be produced by the overexpression of a target enzyme or can occur by a gene mutation that allows an amino acid change in a target enzyme that prevents the herbicide from binding (Powles and Yu 2010).

In the first case, the target overproduction (Fig. GI.3, letter E) increases the herbicide dose needed for weed control. The overproduction of the target can occur either due to an increase in the genomic copy number of the target or due to an enhanced translation of a normal genomic copy number into proteins. Several cases of resistance by target overproduction have been described in recent years, and this mechanism was first identified in glyphosate-resistant plants in Gaines et al. (2010).

In the second case, mutations that provoke structural changes in proteins (Fig. GI.3, letter F) can be selected over generations by pressure from the repetitive use of an herbicide with the same target site (Sabbadin et al. 2017). These structural changes could result in strong negative effects on the ability of the herbicide to bind the target protein, which results in no (Fig. GI.3, letter F-a), moderate (Fig. GI.3, letter F-b) or a marked reduction (Fig. GI.3, letter F-c) in herbicide sensitivity at the protein level. In contrast, structural changes could result in an increased ability of the herbicide to bind the target protein (Fig. GI.3, letter F-d), which increases the sensitivity to the herbicide at the protein level (Délye et al. 2013).

GI.2 GLYPHOSATE

GI.2.1 Overview

Over the past four decades, the herbicide glyphosate has changed modern agriculture. Glyphosate is the common name of the molecule N-(phosphonomethyl) glycine that was first commercialized as Roundup® in 1974. This compound is a unique herbicide that stands alone in many categories (Sammons and Gaines 2008), and due to its properties, it is the most highly used herbicide around the world.

Glyphosate is a water-soluble herbicide that penetrates the leaf cuticle and is symplastically translocated via phloem to apical meristems, although acropetal glyphosate movement through the apoplast has also been reported (Vila-Aiub et al. 2012). This herbicide belongs to the G family of herbicides, which means that its mechanism of action is the specific inhibition of EPSPS, an enzyme that converts shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to 5- enolpyruvylshikimate-3-phosphate (EPSP) in plastids in the biosynthetic AAA pathway (Steinrücken and Amrhein 1980) (Fig. GI.4). In the functioning of EPSPS, the enzyme must first bind to S3P as the first substrate, and the complex formed by EPSPS and S3P (EPSPS-S3P) subsequently binds to PEP as the second substrate. Glyphosate is a competitive inhibitor of PEP. When glyphosate binds to EPSPS, this prevents binding between PEP and the EPSPS-S3P complex, blocking its enzymatic activity (Schönbrunn et al. 2001). This affects the biosynthesis of three aromatic amino acids (AAAs): tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe).

Glyphosate is a total herbicide that, today, can be used as a post-emergence or pre-emergence herbicide.

When glyphosate reached the market in 1974, it was classified as a post-emergence, non-selective herbicide (Duke and Powles 2008). For agricultural purposes, when it is not applied in combination with genetically modified (GM) crops, glyphosate is applied over the cropland only in pre-emergence due to its capability to kill almost all plants (weeds or otherwise) (Duke and Powles 2008; Duke 2018). Glyphosate is the most highly used herbicide for early-season weed control before planting wheat crops (Duke and

Powles 2008), demonstrating the importance of pre-emergence glyphosate use in direct seeding techniques. In addition to agricultural uses, glyphosate has a side use in non-crop areas where the absence of plants is wanted, such as railways or pathways (Gomes et al. 2014; Duke 2018).

The development of genetically modified resistant crops during the 1990s made possible the agricultural use of glyphosate as a post-emergence herbicide in places where the cultivation of GM resistant crops is authorized (Duke 2018). In these cases, glyphosate is sprayed onto the plant foliage (Gomes et al. 2014) to kill weeds exactly at the time that it is needed.

GM crops resistant to glyphosate were obtained using the *Agrobacterium sp.* strain CP4 (CP4-EPSPS), which is naturally resistant to glyphosate (Tétard-Jones and Edwards 2016). All plants and most bacteria have glyphosate-sensitive class I EPSPS, whereas some bacteria, such as *Agrobacterium sp.* strain CP4, have class II EPSPS that are relatively resistant to glyphosate and therefore have been used to generate glyphosate-resistant crops (Funke et al. 2006). To that end, the CP4-EPSPS gene was inserted into plants to encode an alternative EPSPS that was less sensitive to glyphosate than the endogenous EPSP synthase (Tan et al. 2006). As a result, the plant with the inserted CP4-EPSPS had an alternative EPSPS that was less sensitive or insensitive to glyphosate compared to endogenous EPSPS (Tan et al. 2006) and this allowed plants to survive glyphosate treatment.

For post-emergence treatment, management with glyphosate requires glyphosate-resistant crops; therefore, the increase in the use of glyphosate has been closely related to the increasing development of genetically modified GM crops (Vila-Aiub et al. 2012; Duke 2018). Mechanical innovations such as shielded sprayers and devices that wipe the herbicide onto weeds that are taller than the crop have been developed to allow the use of this herbicide for crops that do not have GM glyphosate resistance. The application of glyphosate to GM crops brings a wide range of advantages (Duke and Powles 2008), and thus, crops can take advantage of their resistance to glyphosate and use all the resources for their growth with no competition. In this context, glyphosate is used on several million hectares of cropland (Powles 2008). However, the use of GM herbicide-resistant crops also brings many risks for weed control, such as grain contamination, segregation and introgression of herbicide-resistant

characteristics, weed population shifts between herbicide-resistant crops and weeds, and an increased use of herbicides for weed control (Owen and Zelaya 2005).

The toxic effect of glyphosate cannot be considered only in terms of its interaction at the target site. The inhibition of EPSPS results in a metabolic roadblock, with physiological consequences leading to plant death (Gomes et al. 2014). Thus, even though the changes in physiological plant processes induced by glyphosate have not been considered as primary effects, these changes contribute to the overall toxic effects of glyphosate.

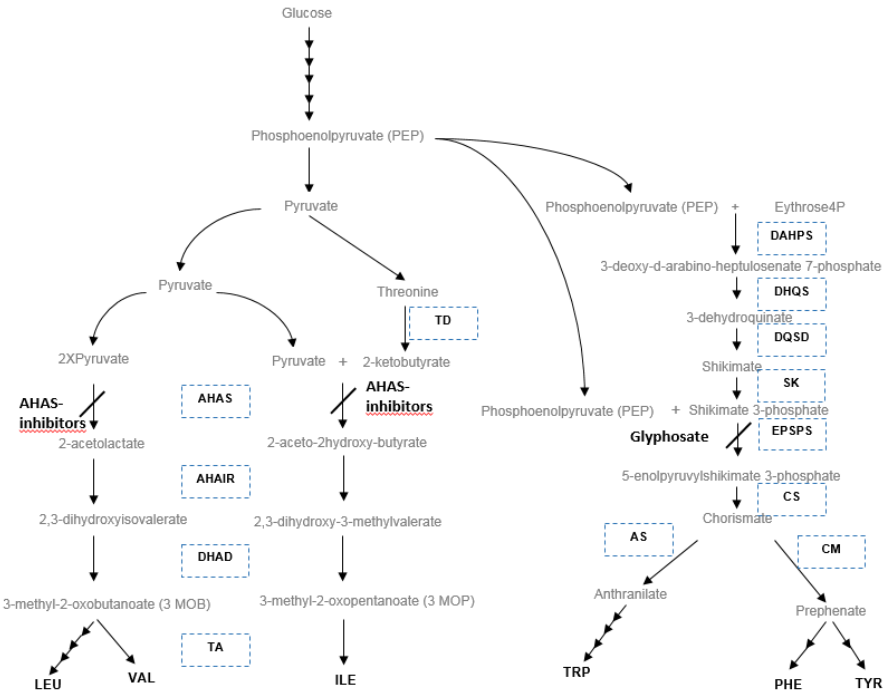

Figure GI.4 Aromatic amino acid pathway (AAA pathway) (in the right side) with their main enzymes: D-arabino-heptulosonate 7-phosphate synthase (DAHPS), dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase/shikimate dehydrogenase (DQ/SD), shikimate kinase (SK), 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), chorismate synthase (CS), chorismate mutase (CM) and anthranilate synthase (AS). Branched-chain amino acid pathway (BCAA pathway) (in the left side) with their main enzymes: Acetohydroxy acid synthase (AHAS), acetohydroxyacid isomerreductase (AHAIR), dihydroxyacid dehydratase (DHAD) and BCAA transaminase (TA).The enzyme that synthesizes 2-ketobutyrate, threonine deaminase (TD), needed to start the reactions in the branch of Isoleucine (ILE) biosynthesis is represented too. Main enzymes of both pathways are represented in bold letters inside a dotted blue box. In black lower case letters the inhibitors of both pathways: Glyphosate in aromatic amino acid pathway and AHAS-inhibitors in branched chain amino acid pathway. The source of carbon for both pathways is phosphoenol pyruvate (PEP) that comes from the photosynthesized glucose through glycolysis. Final products, leucine (LEU), valine (VAL), and isoleucine (ILE) in BCAA pathway, and tryptophan (TRP), phenylalanine (PHE) and (TYR) tyrosine in AAA pathway are represented in bold capital letters and intermediate products are represented in grey lower case letters. (Modified from Tan et al. 2006).

GI.2.2 Glyphosate mode of action

The exact mechanism of plant death after glyphosate treatment is not known. Many studies have included new molecular methods, such as transcriptional comparisons (Yu et al. 2007; Zhu et al. 2008), proteomic approaches (Ahsan et al. 2008) and metabolomic profiling (Maroli et al. 2015; Trenkamp et al. 2009), but to date, the mode of action of this herbicide is not clear.

In general, when the target of an inhibitor is affected, in this case, the EPSPS enzyme affected by glyphosate, death can occur due to different causes:

- ✓ **Accumulation of substrates**

Plant death could be the consequence of an accumulation or increased availability of the substrates of the inhibited enzymatic pathway. These accumulated substances could affect other important pathways in the plant, related in some way in a subtle balance between them.

The main metabolite whose accumulation has been reported upstream of EPSPS is shikimate. Massive levels of shikimate have been detected in plant tissues (Lydon and Duke 1988; Becerril et al. 1989; Gaines et al. 2011) because inhibition of EPSPS causes feedback loops that drive an increased flow of carbon through the shikimate pathway, promoting shikimate accumulation. A decreased activity of the EPSPS enzyme is not enough to consume all the S3P, provoking the upstream accumulation of shikimate. The accumulation of shikimate is a well-known stress marker in plants treated with glyphosate (Zhu et al. 2008; Orcaray et al. 2010; Gaines et al. 2011; Whitaker et al. 2013; Lorentz et al. 2014; Doğramacı et al. 2015; Dillon et al. 2017). Although shikimate accumulation has been proposed to have toxic effects in the plants (De Maria et al. 2006), no evidence confirming this toxicity has been found.

Quinate is another metabolite whose accumulation upstream of EPSPS has been reported (Orcaray et al. 2010). Quinate can enter into the AAA pathway by its conversion to either dehydroquinate or to shikimate by dehydroquinate synthase or 3-dehydroquinate dehydratase/shikimate dehydrogenase, and the inhibition of this main trunk results in an accumulation of quinate (Orcaray et al. 2010). Other metabolites upstream of EPSPS, such

as gallic acid and protocatechuic acid, which are produced in lateral branches of the shikimate pathway, accumulate after glyphosate treatment (Lydon and Duke 1988; Becerril et al. 1989; Hernandez et al. 1999). Nevertheless, it has not been possible to correlate the accumulation of these metabolites with the toxic response of the plant. Death could be the consequence of an accumulation or increased availability of the substrates of the inhibited enzymatic pathway. These accumulated substances could affect other important pathways in plant, related in some way in a subtle balance with them.

- ✓ **Lack of end products**

The lack of end products due to the blockage of a biosynthetic pathway when glyphosate is applied is another possible cause of plant death. If AAA levels are insufficient to maintain necessary protein synthesis, this could trigger injury symptoms and the lack of essential plant compounds, and in the long run, could lead to plant death (Duke and Powles 2008). However, the reduction of AAAs is a tendency that is not always seen in plants when glyphosate is applied. In some cases, a transient decrease occurs, but after that, the AAA content does not decrease significantly (Orcaray et al. 2010). The recovery in AAA levels could be due to increased protein turnover, with a resultant total free amino acid accumulation, including AAAs, and a decrease in soluble protein levels (Zulet et al. 2013a, 2015).

- ✓ **Deregulation of the pathway**

Lethality could be associated with the deregulation of the pathway. When the pathway is deregulated because of the inhibition of the target, several side reactions are triggered, and these side reactions can have effects on different metabolic pathways that are related to the affected pathway (Maroli et al. 2015). EPSPS can directly affect carbon metabolism because PEP is not consumed when EPSPS is inhibited (Fig. GI.4), which causes deregulation at this level (Colombo et al. 1998; De Maria et al. 2006). This deregulation, provoked by glyphosate in a key product of carbon metabolism, could affect carbohydrate biosynthesis and translocation. In relation to this, it has been reported that treatment with glyphosate produces carbohydrate accumulation in leaves and roots of treated pea plants and an enhanced ethanol fermentative metabolism

(Orcaray et al. 2012). Another point of possible deregulation is in the biosynthesis of amino acids.

GI.2.3 Resistance to glyphosate

In 1997, glyphosate-resistant weeds were reported for the first time (Heap 2018; Powles et al. 1998), twenty-three years after the initial use of this herbicide in 1974 (Powles 2008). The introduction of genetically modified crops such as corn, cotton, and soybean exacerbated the evolution of resistance to glyphosate due to its extended use (Nandula 2010; Vila-Aiub et al. 2012). To date, resistance to glyphosate has been documented in 41 species (Fig. GI.5.) (Heap 2018).

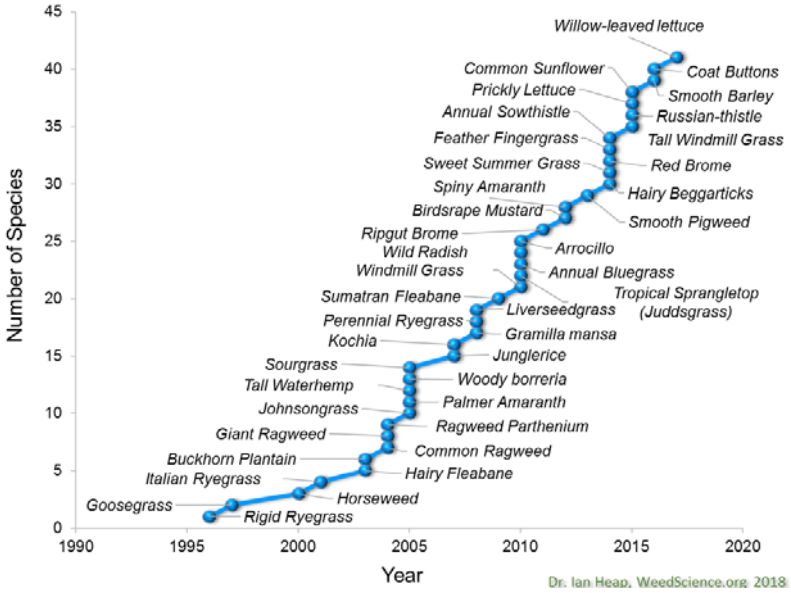

Figure GI.5 Evolution of the number of species that have at least one population resistant to glyphosate in the last 20 years (Heap 2018).

Glyphosate-resistant weeds are a serious challenge to modern agricultural practices and are likely to increase the cost of production (Livingston et al. 2015). Glyphosate resistance individually would not be a major issue because it is controllable by usual herbicide management practices. However, glyphosate was applied to fields that contained weeds that were resistant to other herbicides and, as a result, multiple resistances, which are ever more difficult to control, have arisen (Heap and Duke 2018). Thus, some

important weeds, such as *Amaranthus rudis* (Legleiter and Bradley 2008), *Conyza canadensis* (Davis et al. 2009) and *Lolium rigidum* (Neve et al. 2004), which are simultaneously resistant to glyphosate and another herbicides, have appeared (Powles and Preston 1995; Yu et al. 2009). A major lesson is that maintenance of diversity in weed management systems is crucial for glyphosate to be sustainable (Powles 2008).

Over the years, target site and non-target site resistance and the combination of these have emerged (Nandula et al. 2013).

- ✓ **Non-target site resistance to glyphosate**

Non-target site resistance to glyphosate could be an important evolutionary mechanism of weed resistance (Yuan et al. 2007). Glyphosate provokes low variations for target-site resistance alleles, which leads to a limited number of instances of target-site resistance and confers special importance to the appearance of non-target site resistance.

However, a few cases in which the cause of resistance is at least a partially non-target site mechanism have been described. The main non-target site resistance to glyphosate that has been described is an alteration in the pattern of glyphosate translocation; thus, in resistant plants, less herbicide is translocated to the sinks, and it is blocked in treated leaves in *Sorghum halepense* (Vila-Aiub et al. 2012). In *Lolium rigidum*, there is an increased transport of the herbicide to the leaf tips, with 3-fold less glyphosate being translocated from the leaves to other plant organs (Lorraine-Colwill et al. 2002; Yu et al. 2009). The preferential accumulation of glyphosate in the leaf tips of treated leaves in resistant plants suggests that more glyphosate is travelling in the xylem than is moving into the phloem. Although these authors do not propose vacuolar sequestration in this case, rapid vacuole sequestration of glyphosate has been described in several species as contributing to glyphosate resistance (Shaner 2009; Sammons and Gaines 2014). Reduced glyphosate foliar uptake enhanced reduced translocation and was reported in glyphosate-resistant *Lolium multiflorum* (Nandula et al. 2008) and in *Conyza bonariensis* (Dinelli et al. 2008).

- ✓ **Target site resistance to glyphosate**

-Target mutation: In the structure of the EPSPS enzyme, the substitution of proline in position 106 with other amino acids changes the physical-chemical properties of the enzyme. Pro-106 substitutions cause a slight narrowing of the glyphosate/PEP binding site cavity, which endows glyphosate resistance but preserves EPSPS functionality (Healy-Fried et al. 2007). Other substitutions in amino acid positions, such as glycine-101 and threonine-102, have also been reported (Powles and Yu 2010), but they cause such a great reduction in binding that neither glyphosate nor PEP can bind to EPSPS, which implies that EPSPS losses its functionality (Powles and Yu 2010).

The first report of this type of resistance was in *Eleusine indica* (Lee 2000), where proline was changed for serine (Pro-106-Ser) (Powles and Yu 2010). Subsequently, threonine substitution (Pro-106-Thr) in *Lolium rigidum* (Wakelin and Preston 2006) and alanine substitution (Pro-106-Ala) (Yu et al. 2007) were reported in a variety of weeds. Recently, leucine (Pro-106-Leu) and serine substitutions (Pro-106-Ser) were reported in *Chloris virgate* (Ngo et al. 2018a). These mutations endow only a limited resistance to glyphosate (Powles and Yu 2010) unless they are combined with other resistance mechanisms.

-Target overproduction: An increase in the copy number of the gene that encodes the EPSPS enzyme allows its overproduction, which confers a high value of resistance to glyphosate (Gaines et al. 2010; Ngo 2018b). This *EPSPS* gene amplification is heritable and correlates with the expression level and glyphosate resistance (Powles and Yu 2010). Plants with higher amplification of the *EPSPS* gene showed higher levels of glyphosate resistance, whereas less amplification of the *EPSPS* gene endowed a lower level of glyphosate resistance (Vila-Aiub et al. 2014; Ngo 2018b). In addition, it has been demonstrated that *EPSPS* overproduction does not have an associated fitness cost (Vila-Aiub et al. 2014).

This type of resistance was first detected in *Amaranthus palmeri*, with a copy number variation (CNV) between 30 and 50 times higher in resistant populations than in sensitive ones (Gaines et al. 2010). The CNV of *EPSPS* is the mechanism of resistance in several other weed species, including *Lolium multiflorum* (Salas et al. 2012) and *Kochia scoparia* (Wiersma et al. 2015) and

in *Amaranthus,* species such as *Amaranthus tuberculatus* (Lorentz et al. 2014) and *Amaranthus spinosus* (Nandula et al. 2014).

GI.3 AROMATIC AMINO ACID BIOSYNTHETIC PATHWAY

GI.3.1 Overview

Aromatic amino acids (AAA) can only be synthesized by bacteria, fungi and plants and are essential amino acids for humans. In plants, the AAA biosynthetic pathway is located in plastids (Schmid and Amrhein 1999; Délye et al. 2013). The importance of this pathway for the plant is well known, in fact, under normal conditions, this pathway can fix approximately 20% of the total carbon fixed by the plant (Haslam 1993).

To understand the complex mechanisms that are triggered in a plant after glyphosate treatment, the entire AAA biosynthetic pathway must be understood, from the carbon inputs to the products of this pathway, which are the three aromatic amino acids Phe, Tyr, and Trp. The AAA pathway uses carbon from primary metabolism derived from glycolysis and the pentose phosphate pathway (Maeda and Dudareva 2012) to form chorismate, the precursor of the essential AAAs (Tzin and Galili 2010) (Fig. GI. 4). The AAA synthesis pathway can be subdivided into two steps: (i) the pre-chorismate pathway (also known as shikimate pathway), which provides the precursor chorismate used for the synthesis of all AAAs; and (ii) the post-chorismate pathway, which can lead to the synthesis of either Phe and Tyr, or Trp, via two different routes (Maeda and Dudareva 2012; Tohge et al. 2013a) (Fig. GI.1).

The synthesis of chorismate is catalysed by seven enzymes acting sequentially (Fig. GI.1): D-arabino-heptulosonate 7-phosphate synthase (DAHPS), dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase/shikimate dehydrogenase (DQ/SD), shikimate kinase (SK), EPSPS, and chorismate synthase (CS). Enzymes DQ and SD form a bifunctional DHQ-SDH dimer in plants that acts in close coordination (Maeda and Dudareva 2012); therefore, in this work, they were treated as one enzymatic complex rather than two different enzymes, and this complex is called DQSD.

By using chorismate as a substrate, there are two enzymes that can act in this pathway, which divides the pathway into two branches. In one branch, the synthesis of Trp starts with the reaction catalysed by anthranilate synthase (AS) using chorismate as a substrate. In the other branch, the synthesis of Phe and Tyr starts with the reaction catalysed by chorismate mutase (CM), again using chorismate as a substrate (Maeda and Dudareva 2012; Tohge et al. 2013a).

Some of the AAAs formed in the pathway are used by plants to form proteins, are precursors for secondary metabolites, and are involved in plant stress tolerance, such as phenylpropanoids, lignans, flavonoids, isoflavonoids, condensed tannins, anthocyanin pigments and some simple phenolic compounds (Janzik et al. 2005; Sato et al. 2006; Maeda et al. 2010; Tohge et al. 2013a).

The synthesis of AAAs is a process that is strongly controlled by many regulators because it is a key pathway in the biology of the plant (Bentley and Haslam 1990; Tzin and Galili 2010a; Tohge et al. 2013b; Galili et al. 2016). There are four main points in the pathway that can be regarded as checkpoints:

1. The entrance of the pathway. The enzyme DAHPS catalyses the first reaction of the pathway: an aldol condensation of PEP and erythrose 4-phosphate to form 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) (Shumilin et al. 2003). This enzyme is highly regulated by different mechanisms, as this reaction can either increase or decrease the entrance of carbon into the entire pathway (Sato et al. 2006; Maeda and Dudareva 2012).

2. The target site of glyphosate. The enzyme EPSPS, whose gene overexpression or mutation confers resistance to glyphosate, may be regulated by a variety of mechanisms at either the pre- or post-transcriptional level, which coordinates the plant response to herbicide treatment (Maeda and Dudareva 2012; Gaines et al. 2013). EPSPS is the sixth enzyme in the AAA pathway (Fig.GI. 4) and is a nuclear-encoded protein that is transported into the plastids (Della-Cioppa et al. 1986; Délye et al. 2013). This enzyme is monomeric and has two differentiated domains joined by protein fibres (Schönbrunn et al. 2001). These fibres can function like a hinge that brings the two protein domains

closer together. When a substrate binds to the enzyme, the two parts of the active site of the enzyme close over the substrate (Schönbrunn et al. 2001).

3. The branch point in the AAA pathway. The two enzymes that use chorismate as a substrate, CM and AS, could also be regarded as checkpoints. Any preferential flux to one branch or another could be related to different signals in the pathway and could give information regarding which amino acid has a regulatory function, what type of regulation is occurring, and what possible pre- and post-transcriptional mechanisms are involved in these enzymatic activities and other intermediate metabolites (Tzin and Galili 2010b; Maeda and Dudareva 2012).

4. The exits of the pathway. Phe serves as a precursor for a large family of secondary metabolites, of which the main group is the phenylpropanoids (flavonoids, monolignols, flavonoids, coumarins, stilbenes, xanthones, phenolic esters, benzoic derivatives, anthocyanins, and cell wall components) (Sato et al. 2006; Boudet 2007) whose biosynthesis is initiated by the activity of phenylalanine ammonia lyase (PAL). The phenylpropanoids possess multiple functions, in particular, protection against various abiotic and biotic stresses, and their production is generally stimulated by such stresses.

Tyr serves as a precursor to several secondary metabolites, including plastoquinones, isoquinolines, alkaloids and non-protein amino acids, which can be catalysed with PAL enzyme that is the principal exit of the pathway. The secondary metabolites formed with this amino acid are of the utmost importance for the plant. Tyr is catalysed by tyrosine ammonia lyase (TAL) or by Tyr-aminotransferase (TAT) (MacDonald and D'Cunha 2007).

Trp is catalysed into many indol-containing secondary metabolites, such as IAA, phytoalexins and terpenoids. There are many enzymes proposed that use Trp as a substrate, such as two cytochrome P450s, Trp decarboxylase and Trp aminotransferase, and it is not clear if one of these is more prominent (Tzin and Galili 2010b).

GI.3.2 Regulation of AAA pathway

All enzymes involved in the AAA pathway have been biochemically characterized, and the corresponding genes have been identified in both microbes and plants (Maeda and Dudareva 2012). Microbial enzymes of the pathway have been extensively studied with genetic analyses that have clarified AAA pathway regulation. In contrast, only limited genetic studies have been performed in plant enzymes of the AAA pathway, and thus, the regulation of the AAA pathway remains poorly understood in plants (Maeda and Dudareva 2012). Although the regulation of the AAA pathway is not completely clear, recent advances have elucidated some possible transcriptional and post-transcriptional regulations.

- ✓ **Transcriptional regulation**

It has been reported that the presence of many transcription factors co-regulate gene expression of the AAA pathway. This regulation is based in several transcription factors: ODORANT1 (ODO1), EMISSION OF BENZENOIDS II (EOBII) (Verdonk 2005) and EPF1, a C2H2-type zinc finger DNA-binding protein (Takatsuji et al. 1992) (Fig. GI.6).

ODO1 and EOBII are closely related; in fact, it has been proposed that EOBII activates ODO1 in *Petunia hybrida* (Van Moerkercke et al. 2011). The suppression of both transcription factors decreased the expression of *CM*, and EOBII suppression resulted in partial *ODO1* downregulation (Spitzer-Rimon et al. 2010) (Fig. GI.6). In contrast, the overexpression of *EOBII* did not significantly alter the expression of *ODO1* or *CM* (Spitzer-Rimon et al. 2010); therefore, additional factors are likely involved (Maeda and Dudareva 2012). ODO1 affected the expression of several enzymes in the AAA pathway; indeed, *DAHPS* and *EPSPS* expression were affected by this transcription factor (Verdonk 2005) (Fig. GI.6).

It was demonstrated that in *Petunia hybrida*, EPF1 exhibited a very similar activation pattern in expression compared with the *EPSPS* activation pattern (Takatsuji et al. 1992). This common pattern was proposed as evidence of specific regulation, in which *EPF1* is a positive transcription factor of EPSPS (Takatsuji et al. 1992) (Fig. GI.6).

It was reported in *Arabidopsis thaliana* that other transcription factor members of the MYB family (MYB 51 and MYB 34) activated genes encoding *DAHPS* (Bender and Fink 1998) and *AS* (Gigolashvili et al. 2007) and that *AS* had complementary regulation with ORCA3 (another MYB) in *Catharanthus roseus* (Van Der Fits and Memelink 2000) (Fig. GI.6).

Other transcription factors, such as NST1, NST3, and MYB 8, had a general effect on the transcription of all the enzymes in the pre-chorismate part of the AAA pathway (Maeda and Dudareva 2012).

- ✓ **Post-transcriptional regulation**

It has been proposed that a general feedback regulation is carried out by the final products of the AAA pathway, with Trp regulating DAHPS and AS, and Tyr and Phe regulating CM (Tzin and Galili 2010b; Galili et al. 2016), although in the case of DAHPS inhibition by Trp, feedback inhibition remains controversial. In fact, other studies have reported the stimulation of this enzyme when Trp levels increase (Fig. GI.6) (Pinto et al. 1988; Maeda and Dudareva 2012). In addition, it has been proposed that Trp stimulates CM enzyme activity (Maeda and Dudareva 2012; Galili et al. 2016), in which case this enzyme acts in the opposite direction as the other two aromatic amino acids (Fig. GI.6).

In the case of Tyr and Phe, it has been proposed that there is not only allosteric inhibition of CM but also inhibition of the final enzymes whose products are the other two AAAs – arogenate dehydrogenase (ADT) in the case of Phe, and arogenate dehydratase (ArDH) in the case of Tyr (Maeda and Dudareva 2012; Galili et al. 2016) (Fig. GI.6).

Currently, it has not been confirmed whether or not there is any kind of post-transcriptional regulation over EPSPS, although an additional control of transcriptional regulation has been proposed (Mazzucotelli et al. 2008; Délye 2012).

A general coordination of transcriptional and post-transcriptional regulation in some important amino acid pathways, including the AAA pathway, has been proposed (Less and Galili 2008). These authors proposed that the stimulation or repression produced by stress causes a reduction or increase in the level of amino acids. This provokes a concomitant elevation or reduction in the allosteric feedback inhibition (red and green arrows in Fig. GI.6), resulting

in an acceleration or slowdown of the carbon flux into the pathway. Only when the levels of the biosynthetic enzymes are not sufficient to maintain flux through the pathway in response to a stress (e.g., glyphosate) would the expression of the genes also be stimulated (or repressed) (Less and Galili 2008).

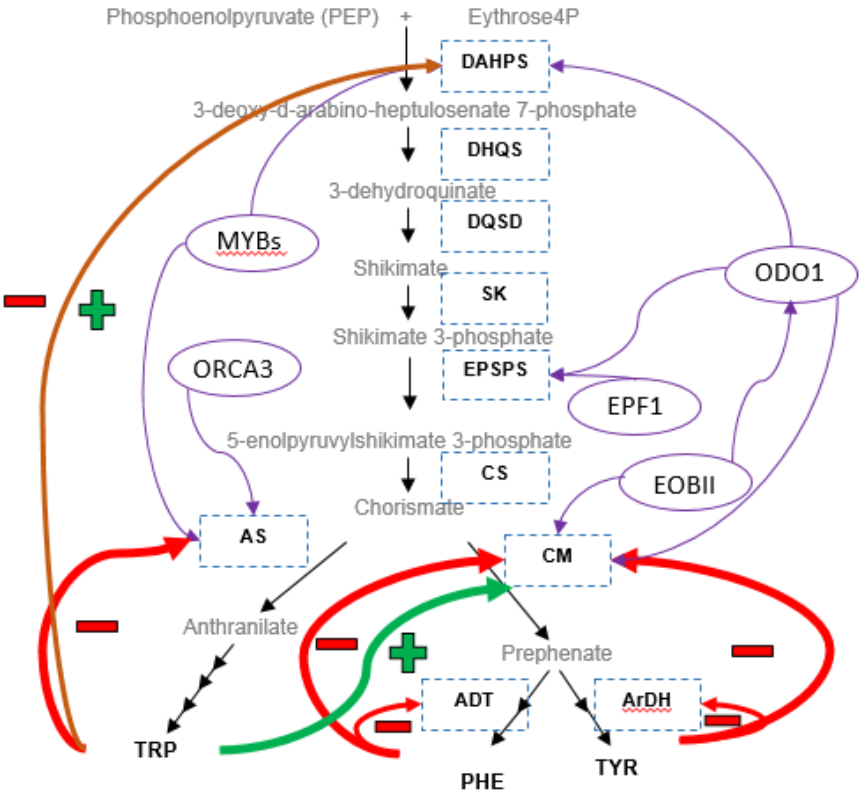

Figure GI.6 Regulation of the AAA pathway with their main enzymes: D-arabino-heptulosonate 7-phosphate synthase (DAHPS), dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase/shikimate dehydrogenase (DQSD), shikimate kinase (SK), 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), chorismate synthase (CS), anthranilate synthase (AS), chorismate mutase (CM) and two post-prephenate enzymes with a regulatory role:arogenate dehydratase ADT and arogenate dehydrogenase (ArDH). Arrows in purple: transcriptional regulation produced by transcriptional factors. Arrows in red, green and brown: Post-transcriptional regulation, being red for inhibition loop, green for enhanced loop, and brown for controversial behavior. In purple circles transcriptional factors: myelobastosis family (MYBs), octadecanoid-derivative responsive Catharanthus AP2-domain 3 (ORCA3), ODORANT1 (ODO1), EPIDERMAL PATTERNING FACTOR 1 (EPF1) and EMISSION OF BENZENOIDS II (EOBII) and in blue boxes enzymes of the pathway. (Based on Maeda and Dudareva 2012).

The stimulation of the genes is probably mediated by transcription factors (Maeda and Dudareva 2012) (purple arrows in Fig. GI.6). It has been proposed a general feedback regulation produced by the final products of the AAA pathway, the three aromatic amino acids (Trp over DAHPS and AS; and

Tyr and Phe over CM) (Tzin and Galili 2010b; Galili et al. 2016) although in the case of DAHPS inhibition by Trp, feedback inhibition is controversial. In fact, other studies reported the stimulation of this enzyme when Trp levels increase (Fig. GI.6) (Pinto et al. 1988; Maeda and Dudareva 2012). In addition, it has been proposed a stimulation of CM enzyme activity by Trp (Maeda and Dudareva 2012; Galili et al. 2016), that acts over this enzyme in the opposite direction comparing to the other two aromatic amino acids (Fig. GI.6).

GI.4 BRANCHED-CHAIN AMINO ACID BIOSYNTHETIC PATHWAY

GI.4.1 Overview

Branched-chain amino acids (BCAAs) can only be synthesized by microorganisms and plants and are essential amino acids for humans. In plants, the BCAA biosynthetic pathway is located in plastids (Wittenbach and Abell 1999).

This pathway is composed of two parallel branches, where Ile is synthesized in one branch and Val and Leu in the other branch. Both branches consist of a single set of four enzymes that develop their function one by one (Galili et al. 2016). These reactions start with the enzyme AHAS, which is a nuclear-encoded protein that is transported into plastids (McCourt and Duggleby 2006). AHAS catalyses the condensation of either two molecules of pyruvate to form acetolactate, or one molecule of pyruvate and one molecule of 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate (Singh 1999; Binder et al. 2007) (Fig. GI.4). 2-Ketobutyrate is synthesized from threonine with the intermediation of the enzyme threonine deaminase (TD) (Halgand et al. 2002) (Fig. GI.4). Once 2-acetolactate or 2-aceto-2-hydroxy-butyrate are formed, the next enzymes act sequentially until the biosynthesis of the BCAAs is complete (Fig. GI.4). Acetohydroxyacid isomerreductase (AHAIR) catalyzes the reductive isomerization of acetolactate to 2,3-dihydroxy-3-isovalerate or the conversion of 2-aceto-2-hydroxybutyrate to 2,3-dihydroxy-3-methylvalerate (Durner et al., 1993). Dihydroxyacid dehydratase (DHAD) catalyses the dehydration of 2,3-dihydroxy-3-isovalerate or 2,3-dihydroxy-3-methylvalerate to the 2-oxo acids 3-

methyl-2-oxobutanoate (3MOB) or 3-methyl-2-oxopentanoate (3MOP). 3-MOB and 3-MOP are 2-oxo acids that can be converted into the three BCAAs; 3-MOB serve as a substrate for the biosynthesis of valine and leucine, and 3-MOP is the substrate for the biosynthesis of isoleucine (Binder 2010). The last step in the biosynthesis is the action of BCAA transaminase (TA), which catalyses 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 (Fig. GI. 4) (Singh 1999).

GI.4.2 BCAA regulation

For BCAA regulation, a predominant mechanism of allosteric post-transcriptional regulation with a general feedback loop produced by BCAA accumulation has been proposed, with other possible transcriptional and post-transcriptional mechanisms that are not yet elucidated (Binder et al. 2007; Galili et al. 2016). An increase in BCAA levels inhibits the AHAS enzyme, which blocks the pathway at the first step. In addition, TD is inhibited by high levels of Ile, although this inhibition of the TD enzyme is weakened by an increase in Val levels (Halgand et al. 2002).

Each AHAS catalytic subunit comes with two separate repeats of its regulatory subunit, and it is speculated that these duplicate regions provide two separate regulatory sites, one for leucine and another for valine/isoleucine, taking into account the synergistic inhibition between leucine and valine/isoleucine (Duggleby et al. 2008), rather than a general inhibition by BCAAs for both branches of the pathway.

GI.4.3 BCAA inhibitors

BCAA inhibitors belong to the B family of herbicides according to HRAC classification, as was explained in GI.1.1. AHAS catalyses the synthesis of acetolactate, a precursor of valine and leucine, and acetohydroxybutyrate, a precursor of isoleucine (Binder 2010). As AHAS is the entrance point of the BCAA biosynthetic pathway, it has been intensely used as a target point for

herbicides (Tan et al. 2006). Indeed, AHAS inhibitors are the only known commercialized herbicides belonging to this family.

AHAS has a catalytic subunit that is normally activated by the cofactor thiamine diphosphate (TPP, also known as ThDP) and a smaller regulatory subunit that controls the catalytic site (Li et al. 2013). It is in this regulatory subunit where the feedback inhibition by BCAAs takes place (Duggleby et al. 2008). The mechanism of AHAS inhibition is not the same as in EPSPS, where glyphosate mimics PEP and binds to the enzyme causing a blockage of the active site of the enzyme. In the case of AHAS, the inhibitors block the channel that allows either the two molecules of pyruvate or one of pyruvate and another of 2-ketobutyrate to enter the active site in the catalytic subunit. This is possible because the active site is deep inside a narrow channel that can be obstructed by the herbicide (McCourt and Duggleby 2006).

Very little is known about AHAIR and DHAD (Zhang et al. 2015). AHAIR has been studied as a target of herbicides, but the inhibition of this enzyme is less efficient than the inhibition of AHAS (Dumas et al. 1994), making AHAS inhibitors much more effective at altering plant metabolism than AHAIR inhibitors (Zabalza et al. 2013). There are two main problems for AHAIR inhibitors. First, there is a larger amount of AHAIR than AHAS; therefore, higher concentrations of herbicide are needed to obtain the same results. Second, binding between AHAIR herbicides and the enzyme are reversible, while AHAS-inhibitor binding is irreversible (Durner et al. 1993). Even fewer studies are available regarding the DHAD enzyme, although the *DHAD* gene is an essential gene whose decreased expression has been related to slowed root growth, and its disruption has been related to a higher susceptibility to salinity in *Arabidopsis thaliana* (Zhang et al. 2015). This lack of knowledge makes it difficult to design inhibitors, and to date, none are present in the market.

TAs have been studied only in a few plant species (Maloney et al. 2010). Six *TA* genes were identified in *Arabidopsis thaliana* (Diebold et al. 2002) and in *Solanum lycopersicum* (Maloney et al. 2010). The location of TAs and their function have been reported in many studies as being highly variable (Diebold et al. 2002; Schuster et al. 2006; Binder et al. 2007; Maloney et al. 2010). This complexity makes very difficult to design an inhibitor for commercialization, and to date, none are commercially available.

GI.4.4 Resistance to herbicides inhibiting AHAS

The primary use of this type of herbicide concerns non-transgenic crops, where they are effective, but their continuous use has resulted in the development of resistance in weeds (Powles and Preston 1995) as has occurred in the case of glyphosate and any other herbicide used in excess. Currently, there are 159 AHAS weed species with at least one population that has shown resistance to AHAS inhibitors, and 39 of them are resistant to imazamox (Heap 2018). This is the highest number of species with at least one population resistant to this group of herbicide (Délye et al. 2016). *A. palmeri* is now included among these species (Gaedert et al. 2017; Küpper et al. 2017).

The main mechanisms of resistance to this family of herbicides are target site mechanisms based on AHAS protein mutations (Ala-122, Pro-197, Ala-205, Asp-376, Trp-574, Ser-653 and Gly-654) (Powles and Yu 2010). This family of herbicides is well known for their ability to select resistant weed populations (Tranel and Wright 2002); therefore, their repetitive use has likely caused the appearance of these mutations and the subsequent resistance in weeds (Sabbadin et al. 2017). Because of this, these herbicides should not be used individually as the sole means of weed control in any field over a long period of time, and mixtures of herbicides could be a possible alternative (Shaner 1999).

Non-target site resistance mechanisms to AHAS inhibitors based on cytochrome P450 monooxygenases have been described (Busi et al. 2011), and it is proposed that these can appear in a wide range of species and can be very specific to a given herbicide (Yu and Powles 2014). These mechanisms are another cause of resistance to AHAS inhibitors in weeds (Délye et al. 2013), although to date they have only been demonstrated in three broadleaf weed species (Délye et al. 2016).

Differential herbicide uptake has been identified as a resistance mechanism to AHAS inhibitors in *Helianthus annuus* (White et al. 2002) and in *Sorghum bicolor* (Anderson et al. 1998). Regarding translocation, only *Echinochloa crus-galli* (Riar et al. 2013) has been reported to have this effect in its resistance against imazamox.

Growers have rapidly adopted glyphosate-resistant crops, in part to control weeds that had evolved resistance to other herbicide chemistries, in

particular, weeds resistant to AHAS inhibitors, ACCase (Acetyl CoA carboxylase) inhibitors and triazines (Heap and Duke 2018), and they have treated the crops only with glyphosate. With the rise of glyphosate-resistant weeds, multiple resistances to glyphosate and other herbicides are presenting a large problem, particularly the multiple resistances to AHAS inhibitors and glyphosate. Today, there are no new herbicide mechanisms of action known, and few new chemistries to work with, which makes the increasing number of glyphosate multiple resistant weeds a particularly difficult problem (Heap and Duke 2018).

GI.4.5 Common physiological effects of AHAS-inhibitors and glyphosate

The targets of the herbicides in the BCAA and AAA biosynthesis pathways are well known. In the AAA pathway EPSPS is blocked, while in the BCAA pathway AHAS is blocked (Binder 2010) (Fig. GI.4). Despite knowing these mechanisms of action, it is still unclear how the inactivation of AHAS or EPSPS results in plant death. Previous studies have reported that in both cases growth arrest is followed by a slow death of the herbicide-treated plants (Gruys and Sikorski 1999; Wittenbach and Abell 1999), and those common effects occur even though glyphosate and AHAS inhibitors act through different pathways. At a molecular level, it has been reported that with both herbicides, an accumulation of free amino acids (Maroli et al. 2015; Orcaray et al. 2010; Zabalza et al. 2017; Zulet et al. 2015), a decrease in the general soluble protein content (Maroli et al. 2015, 2016; Zulet et al. 2013), an accumulation of carbohydrates (Orcaray et al. 2012; Maroli et al. 2015; Zulet et al. 2015) and an increase in fermentative enzymatic activity of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Orcaray et al. 2012) are present. Although the herbicides target different enzymes, these common physiological effects suggest that they kill plants by similar mechanisms and that there is a close cross relation between both pathways.

Herbicide mixtures can interact in three different ways: antagonistic, additive, and synergistic (Barrett 1993). The close relationship between AAAs

and BCAAs could cause notable synergistic effects; therefore, it is important to clarify whether this occurs.

The efficacy of glyphosate-AHAS inhibitor mixtures have been repeatedly tested (Hydrick and Shaw 1994; VanLieshout et al. 1996; Lich et al. 1997; Starke and Oliver 1998; Johnson, W.G. et al. 1999; Li et al. 2002; Nelson and Renner 2002; Shaw and Arnold 2002). Although some additive results have been obtained (Starke and Oliver 1998; Li et al. 2002; Nelson and Renner 2002), other experiments showed antagonistic behaviour (Hydrick and Shaw, 1994; Johnson, et al., 1999; Lich et al., 1997; VanLieshout et al., 1996); thus, more studies are needed to more thoroughly understand what kind of effects are produced in the plant by the mixtures and optimize them.

In the case of *A. palmeri,* it is a crucial time because some populations of this weed have started to show resistance not only to glyphosate but also to AHAS inhibitors (Gaedert et al. 2017; Küpper et al. 2017).

GI.5 CROSS-REGULATION BETWEEN AMINO ACID BIOSYNTHETIC PATHWAYS

The hypothesis of the existence of cross-regulation of amino acid metabolic pathways at the transcriptional level has been proposed (Pratelli and Pilot 2014). Some direct interactions were reported in that paper, such as the requirement of Glu for transamination reactions in the synthesis of BCAAs, the diaminopimelate amino transferase for Lys, the histidinol-phosphate aminotransferase for His, prephenate amino transferase (PAT) for Phe and Tyr, and glutamine (Gln) for the synthesis of anthranilate from chorismate for Gln (mediated by AS) (Pratelli and Pilot 2014). Moreover, the carbon skeleton of some amino acids is used to synthesize other amino acids (e.g., Ser is used for the synthesis of Trp, Cys for the synthesis of Met, and Asp for the synthesis of Arg). It was proposed that these close relationships imply that the donor metabolites are synthesized concordantly with their use in the other amino acid biosynthetic pathways (Pratelli and Pilot 2014).

In contrast, it has been proposed that the apparent cross-regulation of the pathways is the consequence of a stress response triggered by alterations

in the activity of specific amino acid pathways that modify normal amino acid levels that were triggered by amino acid perturbation (Hey et al. 2010).

Some cases of specific coordination between the AAA and BCAA pathways have been reported. Plants expressing two decarboxylases specific for Trp or Tyr showed modification of the content in amino acids from most of the other pathways (Guillet et al. 2000). The contents of many minor amino acids vary in concert between different amino acid biosynthetic families (Noctor et al. 2002), and it has been reported that the closest amino acid biosynthetic pathway to the AAA pathway is the BCAA pathway (Noctor et al. 2002; Orcaray et al. 2010) (Fig GI.4). Treatment of *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).

The common physiological effects of BCAA and AAA inhibitors (GI.4) suggest that the lethal physiological processes triggered could be similar. Additionally, specifically suggested cross interactions (Pratelli and Pilot 2014) make it possible to hypothesize the existence of cross-regulation between the AAA and BCAA biosynthesis pathways (Guyer et al. 1995; Mohapatra et al. 2010; Pratelli and Pilot 2014).

GI.6 *AMARANTHUS PALMERI*

One of the most damaging glyphosate-resistant weed species is *Amaranthus palmeri* S. Wats (Culpepper et al. 2006; Powles and Yu 2010; Whitaker et al. 2013), located in places as diverse as the southern Canadian border and Brazil, which have quite different climates, soil types and agricultural techniques.

A. palmeri is a common C_4 summer annual of the Sonoran Desert. Under conditions of high soil water availability, its photosynthetic capacity exceeds 70 μmol CO_2 m^{-2} s^{-1} at a leaf temperature optimum of 42°C (Ehleringer 1983). It is dioecious with its inflorescences being a terminal spike, with male and female flowers on separate plants (Keeley et al. 1987). This erect annual weed can grow up to 2 m in height, and its terminal seed heads can reach up to 0.5 m (Culpepper et al. 2006). Compared with other *Amaranthus*

species, *A. palmeri* has the largest plant volume, dry weight, leaf area (Horak and Loughin 2000) and rate of height increase per growing degree day (24 to 62% greater) (Culpepper et al. 2006). A rapid growth rate and tall stature make *A. palmeri* extremely competitive with crops (Culpepper et al. 2006).

A. palmeri can cause severe losses in yields in a wide range of crops; representative cases are summarized in Culpepper et al 2006, such as *Zea mays* L. yields, with values reduced from 11 to 91% with 0.5 to 8 plants m^{-1} of row, respectively (Massinga et al. 2001), *Glycine max* yields, with values reduced from 17 to 68% with 0.33 to 10 plants m^{-1} of row, respectively (Klingaman and Oliver 2018) and *Gossypium hirsutum* yields, with values decreasing linearly from 13 to 54% as Palmer amaranth density increased from 1 to 10 plants in 9.1 m of row, respectively (Morgan et al. 2001).

The first case of resistance in *A. palmeri* was reported in 1989 and was to the herbicide family K1 (microtubule inhibitors). The resistances reported until 2005 were to herbicide families K1, C1 (photosystem II inhibitors) and B (AHAS inhibitors). After the first case of resistance was reported against the G family of herbicides (the EPSPS inhibitor, glyphosate) (Culpepper et al. 2006), in most cases subsequently reported resistances in the United States were to glyphosate (Table GI.1). Moreover, resistance to glyphosate was also reported in Brazil and Argentina, probably due to the use of GM crops in these three countries (Table GI.1). Outside of America, only one case of resistance of *A. palmeri* has been reported (in Israel in 2008), but it was to AHAS inhibitors instead of to glyphosate (Heap 2018). Since 2008 there has been a notable increase in cases of multiple resistances to more than one site of action with glyphosate involved (Table GI.1), and there have even been cases of multiple resistances without the involvement of glyphosate, which highlights the necessity to improve the control of this weed.

The mechanism of resistance to glyphosate in some of these populations has been identified as the gene amplification of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Fig. GI.7) (Gaines et al. 2010; Chandi et al. 2012).

	Country	Year	Site of action
1	United States (Georgia)	2005	G
2	United States (North Carolina)	2005	G
3	United States (Arkansas)	2006	G
4	United States (Tennessee)	2006	G
5	United States (South Carolina)	2006	G
6	United States (New Mexico)	2007	G
7	United States (Mississippi)	2008	MR: G+B
8	United States (Alabama)	2008	G
9	United States (Missouri)	2008	G
10	United States (Georgia)	2008	MR: G+B
11	United States (Tennessee)	2009	MR: G+B
12	United States (Louisiana)	2010	G
13	United States (Illinois)	2010	G
14	United States (Ohio)	2010	G
15	United States (South Carolina)	2010	MR: G+B
16	United States (Georgia)	2010	MR: B+C+G
17	United States (Kentucky)	2010	G
18	United States (Michigan)	2011	G
19	United States (Virginia)	2011	G
20	United States (Kansas)	2011	G
21	United States (Texas)	2011	G
22	United States (Arizona)	2012	MR: G+B
23	United States (Delaware)	2012	G
24	United States (Indiana)	2012	G
25	United States (Pennsylvania)	2013	G
26	United States (Illinois)	2013	MR: G+B
27	United States (Florida)	2013	G
28	United States (Florida)	2013	MR: G+B
29	United States (Wisconsin)	2013	G
30	United States (Delaware)	2014	MR: G+B
31	United States (Maryland)	2014	MR: G+B
32	United States (New Jersey)	2014	G
33	Argentina	2015	G
34	Brazil	2015	G
35	United States (Tennessee)	2015	MR: G+E
36	United States (California)	2015	G
37	Brazil	2016	MR: G+B
38	United States (Nebraska)	2016	MR: G+C
39	United States (Illinois)	2016	MR: G+E
40	United States (Oklahoma)	2018	G

Table GI.1 Cases of resistance to glyphosate in *Amaranthus palmeri* populations since the first case detected in 2005. In orange, cases of multiple resistance to 2 sites of action and in purple to 3 sites of action. Capital letters indicate multiple resistance (MR) and the name of the herbicide family: AHAS-inhibitors (B), Photosystem II inhibitors (C), protoporphyrinogen oxidase (PPO) inhibitors (E), and glyphosate (G). (Based on Heap 2018).

The recommended field dose is not sufficient to inhibit EPSPS activity when this enzyme is overexpressed, and thus the plants survive, making the management of this weed more complicated. The resistance to glyphosate of *A. palmeri* was first reported in 2006 (Culpepper et al. 2006), and a few years later the mechanism of resistance was elucidated. This reported mechanism of

gene amplification was important because it was the first study where gene amplification in field-evolved resistance to an herbicide was confirmed (Sammons and Gaines 2014).

It is known that the CNV can vary by 2- to >63- fold compared with sensitive populations in different species, and even between individuals of a single population (Sammons and Gaines 2014). Notably, no fitness cost has been associated with the increase of *EPSPS* gene expression and subsequent increase in enzymatic activity in GR populations (Giacomini et al. 2014; Vila-Aiub et al. 2014). The gene amplification resistance mechanism found in *A. palmeri* (Fig. GI.7) offers us the opportunity to study the effect of glyphosate application, the regulation of the AAA pathway, the effect of *EPSPS* overexpression due to extra *EPSPS* gene copies, and possible cross-regulation mechanisms with other biosynthetic amino acid pathways.

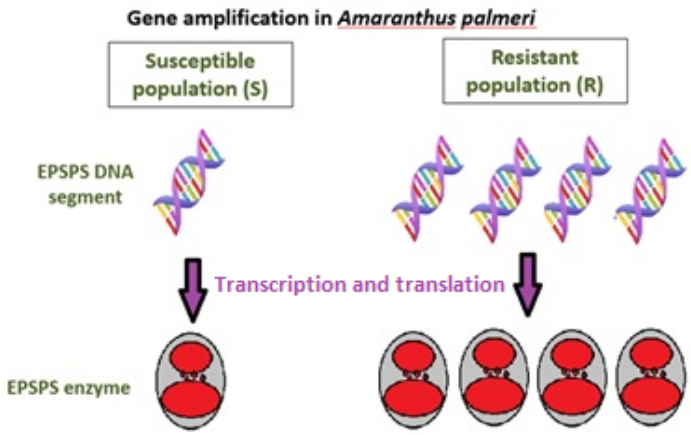

Figure GI.7 Mechanism of target site resistance in *Amaranthus palmeri*. Target overproduction in resistant population (R) comparing to susceptible (S) one. And overproduction at *5-enolpyruvylshikimate 3-phosphate synthase* (*EPSPS*) segment of DNA (helix in purple) results in an increase in EPSPS enzyme (Oval grey with two domains joined by protein fibers, in red)

OBJECTIVES

Although the target site of glyphosate is the well-known inhibition of the EPSPS enzyme in the AAA biosynthetic pathway, its mode of action is not completely clear. The main objective of this work was **to evaluate the physiological effects triggered by glyphosate that lead to the death of *A. palmeri* plants and to clarify whether these effects are different in sensitive and resistant plants**, focusing mainly on the consequences of glyphosate treatment in the AAA and BCAA pathways. To this end, the responses of glyphosate-sensitive and glyphosate-resistant populations (due to EPSPS gene amplification) of *A. palmeri* upon glyphosate treatment were compared at the molecular and biochemical levels. This general aim was approached by three specific objectives that are covered in the three individual chapters of this thesis:

1. To **characterize** the physiology of *A. palmeri*-resistant populations by comparing with sensitive populations after confirming resistance to the herbicide. This information is fundamental for further studies regarding the effects of glyphosate on this weed.
2. To evaluate the impact of *EPSPS* overexpression by gene amplification and of glyphosate treatment on the **regulation of the AAA pathway** and to look for a possible transcriptional **cross-regulation between the AAA and BCAA** biosynthetic pathways.
3. To evaluate whether a mixture of glyphosate and BCAA inhibitors is synergistic, antagonistic or additive and whether the effect is different if the treated plant is resistant to glyphosate.

This thesis consists of a general introduction, a general materials and methods section, three chapters that are original research works (two of them already published) and one general discussion for the three chapters, with a short conclusion of the thesis. The bibliography is presented at the end and covers the three chapters.

MATERIALS AND METHODS

MM.1 PLANT MATERIAL AND TREATMENT APPLICATION

MM.1.1 Plant material

To develop the experiments in all chapters, resistant and sensitive populations of *A. palmeri* originally collected from North Carolina (United States) with a described copy number variation between 22 and 63 fold more in GR population in field respect to GS population (Chandi et al. 2012), were used. The seeds of these populations were provided by Dr. Todd Gaines (Colorado State University, Fort Collins, Colorado).

A. palmeri plants were grown in similar conditions in the three chapters. In all chapters plants were treated when they were homogenous in height (between 5 and 7 cm) (Culpepper et al. 2006). Firstly, seeds were surface sterilized prior to germination (Labhilili et al. 1995).

For germination, seeds were incubated for 7 days at 4 °C in darkness and then maintained for 48 h in a light/darkness cycle of 16 h/8h at temperature of 30 °C in light and at 18 °C in darkness (Fig. MM.1).

Seeds were then transferred to aerated 2.7 L hydroponic tanks in a phytotron (day/night, 16 h/8 h; light intensity, 500 μmol s^{-1} m^{-2} PAR; temperature, 22/18 °C; relative humidity of the air, 60/70%) (Fig. MM.1). Throughout the course of the experiment, the plants remained in the vegetative phenological stage. The nutrient solution (Hoagland and Arnon 1950) was supplemented with 15 mM KNO_3.

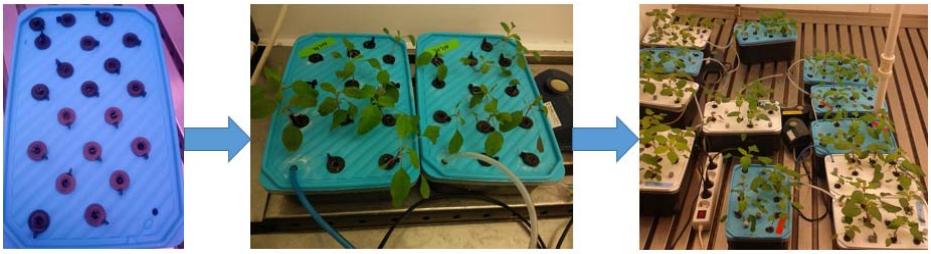

Figure MM.1 Growth process of *A. palmeri* plants. In the left, plants after 48 hours of incubation, in the middle, in an intermediate state of growth in the phytotron and in the right, plants in phytotron just the day of treatment.

Three week-old plants (after reaching the growth stage defined as BBCH 14 (Hesset al., 1997)) were treated with glyphosate (commercial formula, Glyfos, 360 g a.e. L^{-1}, isopropylamine salt, BayerGarden, Valencia, Spain) (Fig. MM.1), and in chapter three with a simultaneous treatment of imazamox (1.5

mg active ingredient L^{-1}) 4% P/V (Pulsar 40®, BASF, Barcelona, Spain). Glyphosate treatment was performed using an aerograph (Junior Start model; Definik; Sagola) at a rate of 500 L ha^{-1}, while imazamox was added to the nutrient solution.

MM.1.2 Dose response study

This experiment was developed only in chapter one to verify the previously reported resistance of *A. palmeri* population GR and to stablish comparable doses between resistant and sensitive populations in order to perform the physiological studies.

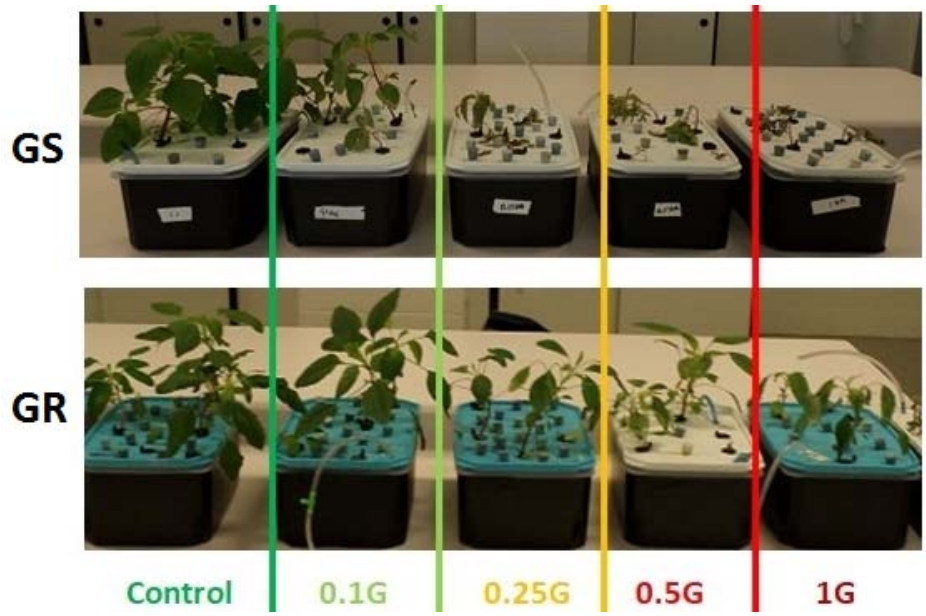

Figure MM.2 Control, 0.1G (0.084 kg ha^{-1}), 0.25G (0.21 kg ha^{-1}), 0.5G (0.042 kg ha^{-1}) and 1G (0.84 kg ha^{-1}) being G recommended field dose (0.84 kg ha^{-1}) treated plants of sensitive (GS) and resistant (GR) populations after five days of treatment. Pictures show that in GS population glyhposate dose affects more aggressively plants than in GR population. 0.05G (0.042 kg ha^{-1}) dose was tested only in GS population so it is not include in this comparative picture between both populations (GR and GS). 2G (1.68 kg ha^{-1}) and 3G (2.52 kg ha^{-1}) treatment kill all sensitive plants of sensitive after five days so these treaments were removed from the picture.

The dose-response relationship was established as in previous studies (Seefeldt et al. 1995). For each population, plants of uniform size and appearance were selected, and three tanks with four to five plants each were treated with each glyphosate rate.

Both populations were treated when plants were approximately three weeks old. Glyphosate was sprayed at these rates: 0.0, 0.042 (only for GS), 0.084, 0.21, 0.42, 0.84, 1.68 and 2.52 kg ha^{-1}, and were represented (Fig. MM.2) as a percentage of the recommended field dose (0.84 kg ha^{-1}) : control, 0.1G, 0.25G, 0.5G, 1G, 2G and 3G.

Their effects were evaluated in terms of biomass weight, and completed with shikimate content data. For biomass evaluation, the shoot and root fresh weights of each plant were determined five days after treatment. The material was dried for 48 h at 75−80 °C to obtain the dry weight. Data were analyzed with a log-logistic adjustment that is a good model to evaluate herbicide effects (Seefeldt et al. 1995; Ritz et al. 2015).

MM.1.3 Physiological studies

With the information obtained in the dose response study, different doses of glyphosate were used according to the objectives of each chapter. In the one hand, in chapter one and two, recommended field rate (1G = 0.84 kg ha^{-1}) (Culpepper et al. 2008) and three times that rate (3G = 2.52 kg ha^{-1}) were used (Fig. MM.3), to better observe the variations in AAA pathway and physiological effects produced by increasing the dose. In the other hand, in chapter three, as the main objective was determine the effect of mixtures, lower doses of glyphosate were used. Too High doses of glyphosate would have covered up the effect of imazamox in mixtures, and the results probably had been similar to those with glyphosate applied individually. For this, in chapter three 0.25 times recommended field rate (0.25G=0.21 kg ha^{-1}) and recommended field rate (1G=0.84 kg ha^{-1}) were used.

Imazamox was only used in chapter three, applied directly in the nutrient solution at 1.5 mg L^{-1} rate, at the same time of aerial glyphosate treatment in mixtures. Preliminary studies were performed to determine a dose of imazamox that allowed to display the response to the herbicide without kill the plants too fast.

Three days after treatment, leaves and roots were collected, frozen with liquid N_2 and stored at -80 ºC. Three days of treatment were selected because of this period of time allowed plants to show the effects of relative high doses

of herbicides in a short term preventing from a massive death of plants (over all in GS population). After that, the samples were ground to a fine powder under liquid N_2 using a Retsch mixer mill (MM200, Rescht, Haan, Germany) maintaining separately individual plants as biological replicates. Additionally, leaf disks were excised at the same time of leaf collection to determine shikimate content three days after treatment. Three leaf disks were excised from each plant using one Harris Uni-Core puncher (4 mm-diameter) (Healthcore, Bucks, UK), trying to avoid the nerves of the leaves, and then were frozen with liquid N_2 and stored at -80 ºC.

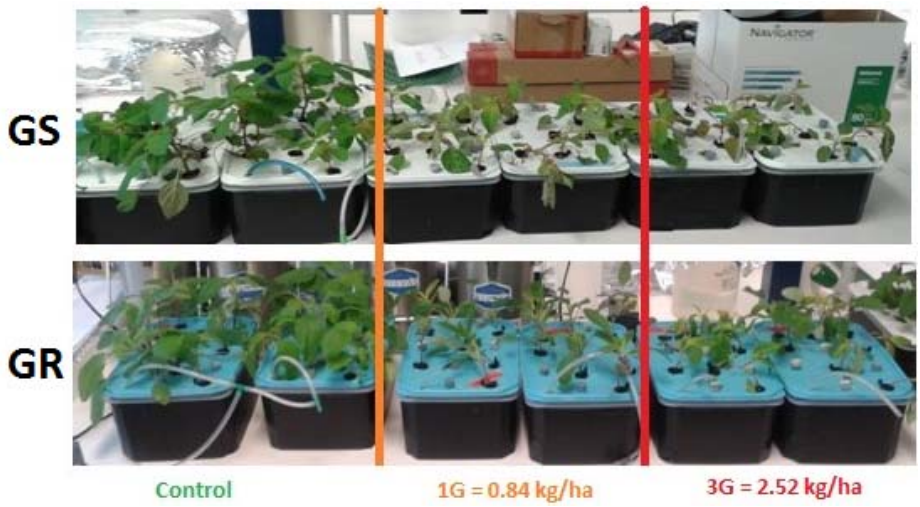

Figure MM.3 Control, 1G (glyphosate recommended field dose) and 3G (three times glyphosate recommended field dose) treated plants of sensitive (GS) and resistant (GR) populations after three days of treatment. It can be seen that in GS population glyhposate dose affects more agresively plants than in GR population.

MM.2 ANALYTICAL DETERMINATIONS

Physiological responses to the treatment (amino acid content variation, carbohydrate content variation and fermentative enzymatic activities variation), were studied in roots and leaves in chapter one, while in chapter two and three, it was preferred to develop the experiments only in leaves (amino acid content and carbohydrate content).

MM.2.1 Shikimate assays

In this study, shikimate levels in plant were quantified with *in vivo* shikimate assays (chapter one) and shikimate determinations in plants tissues after glyphosate treatment (chapter one, two and three).

MM.2.1.1 *In vivo* shikimate assay

This experiment was developed in chapter one to verify plant sensitivity to glyphosate by incubating excised leaf disk tissue in a 96-well microtiter. Shikimate accumulation following glyphosate treatment was compared between GR and GS as described previously (Shaner et al. 2005). Leaf disks with a 4 mm-diameter (6−10 disks) were excised from the youngest leaf of three to six plants of each biotype using a Harris Uni-Core puncher (Healthcore, Bucks, UK). One disk was placed in a well of a 96-well microtiter plate. Each well contained 100 µL of a solution containing 169, 84.5, 42.3, 21.1, 10.6, 5.3, 2.6, 1.3, or 0 mg glyphosate L^{-1}. Glyphosate was diluted from commercial formula (Glyfos, BayerGarden, Valencia, Spain). Each row of the microtiter plate contained a different glyphosate concentration. The plates were incubated at 22 °C under continuous light for 16 h. After incubation, the plates were placed in a freezer (−20 °C). Microplates were thrown at room temperature and the concentration of shikimate in each well was measured according to the procedure of Cromartie and Polge (Cromartie and Polge 2000).

Shikimate was extracted from the frozen-thawed leaf disks by adding 25 µL of 1.25 N HCl and incubating the plates at 60 ºC for 15 min. Aliquots of 25 µL of each well were transferred to a new plate, and 100 µL of 0.25% periodic acid/0.25% metaperiodate was added to each well. After the periodic acid-metaperiodate incubation (60 min in the dark), 100 µL of 0.06 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 Sinergy™ HT Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA) was used. A shikimate standard curve was developed by adding known amounts of shikimate to wells containing leaf disks not exposed to glyphosate (3, 6, 12, 25, 50, and 100 µg mL^{-1}).

MM.2.1.2 Shikimate determination after treatment

Shikimate accumulation is a well-known marker of glyphosate damage, so it was determined in the three chapters. In chapter one, shikimate content was determined at 5 days after glyphosate treatment, because it was needed to know how damaged were the plants at that time of treatment to support the dose-response study. In chapter two and three, all experiments were developed with tissues collected three days after herbicide treatment, so it was needed to determine shikimate content after 3 days of treatment. Three leaf disks (4 mm diameter) were excised from the youngest leaf of each plant three days after treatment. Leaf disks were placed in a screw-top 2 mL epitube, frozen, and stored at -80ºC until analysis. Shikimate was extracted as it was described (Koger et al. 2005a). After addition of 100 μL of 0.25 N HCl per disk to each vial, samples were incubated at 22ºC for 1.5 h and mixed by vortexing. Shikimate content was quantified spectrophotometrically as determined in MM.2.1.1 section.

MM.2.2 Nucleic acid determinations

In this study, genomic DNA for *EPSPS* enzyme (in chapter one) and *m*RNA levels for all main enzymes in AAA and BCAA pathway (in chapters two and three) were determined.

MM. 2.2.1 *EPSPS* relative genomic copy number

This data, determined in chapter one, was used in the three chapters, as a reference mean value of EPSPS relative genomic copy number of GR population. Quantitative realtime PCR was performed to determine the *EPSPS* genomic copy number relative to *AHAS* in untreated GS and 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 20,000 *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 20,000 *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. The DNA quality was also checked using a 1% agarose gel. Ten-fold diluted DNA samples were loaded onto a 1% agarose gel and run at 75 mA for 35 min. The gels were visualized using a Gel Doc 2000 system (Bio-Rad Laboratories Inc., Hercules, CA, USA). 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′); AHAS forward (5′ -gctgctgaaggctacgct-3′) and AHAS reverse (5′ -gcgggactgagtcaagaagtg-3) specified in Gaines et al. (2010). To determine the efficiency of the primers, a standard curve using a 1 X, 1/5 X, 1/25 X, 1/125 X, and 1/500 X dilution series of genomic DNA from GR was conducted.

Real-time PCR amplifications 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, 10 biological replicates were performed, and each individual sample was run in triplicate. The average increase in EPSPS copy number relative to AHAS and the standard deviation were calculated for each sample. The increase in EPSPS copy number was expressed as $2\Delta Ct$, where ΔCt = (Ct, AHAS −Ct, EPSPS) (Gaines et al. 2010).

MM.2.2.2 Transcript levels

This parameter was determined in chapter two and three. A stay in Colorado State University (CSU) was carried out to obtain *m*RNA data for chapter two, and, with the procedure learned there and the primers designed, the determination was repeated for data of chapter three, to check the response of those enzymes to herbicide mixtures. This was the first time where *m*RNA levels of all the main enzymes of AAA and BCAA pathway were determined, in *A. palmeri*, so all primers except EPSPS primer, were designed for the experiment.

As *A. palmeri* was not sequenced, primers were designed using a near sequenced species of Amaranthaceous family, *Amaranthus hypochondriacus*. The data base of the genome of *A. hypochondriacus* was crossed with the one of *Arabidopsis thaliana*, which is sequenced and their genes were related with their function in *The Arabidopsis Information Resource* (TAIR) website. To cross genes of both species a variety of informatics tools were used. Notepad++® was used to manage the *A. hypochondriacus* genome, EMBOSS needle website to compare sequences, NCBI page to blast the nucleotides to proteins and to obtain the open reading frames (ORFs) *A plasmid editor* (APE®) software.

EPSPS primer was modified from Gaines et al. (2010). The protocol followed was the same for data in chapter two and three, although experiment in chapter two was developed in CSU and in chapter three in UPNA. RNA was extracted from leaf tissues using the Macherey-Nagel NucleoSpin® RNA Plant kit following manufacturer's instructions. Total RNA concentration was measured with Gen 5.1.11 (Biotek Instruments, Inc., Winooski, VT, United States) and RNA quality was assessed using RNA gel electrophoresis. The gels were visualized using a Gel Doc 2000 system (BIORAD Laboratories Inc., Hercules, CA, USA).

cDNA (complementary deoxyribonucleic acid) extraction was performed using Biorad iScript™ 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 ºC, 30 min at 42 ºC, 5 min at 85 ºC and hold at 4 ºC until their storage at -20 ºC.

GENE	FORWARD	REVERSE	Annealing temperature	Efficiency
Aromatic amino acid biosynthetic pathway				
DAHPS	cctcataggatgataagggc	ctttgcatggcagcataacc	55 ºC	96 %
DHQS	gcattgttggctagggatcc	aacctcggccttgttttcac	61 ºC	91 %
DQSD	ggtgtactcaagcaaggagc	tgtggactcttactatggcc	57 ºC	84 %
SK	gattctgaagcacaaagcagc	cagttgttttcccagagccc	55 ºC	91 %
EPSPS	aatgctaaaggaggccttcc	tcaatctccacgtctccaag	61 ºC	93 %
CS	cttgatagaaggaggcctgg	gtttctttcctaggagtagtg	57 ºC	90 %
CM	gaatacattatggcaagtatgt	gtcataagtcgctccttgtc	52ºC	97 %
AS	tttggagggaaggttgtgcg	ctggtgagctttttccatgc	57 ºC	88 %
Branched chain amino acid biosynthetic pathway				
AHAS	cttcctcgacatgaacaagg	attagtagcacctggacccg	57 ºC	84 %
AHAIR	atggctcagattgagatcttg	ccacggcttcaatcacactc	52 ºC	90 %
DHAD	taccatggcatcagctatcg	ggtgttgacgagctgtaagg	55 ºC	96 %
TA	gtgaagatgatcttcgtcggc	tcacaatcagacttgaaagatg	52 ºC	99 %
Normalization gene				
Beta tubulin	gatgccaagaacatgatgtg	tccacaaagtaggaagagttc	55 ºC	90 %

Table MM 1 Primers of genes from aromatic amino acid (AAA) pathway enzyems: D-arabino-heptulosonate 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 (BCAA) pathway: Acetohydroxy acid synthase (AHAS), acetohydroxyacid isomerreductase (AHAIR), dihydroxyacid dehydratase (DHAD) and BCAA transaminase (TA) and normalization gene selected for this study. For each primer is shown the anealing temperature and the efficiency.

Quantitative RT-PCR (qRT-PCR) was performed using a Thermocycler Biorad CFX Connect TM Real-Time System (Biorad Laboratories Inc., Hercules, CA, USA). The reaction kit used for qPCR was PerfeCTa SYBR® Green SuperMix (Quantabio, Beverly, MA, United States). Each reaction was performed using 1 μL of cDNA template, 10 μL of Perfecta SYBR green supermix (2x), 1 μL of a solution with forward and reverse primers (10 μMol for

primer forward and another 10 μMol for primer reverse) in nuclease-free water and 8 μL of nuclease-free water. The following thermal profile was used for all PCRs: denaturation at 95ºC for 2 min, 40 cycles of 95 ºC for 15 s and 52–61ºC 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 table MM.1. 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 five biological replicates. Relative transcript level was calculated as in previous protocols: $E_{GOI}^{CP_{GOI}\ control-CP_{GOI}treated}/E_{REF}^{CPREF\ control-CPREF\ treated}$ (Pfaffl 2001), where GOI = gene of interest, REF = reference gene (beta tubulin was used as normalization gene), and CP = crossing point, the cycle at which fluorescence from amplification exceeded the background fluorescence. In chapter two, values of all treatments in both populations were calculated respect to GS mean control, but in chapter three it was decided to calculate the GR values respect to their own control.

MM.2.3 Enzymes (EPSPS, DAHPS and PAL) content measurements

In this study, protein contents of the enzymes EPSPS (chapters one, two and three), DAHPS (chapters two and three) and PAL (chapter two) were determined. All protein determinations were done in leaf tissues of sensitive and resistant populations.

MM.2.3.1 Protein extraction

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)) for each one of the three enzymatic contents analyzed. Then it was centrifuged at 4ºC 18,000 *g* for 30 min, and the supernatant was collected for protein content determination.

MM.2.3.2 Soluble protein content

Protein levels of the samples were measured following Bradford protocol (Bradford 1976). Protein aliquots were diluted with deionized water to 1:60, and 60 μL of the dilutions were mixed with 200 μL Biorad Protein Assay Dye Reagent (Biorad Laboratories Inc., Hercules, CA, USA). The mixtures 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 a given concentration per well of bovine serum albumin (BSA) was performed to calibrate the protein determination of each sample. For absorbance measurements was used a Sinergy™ HT Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA).

MM.2.3.3. SDS-PAGE transference and immunoblotting

Proteins were separated by 4.6% stacking gel and 12.5% resolving gel SDS-PAGE. The gel was run in a vertical electrophoresis cell (Mini protean III; (Biorad 170, Biorad 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 75 min at 100 V in a Mini-trans blot electrophoretic transfer cell (Biorad 170, Biorad 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) for 1 h. 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. Except for EPSPS protein content in chapter 1 (in which different method of band identification was used), An anti-rabbit AP conjugated antibody (Sigma

Chemical, Co., St. Louis, MO, United States) was used as a secondary antibody at a dilution of 1:20,000. 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) (Biorad 170-6412, Biorad Laboratories, Inc., Hercules, CA, United States). Immunoblots were scanned using a GS-800 densitometer, and protein bands were quantified using Quantity One software (Biorad Laboratories Inc., Hercules, CA, United States).

MM.2.3.4 EPSPS specific parameters

Protein loaded for EPSPS enzyme determinations were 60 μg per well of protein in GS samples and 15 and 5 μg per well of protein in GR samples were loaded in chapter 1 and 2, and 80 μg per well in GS and 15 μg per well in GR were loaded in chapter 3. 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).The primary EPSPS antibody dilution was 1:2000. The secondary antibody used is specified in MM.2.3.3 section, except for the secondary antibody used for EPSPS enzyme content in chapter one. For EPSPS enzyme determinations in this chapter, an anti-rabbit immunoglobuline G horseradish peroxidase (IgG HRP) conjugated antibody (Agrisera AB, Vännäs, Sweden) was used as a secondary antibody at a dilution of 1:75000. After 1 hour of incubation, the membrane was washed three times during 5 minutes with 10 % TTBS and after that. Bands were identified using an advanced ECL chemiluminescence detection kit.

MM.2.3.5 DAHPS specific parameters

Protein loaded in each well were 40 μg of protein in GS and GR samples for DAHPS enzyme determinations in chapters 2 and 3. 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).

MM.2.3.6 PAL specific parameters

For PAL enzyme content determinations, 90 μg per well of protein in GS and GR samples were loaded in chapter 1. PAL primary antibody was produced by a custom peptide facility (Biogenes, Berlin, Germany) using a short, conjugated peptide as an antigen(C-GATSHRRTKQGGA). The primary antibody dilution was 1:500.

MM.2.4 Enzymatic activities

In this study, enzymatic activities of the enzymes EPSPS (chapters one and two), CM (chapter two), AS (chapter two), PAL (chapter two), PDC and ADH (chapter 1) were determined. All determinations of enzymatic activities were done in sensitive and resistant populations in leaf tissues, except pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in which determinations only were done in roots.

MM.2.4.1 EPSPS activity

The EPSPS extraction and assay were conducted following the procedures of Gaines et al. (Gaines et al. 2010). One g of leaf tissue was ground to a fine powder in a chilled mortar and mixed with 10 mL of cold extraction buffer (100 mM MOPS, 5 mM EDTA, 5mM KCl, 10% (v/v) glycerol, 0.5 mM benzamidine, 7.3 μM pepstine, 2.5 mg tripsine inhibitor and 4.2 μM leupeptine and pH 7). 1% (p/v) polyvinylpolypyrrolidone (PVPP) and 0.07 % (v/v) mercaptoethanol were added in fresh. Samples were centrifuged 40 min at 7500 *g* and 4 ºC. After centrifugation, the supernatant was precipitated with ammonium sulfate (45−70%). Then samples were centrifuged 30 min at 10,000 *g* and 4ºC. Pellets formed were resuspended with resuspension buffer (10mM MOPS, 0.5mM EDTA, 5% (v/v) glycerol, 0.07% (v/v) mercaptoethanol and pH 7) and desalted in Zeba desalt columns (Zeba, 7K MWCO, Thermo Scientific; Pierce Biotechnology, Rockford, IL, USA) with centrifugation at 1000 *g*.

A phosphate detection kit (Molecular Probes, Eugene, OR, USA) was used for the continuous measurement of inorganic phosphate release for EPSPS activity. Protein levels of the desalted samples were measured following Bradford protocol (Bradford 1976) as it was indicated in MM.3.2 section. The total soluble protein in the reaction mixtures were 12.5 μg per well

for the GS population and 1.25 µg per well for the GR population. The volume of desalting samples needed to obtain the amount of desired protein were added: 125 µL 2x reaction buffer (100 mM MOPS, 10 mM Mg_2Cl, 10 % (v/v) glycerol, 2 mM Na_2MoO_4, 200 µL NaF and pH 7), 50 µL of 1mM 2-amino, 6-meracptho, 7-methyl-purine riboside (MESG), 2.5 µL of 100 units/mL purine-nucleoside phosphorylase (PNP), 6.25 µL of PEP, and mili-Q water until a total sample volume of 250 µL.

After obtaining a background phosphate release level (10 min) at 360 nm, the final step was the addition of 12.5 µL of 10 mM shikimate-3-phosphate (S3P). Phosphate release was measured spectrophotometrically for an additional 10 min at 360 nm.

MM.2.4.2 CM and AS activities

Enzyme extraction for CM and AS activity assays was developed as described in Singh and 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. After vortexing and 10 min in ice, samples were centrifuged 20 min at 15,000 *g* and 4 ºC. The supernatants of each sample were desalted with desalting columns PD MiniTrap™ G-25 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein levels of the samples were measured following Bradford protocol (Bradford 1976) as it was indicated in MM.3.2 section.

CM enzymatic activity was measured as described in Goers and Jensen (1984). Eighty µL of reaction buffer and 100 µL of 2.7 mM chorismate were added to 20 µL of desalted extracts and incubated for 30 min. Chorismate was added in the form of chorismic acid barium salt obtained from *Enterobacter aerogenes* with between 55-80% of richness purchased to Sigma-Aldrich® (Sigma-Aldrich Co., St. Louis, MO, USA). 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 ºC. 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, heigth 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 ºC. 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 18,000 *g*. In a fluorescence Black cliniplate (Thermo Scientific, Thermo-Fisher Scientific, Vantaa, Finland) 150 μL of each supernatant were loaded. AS activity 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.

MM.2.4.3 PAL activity

PAL activity was carried out mainly according to Politycka and Mielcarz (2007). Protein extraction was performed using 0.1 g of ground leaf tissue in 650 μL of extraction buffer (100 mM Tris-HCl pH 8.9, 10 mM mercaptoethanol and 2% (p/v) PVPP) in each sample. Samples were sonicated and then centrifuged at 12,000 *g*, 20 min, 4ºC (Maroli et al. 2015). The supernatant was collected and the protein content was determined in them following Bradford protocol (Bradford 1976) as it was indicated in MM.3.2 section. For first incubation 500 μL of 50 mM Na-borate (pH 8.8) were added to 100 μL of extracted samples, for 5 min at 37 ºC. Reaction was started by the addition of 25 μL of 50 mM l-phenylalanine (Maroli et al. 2015). Controls (without l-phenylalanine) were prepared to determine endogenous levels of transcinnamic acid (t-CA). Incubation was performed for 1 h at 37ºC (Sarma et al. 1998; Wang et al. 2007). The reaction was stopped with the addition of 600 μL of 5N HCl. After reaction was stopped, samples were centrifuged for 5 min at 5,000 *g* and 4ºC (Rivero et al. 2001). In an ultraviolet (UV) plate Costar® (Corning Inc., Corning, NY, USA) 200 μL of supernatants were charged and absorbance was measured

spectrophotometrically at 290 nm. Cinnamic acid at 10-100 µM was used as standard.

MM.2.4.4 Fermentative enzymatic activities (PDC and ADH)

PDC and ADH activities were assayed in desalted extracts as described previously (Gaston et al. 2002).

About 0.1 g of root tissue was ground and homogenized with 650 µL of the following extraction buffer (50 mM MOPS, 5 mM $MgCl_2$, 20 mM KCl, 1mM EDTA Na_2 and pH 7) and 1 mM DTT added in fresh. Homogenates were centrifuged for 30 min at 18,000 *g* and 4ºC. After centrifugation, supernatants were collected and soluble protein content was determinate following Bradford protocol (Bradford 1976) as it was indicated in MM.3.2 section.

They were desalted 200 µL of the supernatants in a Whatman UNIFILTER^{TM} filtration microplate (Whatman Inc., Clifton, NJ, USA) containing 600 µL of Sephadex® G-50. The columns were equilibrated with a desalting buffer (50 mM MOPS (pH 7.0), 5 mM $MgCl_2$ and 20 mM KCl). To measure each one of PDC and ADH enzymatic activities 20 µL of desalted samples were collected. After that, soluble protein content was determinated again in the desalted extract following Bradford protocol (Bradford 1976).

In the case of PDC activity, 150 µL of reaction buffer (100 mM Tricine (pH 6.5), 2 mM $MgCl_2$, 1 mM DTT, 0.2 mM nicotinamide adenine dinucleotide (NADH), 3 Uml^{-1} *Saccaromyces cerevisae's* ADH, 25 mM oxamate, 1 mM TPP and 10 mM pyruvate) were added to 20 µL of the sample. PDC activity was measured in the pyruvate-to-acetaldehyde direction in the presence of 25 mM oxamate to inhibit LDH (Bouny and Saglio 1996). Consumption of NADH was determinated at 340 nm. Blanks were done not adding the substrate of the reaction (pyruvate).

In the case of ADH activity, 150 µL of ADH reaction buffer (50 mM Bicine (pH 8.8), 5 mM $MgCl_2$, 49 µM absolute ethanol and 1 mM NAD^+) were added to the 20 µL of the sample. ADH activity was measured in the ethanol-to-acetaldehyde direction (John and Greenway 1976). The production of NADH is determined at 340 nm. Blanks were done by not adding the substrate of the reaction (ethanol).

MM.2.5 Amino acid content determination

Ground leaf and root samples (0.1 g) were homogenized in 1.5 mL of 1 M HCl for amino acid extraction. Protein precipitation was performed after incubation on ice for 10 min and centrifugation at 18,000 *g* for 15 min at 4 ºC. The supernatant was collected and then, extracts were neutralized to a pH between 7 and 8 by adding NaOH 1 M and 4 M and stored at -20 ºC. 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. 2010).

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 a-cyclodextrin in 80 mM borax buffer (pH 9.2) except for Cys (its content was only determined in chapter 1).

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 N-methyl *N*-glucamine (pH 11.2). Analyses were performed at 20 ºC 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. In chapter three it was no needed this second measurement to obtain these two values.

MM.2.6 Carbohydrate content determination

Content in soluble sugars (glucose, fructose and sucrose) and starch were determined in chapters one in roots and leaves and, in chapter three, only in leaves.

Two different methods were used to determine carbohydrate content. In chapter one, starch and soluble sugar concentrations were determined by

capillary electrophoresis (Zabalza et al. 2004) while in chapter three, ion chromatography (930 Compact IC Flex) following manufacturer´s instructions (Gomensoro scientific instrumentation, Madrid, Spain) was used to improve the accuracy of the measurement method. For both methods, samples were prepared in the same way.

The soluble carbohydrate (glucose, fructose, and sucrose) content was determined in ethanol-soluble extracts, and the ethanol-insoluble residue was extracted for starch analysis. In an epitube, 1.5 mL of ethanol 80% were added to 0.1 g of ground leaf tissue. Samples were boiled for 30 s and then centrifuged at 7,500 *g* and 4ºC for 5 min. This step was repeated three times and then samples were washed one more time with ethanol but at room temperature. The supernatant was collected for soluble sugar determinations and then samples were evaporated in a Turbovap® LV evaporator (Zymark Hopkinton, MA, USA) at 40ºC and 1-1.2 bar. When all the ethanol was evaporated, the dried sample was suspended in 1 mL of deionized water, mixed and centrifuged at 6,000 *g* for 10 min at 4ºC and samples were stored in 2 mL epitubes at -20ºC for capillary electrophoresis or ion chromatography determinations.

The preparation of samples for the determination of starch was performed following the protocol of MacRae (1971). The pellet that remains in the epitube after the collection of supernatant was dried in an oven at 70 ºC for 24 h for starch determinations. Dried pellets were resuspended with 1 mL of deionized water. Then the samples were boiled at 100 ºC for 1 h. After that, samples were cooled on ice. To obtain the monomers of glucose whose content was going to be measured, 250 μL of 0.082 % (w/v) amyloglucosidase dissolved in 8.55 mM acetate (pH 4.5) were added to each sample to catalyze the hydrolysis needed. The reaction was incubated at 50 ºC overnight in the darkness with a continuous shaking. The resultant mixture was centrifuged at 7,500 *g* for 15 min at 4 ºC and supernatant was collected and stored in 2 mL epitubes at -20 ºC or capillary electrophoresis or ion chromatography determinations.

To obtain the measurements of soluble sugars and starch by capillary electrophoresis a Beckman Coulter P/ACE™ MDQ (Beckman Coulter Inc., Brea, CA, USA) was used, following Warren and Adams (2000) protocol. The

background buffer consist of a 10 mM benzoate (pH 12.0) 0.5 mM myristril trimethyl ammonium bromide (MTAB). The sample dilution used for soluble carbohydrates and starch was 1:2. The applied potential was -15 kV and the indirect UV detection wavelength was set at 225 nm.

The ion cromatograph 930 Compact IC Flex (930 Compact IC Flex, Metrohm AG, Herisau, Swizerland), was used to obtain the measurements of carbohydrate content, following manufacturer's instructions (Gomensoro scientific instrumentation, Madrid, Spain). To prepare the samples, the eluent used was 300 mM NaOH/1 mM sodic acetate in mili q water solution. The sample dilution used for soluble carbohydrates was 1:10 and for starch 1:50. The applied current for the amperometric detection was 200-500 mA, with pressure of 1000-1200 psi and temperature between 30 and 35 ºC.

MM.2.7 Statistical analysis

The dose-response study was constructed using the program Sigma Plot 12.0 to calculate the four-parameter sigmoid log-logistic dose-response model based on fresh/dry weight.

In all chapters statistical analyses were performed using IBM SPSS statistics (from 18.0 to 24.0 version) (IBM, Corp., Armonk, NY, United States). One-way ANOVA (analysis of variance) with a multiple-comparison adjustment for least significant difference (LSD) at $p<0.05$ was used to determine significant difference. Analyses were performed using at least 4 biological replicates using samples from different individual plants.

CHAPTER 1: Characterization of the *Amaranthus palmeri* physiological response to glyphosate in susceptible and resistant populations

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Characterization of the *Amaranthus palmeri* Physiological Response to Glyphosate in Susceptible and Resistant Populations

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ABSTRACT

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

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

1.1 INTRODUCTION

Over the past three decades, the herbicide glyphosate has revolutionized modern agriculture. Glyphosate is a once-in-acentury herbicide that stands alone in many categories (Duke and Powles 2008). This broad-spectrum, systemic herbicide is the most widely used herbicide in the world. Glyphosate inhibits the biosynthesis of the aromatic amino acids (AAAs) tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe). The primary site of action of glyphosate is the specific inhibition of a key step in the shikimate pathway catalyzed by the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19), which converts shikimate-3-phosphate and phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate in plastids (Steinrücken and Amrhein 1980).

Although chemical management has been very effective for weed control, it has also resulted in the evolution of resistant weeds. Initially, glyphosate controlled most weeds, but as early as 1997, glyphosate-resistant weeds were reported (Heap 2013). Glyphosate was widely used in nonagricultural areas, but the introduction of genetically modified crops such as corn, cotton, and soybean exacerbated the evolution of resistance to glyphosate (Nandula 2010). In this context, glyphosate is used as a stand-alone weed control method on several million hectares of crop land. The intensive use of glyphosate has resulted in the evolution of resistance to this herbicide in several problematic weeds to what had been a very effective herbicide. To date, resistance to glyphosate has been documented in 32 species (Heap 2013). Glyphosate-resistant weeds now pose a serious challenge to modern agricultural practices and are likely to increase the cost of production (Livingston et al. 2015).

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

Despite its widespread use in global crop production, the precise mechanisms by which glyphosate kills plants remain unclear, despite studies

using new molecular methods such as transcriptional comparison, (Yu et al. 2007; Zhu et al. 2008) proteomic approaches (Ahsan et al. 2008), and metabolomic profiling (Trenkamp et al. 2009; Maroli et al. 2015). In general, after the target of an inhibitor has been affected, death can occur due to different causes. First, plant death could result from an accumulation or increased availability of the substrates of the inhibited enzymatic pathway. Second, death could be associated with the lack of end products generated by the inhibited pathway (mainly AAAs). Third, lethality could be associated with several side reactions triggered after the inhibition of the target because the dysregulation caused by the inhibition of this pathway can lead to effects on different metabolic pathways.

The main metabolite that accumulates upstream of EPSPS is shikimate. Massive levels of shikimate have been detected in plant tissues (Lydon and Duke 1988; Becerril et al. 1989) because inhibition at the level of EPSPS causes feedback loops that drive an increased flow of carbon through the shikimate pathway, thereby exacerbating the accumulation of shikimate. Moreover, toxic effects of shikimate accumulation have been proposed (De Maria et al. 2006).

Some authors assume that AAA production at a level insufficient to maintain necessary protein synthesis is the main effect of glyphosate exposure, and this mechanism is consistent with the slow development of injury symptoms and the lack of essential plant compounds leading to plant death (Duke and Powles 2008). However, although a transient decrease has sometimes been reported (Orcaray et al. 2010), the AAA content does not decrease significantly (Orcaray et al. 2010) due to an increase in protein turnover and concomitant total free amino acid accumulation (Zulet et al. 2013a, 2015) and soluble protein decrease.

In relation to the side reactions, the blockage of the shikimate pathway by glyphosate has recently been suggested to reverberate across other biochemical pathways (Maroli et al. 2015). Deregulation at the level of PEP that is not consumed by the inhibited EPSPS in the shikimate pathway can directly affect carbon metabolism (Colombo et al. 1998; De Maria et al. 2006). In this sense, glyphosate can also impair carbon metabolism by interfering with sugar metabolism and translocation. Carbohydrate accumulation has been detected

in both the leaves and roots of treated pea plants, and ethanolic fermentative metabolism is enhanced by glyphosate (Orcaray et al. 2012).

Amaranthus palmeri is among the three most troublesome weeds in the main crops in Georgia (cotton, peanut, and soybean) and is among the top five most troublesome weeds in most other southeastern states. This annual weed is highly problematic due to its competitiveness, aggressive growth habit, and prolific seed production (Culpepper et al. 2006). *A. palmeri* was initially controlled by glyphosate in glyphosate-resistant crops but became a major glyphosate-resistant weed that occurs in several states. Glyphosate-resistant *A. palmeri* was first reported in 2006, (Culpepper et al. 2006) and the mechanism of resistance was gene amplification (Gaines et al. 2010). This was the first reported occurrence of gene amplification in a field-evolved resistance to any herbicide (Sammons and Gaines 2014). The EPSPS gene was amplified from 2- to >100- fold compared with sensitive populations. This mechanism of resistance to glyphosate has since been reported in a number of other species, including *Amaranthus tuberculatus* (Lorentz et al. 2014; Chatham et al. 2015), *Amaranthus spinosus* (Nandula et al. 2014), *Kochia escoparia* (Wiersma et al. 2015), *Lolium multiflorum* (Salas et al. 2012, 2015) and *Bromus diandrus* (Malone et al. 2015).

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

1.2 MATERIALS AND METHODS

1.2.1 Plant material and treatment application

The seeds of the *A. palmeri* biotypes (GR and GS) were kindly provided by Dr. Gaines (Colorado State University, Fort Collins, CO, USA) and were originally collected from North Carolina (USA) (Chandi et al. 2012). Seeds were surface sterilized prior to germination (Labhilili et al. 1995). For germination, seeds were incubated for 7 days at 4 °C in darkness and then maintained for 48 h in a light/darkness cycle of 16 h/8h at temperature of 30 °C in light and 8 h at 18 °C in darkness. The seeds were then transferred to aerated 2.7 L hydroponic tanks in a phytotron (day/night, 16 h/8 h; light intensity, 500 µmol s^{-1} m^{-2} PAR; temperature, 22/18 °C; relative humidity of the air, 60/70%). Throughout the course of the experiment, the plants remained in the vegetative phenological stage. The nutrient solution (Hoagland and Arnon 1950) was supplemented with 15 mM KNO_3.

1.2.2 *In vivo* shikimate assay

Shikimate accumulation following glyphosate treatment was compared between GR and GS as described previously (Koger et al. 2005b). Leaf disks 4 mm in diameter (6–10 disks) were excised from the youngest leaf of three to six plants of each biotype using a Harris Uni-Core (Healthcore, Bucks, UK).

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

The concentration of shikimate in each cell was measured according to the procedure of Cromartie and Polge (Cromartie and Polge 2000). 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. Two 25 µL aliquots from each well were transferred to a new plate, and 100 µL of 0.25%periodic acid/0.25%

metaperiodate was added to each well. After the periodic acid–metaperiodate incubation (60 min in the dark), 25 ml of 0.6 M sodium hydroxide with 0.22 M sodium sulfite solution was added. The optical density of the solution at 380 nm was determined spectrophotometrically. A shikimate standard curve was developed by adding known amounts of shikimate to wells containing leaf disks not exposed to glyphosate (3, 6, 12, 25, 50, and 100 µg mL^{-1}). One microtiter plate was used for each population. The study was repeated twice.

1.2.3 Dose–response studies

A dose–response study was performed to verify the previously reported resistance. The dose–response relationship was established according to the method of Seefeld et al (Seefeldt et al. 1995). For each population, plants of uniform size and appearance were selected, and three tanks with four to five plants each were treated with each glyphosate dose. Both populations were treated when plants were approximately 20 days old, after reaching the growth stage defined as BBCH 14.35. The experiment was repeated twice.

Glyphosate is recommended at 0.84 kg ha^{-1} for the control of *Amaranthus sp.* up to 46 cm in height (Culpepper et al. 2006). Glyphosate herbicide (commercial formula, Glyfos, BayerGarden, Valencia, Spain) was applied using an aerograph (model Definik; Sagola) connected to a compressor (model Werther one, Breverrato, 60 W; 10 L m^{-1}; 2.5bar). The herbicide was sprayed at a rate of 500 L ha^{-1}, and thus there commended field dose resulted in 1.68 g glyphosate L^{-1}. The effect of increasing doses of glyphosate up to 3 times the recommended dose (0.0, 0.042 (only for GS), 0.084, 0.21, 0.42, 0.84, 1.68 and 2.52 kg ha^{-1}) was evaluated in terms of biomass and shikimate content, an adequate indicator of glyphosate-mediated plant injury. The control plants were treated with water because the inert proprietary ingredients could not be obtained.

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

Shikimate content was evaluated at 5 days after treatment. Three to six leaf disks (4 mm diameter) were excised from the youngest leaf of each plant. Leaf disks were placed in a screw-top 2 mL epitube, frozen, and stored at -80 °C until analysis. For shikimate determination, the vials were removed from the freezer, and shikimate was extracted as described by Koger et al (Koger et al. 2005b). First, 100 µL of 0.25 N HCl per disk was added to each vial. The vials were incubated at 22 °C for 1.5 h and mixed by vortexing several times. The shikimate content was analyzed spectrophotometrically (Cromartie and Polge 2000).

1.2.4 Analytical determinations

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

- ✓ **Relative Genomic EPSPS Gene Copy Number**

Quantitative realtime PCR was performed to determine the genomic copy number relative to acetolactate synthase (*ALS*) in untreated GS and 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 EDTA (pH 8.0), 4% sarcosyl, and 1% 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 20,000 *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 20,000*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 mMTris-HCl (pH 8.0), containing 30 µgmL^{-1} RNase A). Samples were briefly incubated at 37 °C for 5 min to degrade contaminating RNAs.

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

Quantitative real-time PCR was used to measure *EPSPS* genomic copy number relative to ALS as described by Gaines et al. (Gaines et al. 2010) with some modifications. The following primer sets were used: *EPSPS* forward (5′ -atgttggacgctctcagaactcttggt-3′) and *EPSPS* reverse (5′ -tgaatttcctccagcaacggcaa-3′); ALS forward (5′ -gctgctgaaggctacgct-3′) and ALS reverse (5′ -gcgggactgagtcaagaagtg-3′) (Gaines et al. 2010). To determine the efficiency of the primers, a standard curve using a 1×, 1/5×, 1/25×, 1/125×, and1/500× dilution series of genomic DNA from GR was conducted.

Real-time PCR amplifications were performed in an optical 96-wellplate 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). Melt-curve analysis was conducted with a final denaturation step of 95 °C for 30 s, 60 °C for 15 s, and 95 °C for 15 s.

To calculate the final Ct values, 10 biological replicates were performed, and each individual sample was run in triplicate. The average increase in *EPSPS* copy number relative to *ALS* and the standard deviation were

calculated for each sample. The increase in *EPSPS* copy number was expressed as $2^{\Delta Ct}$, where ΔCt = (Ct, ALS −Ct, EPSPS) (Gaines et al. 2010).

✓ **EPSPS Extraction and Activity Assay**

The EPSPS extraction and assay were conducted following the procedures of Gaines et al. (2010). Briefly, 1 g of leaf tissue was ground to a fine powder in a chilled mortar and mixed with 10 mL of cold extraction buffer. After centrifugation, the supernatant was precipitated with ammonium sulfate (45–70%) and desalted (Zeba desalt spin columns, Pierce Biotechnology, Rockford, IL, USA). A phosphate detection kit (Molecular Probes, Eugene, OR, USA) was used for the continuous measurement of inorganic phosphate release for EPSPS activity. The total soluble protein in the reaction mixture was 12.5 μg mL^{-1} for the GS population and 1.25 μg mL^{-1} for the GR population. After obtaining a background phosphate release level (10 min), the final step was the addition of shikimate-3-phosphate (up to 0.5 mM). Phosphate release was measured for an additional 10 min.

✓ **Immunoblotting of EPSPS**

Extraction and electrophoresis were performed as described previously (Hoagland et al. 2013). First, 0.05 g of leaf tissue was ground with 0.2 mL of extraction buffer. Proteins were separated by 12.5% SDS-PAGE. Western blots were produced according to standard techniques. The EPSPS antibody was produced by a custom peptide facility (Agrisera AB, Vännäs, Sweden) against a sequence of residues (numbers 193–206) of Palmer amaranth EPSPS (GenBankaccession no. FJ861242). The antibody was raised in rabbits using the manufacturer's standard protocols. The primary antibody dilution was 1:2000. Anti-rabbit IgG peroxidase (Agrisera AB, Vännäs, Sweden) was used as a secondary antibody at a dilution of 1:75000. Bands were identified using an Advanced ECL chemiluminescence detection kit (Amersham Life Science, Arlington Heights, IL, USA). Immunoblots were scanned using a GS-800 densitometer, and protein bands were quantified using Quantity One software (Bio-Rad Laboratories). Membrane signals were normalized according to total soluble protein loading quantity.

✓ **Metabolite Analysis and Fermentative Enzymes**

Ground leaf and root samples (0.1 g) were homogenized in 1 M HCl for amino acid extraction. After protein precipitation, the amino acid concentrations in the supernatant were analyzed. After derivatization with FITC, amino acid content was measured by capillary electrophoresis and a laser-induced fluorescence detector as described elsewhere (Zulet et al. 2013b). Cysteine content was determined from the same acid extracts derivatized with 5-iodoacetamide fluorescein and reduced with tributylphosphine, as described previously (Zinellu et al. 2005).

The soluble carbohydrate (glucose, fructose, and sucrose) content was determined in ethanol-soluble extracts, and the ethanol-insoluble residue was extracted for starch analysis (Zinellu et al. 2005). The starch and soluble sugar concentrations were determined by capillary electrophoresis as described by Zabalza et al. (Zabalza et al. 2004).

Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities were assayed in desalted extracts as described previously (Gaston et al. 2002).

1.2.5 Statistical analysis

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

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

1.3 RESULTS AND DISCUSSION

1.3.1 Population dose response to glyphosate

The dose–response experiments confirmed glyphosate resistance in the GR population. Figure 1.1A presents the effect of different doses of glyphosate on shoot dry weigh accumulation 5 days after glyphosate application, expressed as a percentage of untreated plants. Although the increase in shoot dry weight was highly variable within each dose, the effect on each population was described by a significant four-parameter log–logistic dose–response curve.

The root dry weight of GS plants did not exhibit a dose-dependent change over the range of concentrations of glyphosate used (data not shown), so it was not possible to use this biomass parameter in the resistance characterization.

No difference in shoot dry weight accumulation was observed between the populations in the absence of glyphosate (data not shown). The glyphosate concentration that reduced the shoot dry weight accumulation over 5 days by 50% (EC_{50}) was 0.0897 kg ha^{-1} for GS and 0.3310 kg ha^{-1} for GR, 3.7-fold greater. This difference in the EC_{50} is not really a resistance factor. When percent survival or percent reduction in fresh weight after 21 days was used, the EC_{50} estimates were 18–20-fold higher for the GR biotype compared with the GS biotype (Chandi et al. 2012; Whitaker et al. 2013). These differences in values reflect differences in methodology, in the parameter evaluated (i.e., percent survival versus inhibition of biomass growth), and in the time point of assessment (time since treatment). However, glyphosate resistant biotypes from North Carolina have been reported to exhibit levels of resistance ranging from 3- to 22-fold (Culpepper et al. 2008).

The accumulation of shikimate in plant tissue can be used to distinguish resistant and susceptible plants. Shikimate accumulation was observed in leaf disk tissue from GS plants after 5 days of treatment with glyphosate concentrations >0.21 kg ha^{-1} (Fig. 1.1B). The shikimate accumulation was maximized when the plants were sprayed with 0.84 kg ha^{-1} glyphosate, whereas accumulation was lower but still very noticeable at 1.68 and 2.52 kg ha^{-1}. No significant shikimate accumulation was observed in the glyphosate-

resistant biotype GR at any of the doses tested and, on an expanded scale, there was an effect, although much less than with the GS. Previous studies of the same populations have indicated that shikimate increases in both biotypes as the glyphosate concentration increases, with a greater increase in shikimate in the GS biotype (Whitaker et al. 2013). At glyphosate doses of ≥100 g ha^{-1}, the shikimate concentration is always greater in the susceptible biotype (Whitaker et al. 2013). Due to the increased *EPSPS* copy number, shikimate should not accumulate or may accumulate at lower levels compared to the susceptible biotype.

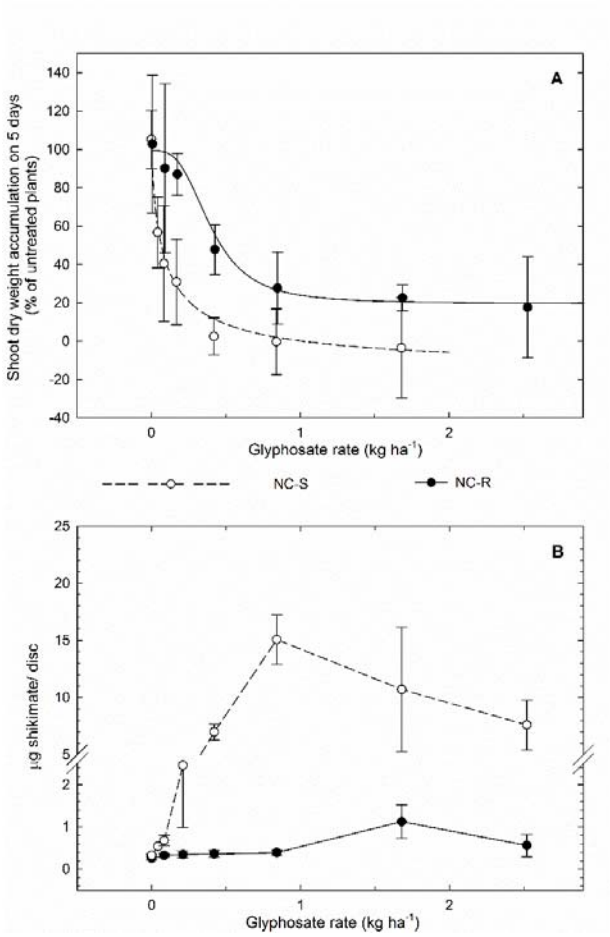


Figure 1.1 (A) Dose−response of the shoot dry weight accumulation in glyphosate-susceptible (GS) and -resistant (GR) *Amaranthus palmeri* plants over the 5 days following glyphosate application, as a percentage of the untreated plants (log−logistic dose−response curves). (B) Shikimate content in the leaves of glyphosate-susceptible(GS) and -resistant (GR) *A. palmeri* plants 5 days after spraying with glyphosate (mean ± SE; n = 4−7).

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

1.3.2 *In vivo* shikimate accumulation in leaf disks

In addition to shikimate quantification in the leaves of plants 5 days after spraying with different glyphosate concentrations (Fig. 1.1B), plant sensitivity to glyphosate was evaluated by incubating excised leaf disk tissue for 24 h with 0.1−170 mgL^{-1}, glyphosate; shikimate accumulation in the leaf disks submerged in different glyphosate concentrations was analyzed in both populations. This second assay has been reported to differentiate resistant from susceptible biotypes in different species (Koger et al. 2005a).

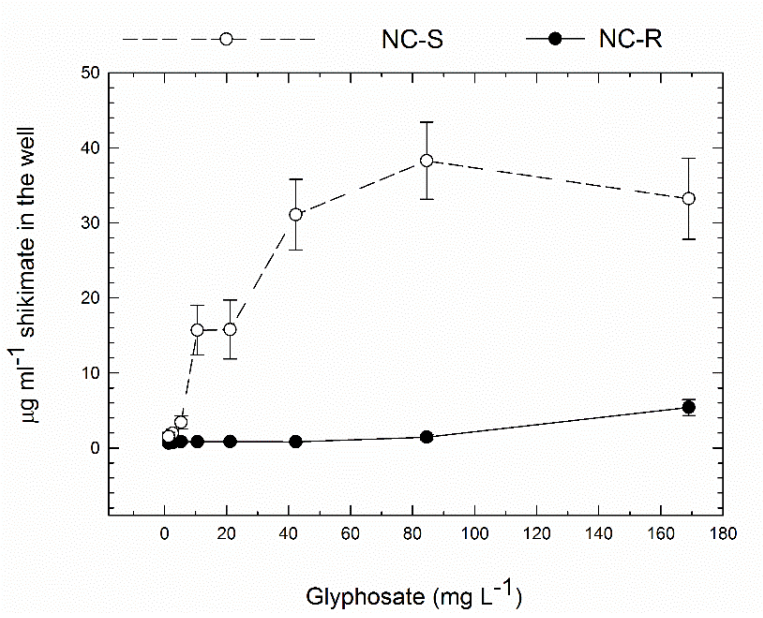


Figure 1.2 Effect of glyphosate concentration on shikimate levels in excised leaf disks from glyphosate-susceptible ((GS) and –resistant (GR) *Amaranthus palmeri* biotypes (mean ± SE; n = 16).

The leaf disks from the resistant biotype had less shikimate accumulation than the susceptible biotype (Fig. 1.2). Shikimate accumulation was similar in leaf disks of GS plants incubated with 5 mg L^{-1} glyphosate and GR plants incubated with 170 mg L^{-1} glyphosate, the highest dose of glyphosate. These results confirm the resistance of the GR population and are consistent with previous research and with figure 1.1.

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

Gene amplification was first reported as a glyphosate resistance mechanism in an *A. palmeri* population from Georgia (Gaines et al. 2010). A North Carolina glyphosate-resistant population of *A. palmeri* with gene amplification of 22–63-fold was subsequently reported. Additional populations have since been identified in New Mexico and Mississippi (Mohseni-Moghadam et al. 2013; Ribeiro et al. 2014). In this study, seeds from the North Carolina population were used. To confirm the resistance mechanism, the *EPSPS* relative genomic copy number was calculated by quantitative real-time PCR using ALS as an internal standard. Consistent with previous studies, the genomes of the GR plants exhibited a mean 47.5-fold increase in the number of copies of the *EPSPS* gene compared to the genomes of GS plants (Fig. 1.3A).

An increased EPSPS copy number results in elevated *EPSPS* expression at RNA transcript levels in *A. palmeri* (Gaines et al. 2010, 2011; Ribeiro et al. 2014), *A. tuberculatus* (Chatham et al. 2015), and *K. scoparia* (Wiersma et al. 2015) but not *B. diandrus* (Malone et al. 2015). In addition to *EPSPS* expression, resistant plants with a greater *EPSPS* copy number have been reported to have a higher quantity of the EPSPS protein (Gaines et al. 2010, 2011; Ribeiro et al. 2014; Wiersma et al. 2015) and higher EPSPS enzyme activity (Gaines et al. 2010, 2011; Salas et al. 2012; Ribeiro et al. 2014; Chatham et al. 2015). In this study, the EPSPS protein was quantified. Immunoblotting with an antibody for EPSPS resulted in a single reaction band at approximately 50 kDa (Fig. 1.3B, top). Greater EPSPS protein abundance was detected in GR plants; the normalized signal for EPSPS was 25-fold higher in untreated GR plants than in GS plants (Fig. 1.3B, bottom). As expected,

untreated GR plants exhibited higher specific EPSPS activity than GS plants (Fig. 1.3C) due to the increased levels of EPSPS protein in the total soluble protein. Interestingly, the activity in GR plants was increased 26.5-fold compared to GS plants, nearly identical to the 25-fold change in EPSPS protein levels.

As widely reported, the glyphosate resistance level appears to increase with higher *EPSPS* genomic copy number (Gaines et al. 2010; Ribeiro et al. 2014; Vila-Aiub et al. 2014; Salas et al. 2015), increased *EPSPS* expression (Gaines et al. 2010; Salas et al. 2012), protein content (Gaines et al. 2010), and specificactivity (Gaines et al. 2010). Two exceptions have been reported: cloned resistant plants of *A. palmeri* from Mississippi (Teaster and Hoagland 2014) that did not exhibit a correlation between resistance and copy number and a line of *Echinochloa colona* in which resistance could not be explained solely by higher EPSPS basal activity (Alarcón-Reverte et al. 2015).

In this study, the effect of glyphosate on the EPSPS protein content (Fig. 1.3B) of GS and GR plant was assessed. In both populations, plants treated with the higher dose exhibited an increase in EPSPS protein levels (Fig. 1.3B top, bottom).These results suggest transcriptional/translational regulation triggered only by a high dose of glyphosate. This study is the first to report an increase in EPSPS protein levels after glyphosate treatment. Increases in *EPSPS m*RNA levels after glyphosate have been reported previously, suggesting transcriptional regulation, in resistant and sensitive biotypes of *Eleusine indica* (Baerson et al. 2002b), *Lolium rigidum* (Baerson et al. 2002a), and tobacco (Garg et al. 2014). By contrast, in *A. tuberculatus* with multiple EPSPS copies, no increase in EPSPS expression was detected after glyphosate application (Chatham et al. 2015).

The effect of glyphosate on the specific activity of EPSPS differed between the two populations (Fig. 1.3C). GR plants exhibited a dose-dependent increase in enzymatic activity with glyphosate treatment, whereas GS exhibited a tendency toward decreased EPSPS activity as the applied dose of glyphosate increased. The increase in the amount of protein in the GR population at the highest dose of herbicide was sufficient for the concomitant increase in EPSPS activity to alleviate the toxicity of the herbicide. Indeed, concomitant increases in *EPSPS m*RNA and activity have been reported previously (Baerson et al.

2002a, b). EPSPS activity in GS plants decreased with glyphosate treatment (Fig. 1.3C). This response differed from that observed in GR plants and demonstrates that although the amount of EPSPS protein was higher in the susceptible population, the increase was not sufficient to counteract the amount of the enzyme inhibitor (herbicide) present.

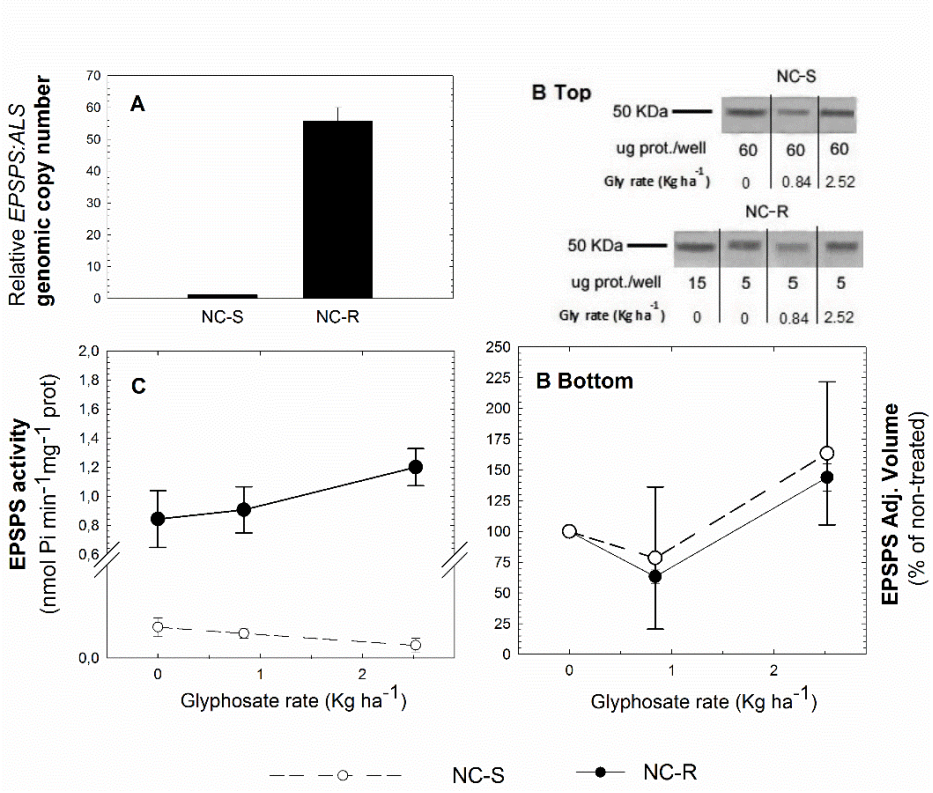


Figure 1.3 (A) *Amaranthus palmeri* genomic copy number of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) relative to acetolactate synthase (ALS) in glyphosate-susceptible (GS) and -resistant (GR) biotypes (mean ± SE; n = 9). (B) EPSPS protein levels in the leaves of glyphosate susceptible (GS) and -resistant (GR) *A. palmeri* populations untreated (0) or treated with 0.84 or 2.52 kg ha−1 glyphosate, 3 days after application (top, representative sample blot with µg protein loaded/well indicated; bottom, normalized EPSPS quantity (mean ± SE; n = 3)). (C) Effect of glyphosate on EPSPS activity in leaves of glyphosate-susceptible (GS) and -resistant (GR) *A. palmeri* populations (3 days after application) (mean ± SE; n = 4).

1.3.4 Free amino acid profile in untreated susceptible and resistant plants.

If EPSPS is a bottleneck in the carbon flow through the shikimate pathway, GR plants should possess higher AAAs biosynthetic capacity. To test this hypothesis, the free amino acid profiles of GR and GS plants were

compared before the herbicide was applied. The total free amino acid (Fig. 1.4A,C) and AAA contents (Fig. 1.5A,C) in leaves and roots were compared. Moreover, the content of each individual amino acid in the leaves (Supplemental Fig. 1.1) and roots (Supplemental Fig. 1.2) was determined, including other groups of amino acids previously reported to be affected by glyphosate (Orcaray et al. 2010): branched-chain (Val, Leu, Ile), acidic (Glu, Asp), and amide (Gln, Asn) amino acids.

The total free amino acid content was similar in the two populations (Fig. 1.4A,C). Interestingly, no differences were detected in the AAA contents between untreated GR plants and GS plants (nor in the leaves or in the roots) (Fig.1.5A,C; Supplemental Fig. 1.1 and 1.2), as reported recently for two other populations of the same species (Maroli et al. 2015). The contents of nearly all individual amino acids in the leaves and roots (shown in Supplemental Figs. 1.1 and 1.2) were similar for untreated plants of the two populations, except Val, Leu, Ala, and Glu in leaves and Tyr, Val, and Asp in roots. GR plants exhibited increases in EPSPS copy number, protein amount, and activity of 47.5-, 23.6-, and 26.5-fold, respectively, compared to GS plants. However, the AAA content was the same in untreated plants of the two populations, indicating that the amount of AAA is independent of the expression of *EPSPS* and suggesting regulation of EPSPS activity at protein level. Plants regulate carbon flux toward AAA biosynthesis at the transcriptional and post-transcriptional levels (Maeda and Dudareva 2012). Although all shikimate pathway genes have been characterized in model plants (Tohge et al. 2013b), information on the effect of AAA levels on the expression of these genes in plants remains limited (Maeda and Dudareva 2012). *In vitro* studies of allosteric regulation have implied that the shikimate pathway in plants is mostly regulated at the gene expression level rather than the post-translational level (Tzin and Galili 2010b).

Some evidence indicates that in plants, the expression of the shikimate pathway and the downstream pathway are coordinately regulated, often by the same transcription factor (Maeda and Dudareva 2012). The transcription factor EPF1 directly binds the EPSPS promoter and controls its spatial and developmental expression (Takatsuji et al. 1992).

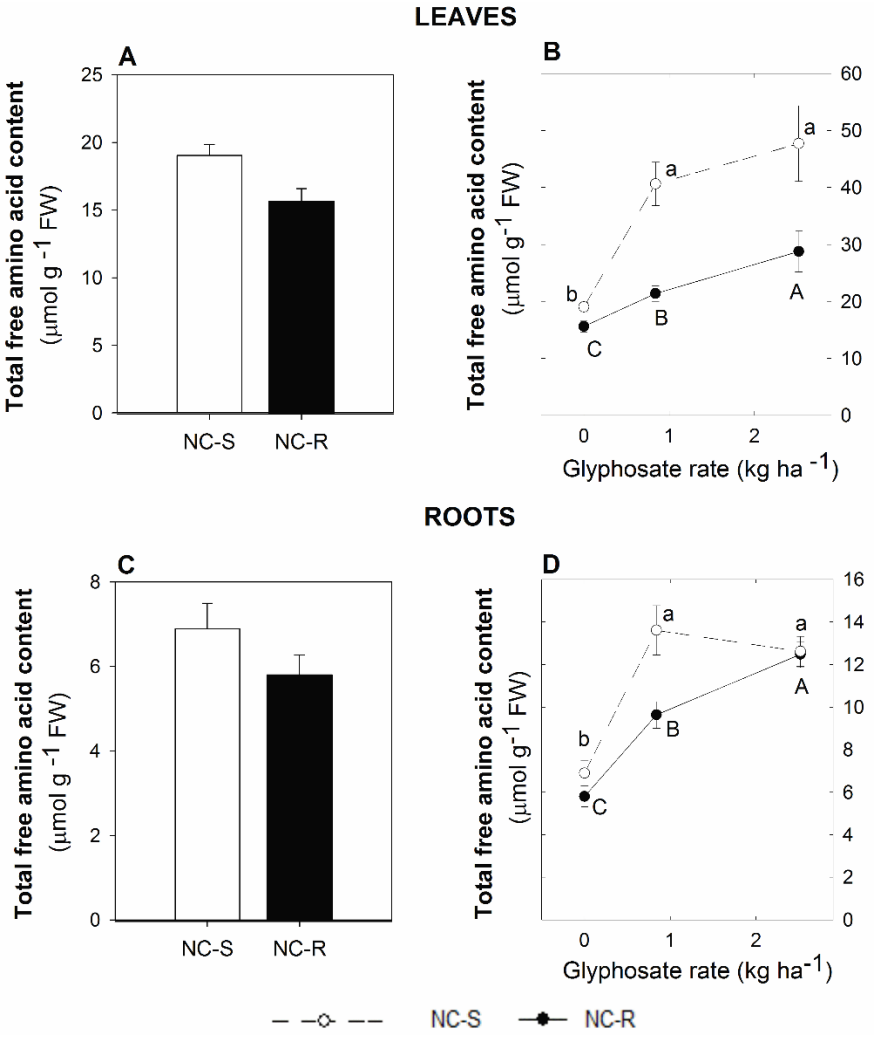


Figure 1.4 Total amino acid content in leaves (A) and roots (C) of untreated plants of glyphosate-susceptible (GS) and -resistant (GR) *Amaranthus palmeri* populations and effect of glyphosate on total amino acid content in leaves (B) and roots (D) 3 days after treatment (mean ± SE; n = 6–9). The asterisk (∗) indicates significant differences between control plants (without herbicide) of each population. Different capital letters in the GR population and different lower case letters in the GS population indicate significant differences between treatments (p value ≤ 0.05).

Although EPSPS has been extensively studied in plants due to its association with glyphosate, the significance of EPSPS activity in the synthesis of AAA has not been sufficiently addressed (Galili and Hofgen 2002). This study of GR plants provides insights into the tight control of the biosynthesis of AAAs by EPSPS activity *in vivo*, in which regulations at levels of gene expression and protein are coordinated.

1.3.5 Free amino acid profile after glyphosate treatment

Changes in the free amino acid profile after glyphosate treatment have been reported (Orcaray et al. 2010, 2012; Zulet et al. 2015), and thus this parameter can also be used as a physiological marker of herbicide activity. The total free amino acid content was higher in GS plants treated with glyphosate than in untreated plants (Fig.1.4B,D).

In both roots and leaves, the increases after treatment with 0.84 or 2.52 kg ha^{-1} glyphosate were similar. Total free amino acid accumulation after glyphosate treatment has been reported previously in several species, including pea (Orcaray et al. 2010; Zulet et al. 2013a), *Arabidopsis thaliana* (Zulet et al. 2015), maize (Liu et al. 2015), and soybean (Moldes et al. 2008; Vivancos et al. 2011). Glyphosate also increased the total free amino acid content in the leaves and roots of GR plants in a dose-dependent manner (Fig. 1.4B,D), although the detected increase in leaves was much lower than that in treated GS leaves. Previous studies have observed small or no effects on this parameter in resistant soybean treated with glyphosate (Moldes et al. 2008; Vivancos et al. 2011). 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). However, an evaluation of the effects of glyphosate on the main proteolytic systems in pea revealed that not all proteolytic systems increased (Zulet et al. 2013a).

The free amino acid profile in GS revealed an important increase in the content of each individual amino acid after 3 days of glyphosate treatment (Supplemental Figs.1.1 and 1.2). In leaves, Val, Leu, Ile, Ala, Asn, Gln, Gly, Asp, Thr, Lys, Ser, Arg, His, Pro, and Cys contents were higher after glyphosate treatment in a dose-dependent pattern (Supplemental Figure 1.1). In roots, Val, Leu, Ile, Ala, Gly, Glu, Thr, Lys, Ser, Arg, and His contents were increased, and in most cases the highest content was detected after treatment with 0.84kg ha^{-1} of glyphosate (Supplemental Fig. 1.2).

Branched-chain amino acid content was greatly increased in the leaves and roots of GS plants after glyphosate treatment, as reported recently (Maroli et al. 2015). The effect of the herbicide on the amide group of amino acids (Gln, Asn) in GS plants varied depending on the organ, increasing and decreasing in

leaves and roots, respectively. γ-Aminobutyric acid (GABA) is a nonprotein amino acid that usually accumulates under stress situations. GABA did not accumulate in glyphosate-treated GS plants. In GR plants, the contents of Val, Leu, Ile, Ala, Asn, Gln, Asp, Thr, Lys, Arg, His and Pro in leaves and roots and the contents of Gly and Cys only in leaves were increased 3 days after the application of the highest concentration of glyphosate. However, the accumulations were not as striking as in the GS biotype (Supplemental Figs. 1.1 and 1.2).

The general increase in the content of each free amino acid due to protein turnover could mask the specific change in each AAA. Thus, each individual AAA and their sum are presented as a percentage of the total free amino acids instead of as absolute values (Fig. 1.5B,D). In general, in the GS population, the relative content of each AAA, and the sum of the three AAA contents were increased after glyphosate application. The detected increase was more striking at the highest dose of glyphosate, following the same pattern as other free amino acids. The absolute contents of Trp, Tyr, and Phe were increased in the leaves and roots of the susceptible plants after glyphosate application (Supplemental Figs. 1.1 and 1.2). Other studies have shown no clear pattern in AAA content in response to glyphosate exposure. In sensitive *A. palmeri* and soybean, only Trp accumulated after glyphosate treatment (Vivancos et al. 2011; Maroli et al. 2015) whereas rapeseed exhibited an increase in Phe concentration in response to low glyphosate concentrations and no change in response to higher glyphosate concentrations (Petersen et al. 2007). The AAA content in GR plants was largely unaffected by glyphosate treatment: it was not affected in roots and was increased in leaves only at a dose of 2.52 kg ha^{-1}. By contrast, metabolic profiling of a resistant biotype of *A. palmeri* revealed perturbations in AAA levels after glyphosate treatment (Maroli et al. 2015).

It is difficult to predict how greatly an increase in one enzyme of the shikimate pathway may affect the AAA content. Transgenic Arabidopsis plants expressing a feedback-insensitive bacterial DHAPS exhibit higher Phe and Trp contents (Tzin et al. 2012). Thus, the *in vivo* roles of two enzymes of the shikimate pathway that are feedback regulated by AAA, chorismate mutase and anthranilate synthase, remain unclear (Tzin and Galili 2010b). In our study, the

comparison of untreated plants of both populations demonstrated that resistant plants with much higher EPSPS protein levels and activity exhibited the same AAA content as susceptible plants. This result suggests a regulatory mechanism after *EPSPS* expression that controls AAA content. The free amino acid content reflects the biosynthesis, catabolism, use rate in protein synthesis, and proteolysis of amino acids. AAAs are used not only in protein synthesis but also in the biosynthesis of many aromatic metabolites, such as phenylpropanoids.

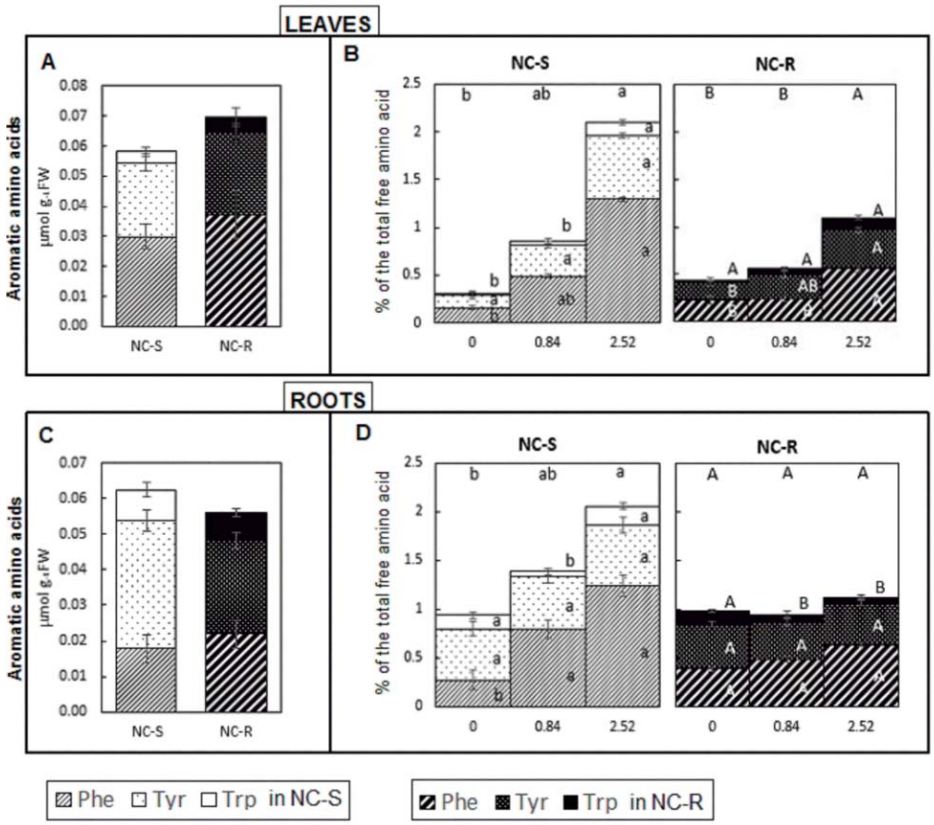


Figure 1.5 Aromatic amino acid (Phe, Tyr, and Trp) content in leaves (A) and roots (C) of untreated plants of glyphosate-susceptible (GS) and-resistant (GR) *Amaranthus palmeri* populations; effect of glyphosate on aromatic amino acid (Phe, Tyr, Trp) content with respect to the total free amino acids (percent of the total free amino acids) in leaves (B) and roots (D) 3 days after treatment (mean ± SE; n = 6−9). Different capital letters for the GR population and different lower case letters for the GS population indicate significant differences between treatments (p value ≤0.05). The letters embedded in the columns indicate differences for each individual amino acid, and letters above the columns indicate difference sbetween the sums of aromatic amino acids.

Glyphosate treatment of the GR population revealed that inhibition of EPSPS activity by the herbicide (even a small amount, as resistant plants possess a large amount of EPSPS protein) induces an increase in EPSPS

protein levels (Fig.1.3B), demonstrating control of gene expression of *EPSPS*. In these resistant glyphosate-treated plants, with higher EPSPS protein amount, the AAA content was largely unaffected (Fig. 1.5B,D), supplemental the interplay of mechanisms at gene expression and protein levels to control EPSPS *in vivo*.

Although the signal eliciting the increase in EPSPS protein synthesis has not been elucidated, our results indicate that AAA content is not involved because AAA levels remained nearly constant after glyphosate treatment. The response-triggering signal is proposed to be related to minor changes in other intermediate metabolites, such as shikimate or chorismate.

The results obtained after glyphosate treatment of the susceptible population confirmed the physiological effects described above for the resistant plants (an increase in EPSPS protein). However, the increase in EPSPS protein in susceptible plants (Fig. 1.3B) was insufficient to overcome the effect of the herbicide, as free amino acid content was clearly increased (Fig. 1.4). Indeed, the AAA content was also increased, most likely due to increased protein turnover, thus confirming that AAA levels are not the signal triggering the increase in EPSPS synthesis.

1.3.6 Carbon allocation and ethanol fermentation after glyphosate treatment

Carbohydrate accumulation is induced by the application of glyphosate (Orcaray et al. 2012; Zulet et al. 2015; Maroli et al. 2015) and thus can be used as a physiological marker of herbicide toxicity. Total soluble sugars (the sum of glucose, fructose, and sucrose) and starch contents were measured in the roots and leaves of both populations of *A. palmeri* 3 days after treatment with glyphosate (Fig. 1.6). Comparison of the control plants (untreated) of the two populations revealed similar carbohydrate levels, although several differences were detected: the control leaves and roots of the resistant biotype exhibited significantly higher contents of starch and total soluble sugars, respectively, than the susceptible plants. In GS plants treated with the lowest concentration of glyphosate, accumulation of total soluble sugars and starch in the leaves and of starch in roots was detected. Sugar accumulation in GS plants exhibited a

trend to decline to control values at the highest concentration, but this behavior does not indicate recovery. On the contrary, the severity of the treatment of these plants makes it difficult to maintain carbohydrate accumulation, corresponding to a possible decline in carbon assimilation (Zabalza et al. 2004; Orcaray et al. 2010). This pattern was not detected in GR plants, where a general increase in carbohydrate content was detected after both concentrations were applied. Carbohydrate accumulation in the leaves and roots of pea and *A. thaliana* plants supplied with glyphosate through the nutrient solution has been described (Orcaray et al. 2012; Zulet et al. 2015). Moreover, the same pattern has been described recently when glyphosate was sprayed onto the leaves, proving that the plant response was similar after foliar or residual applications (Armendáriz et al. 2016).

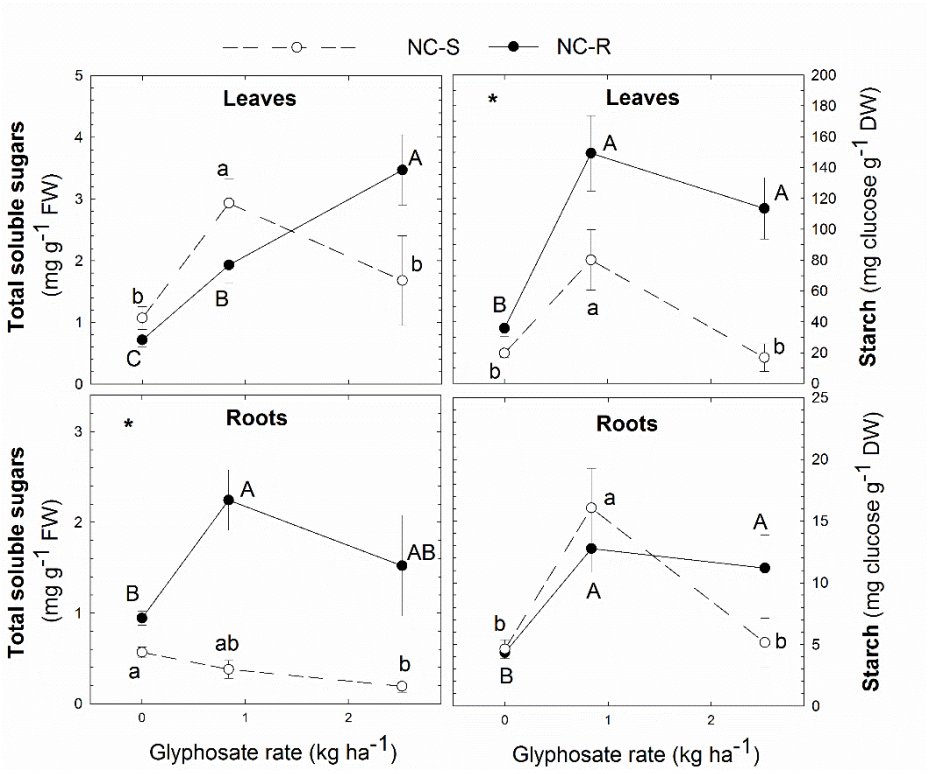


Figure 1.6 Effect of glyphosate on total soluble sugar (fructose, glucose, and sucrose) and starch contents in leaves and roots of glyphosate-susceptible (GS) and -resistant (GR) *Amaranthus palmeri* populations (3 days after application) (mean ± SE; n = 6−9). The asterisk (∗) indicates significant differences between control plants (without herbicide) of each population. Different capital letters for the GR population and different lower case letters for the GS population indicate significant differences between treatments (p value ≤ 0.05).

Glyphosate is a systemic herbicide that is translocated through all the plant body independently of the site of application, and the detected physiological effects were similar. In these studies, accumulation in sinks and sources was attributed to growth arrest. The accumulation of unused carbohydrates in sinks suggests that sucrose is transported from the leaves to the roots at a higher rate than is used in the sinks. Under these conditions, the sugar gradient required for long-distance transport is abolished, and carbohydrates accumulate in the leaves of treated plants because of a decrease in sink strength (Orcaray et al. 2012).

To evaluate another key parameter of carbon metabolism in the roots, ethanol fermentation was assessed in both populations (Fig. 1.7). Previous studies have reported the induction of aerobic fermentation after EPSPS inhibition in pea and *A. thaliana* (Orcaray et al. 2012; Zulet et al. 2015).

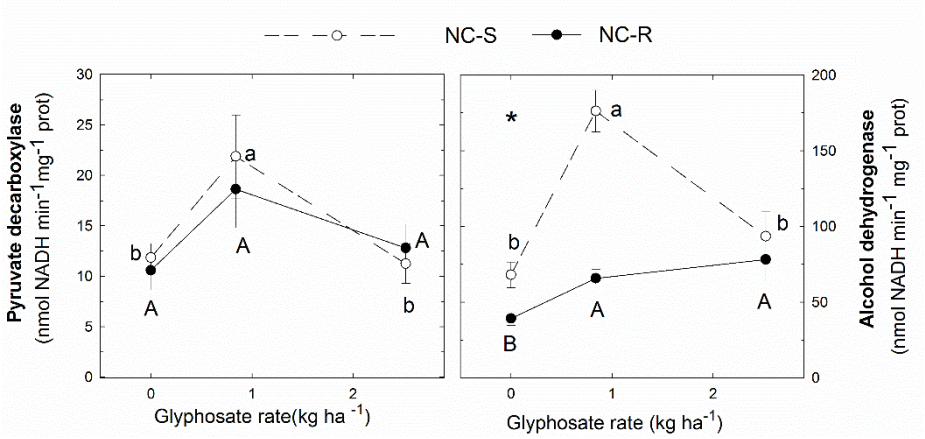


Figure 1.7 Effect of glyphosate on pyruvate decarboxylase and alcohol dehydrogenase enzymatic activities in roots of glyphosate-susceptible (GS) and -resistant (GR) *Amaranthus palmeri* populations (3 days after application) (mean ± SE; n = 6−9). The asterisk (∗) highlights significant differences between control plants (without herbicide) of each population. Different capital letters for the GR population and different lower case letters for the GS population indicate significant differences between treatments (p value ≤ 0.05).

The roots of untreated plants of both populations exhibited similar PDC activity, whereas ADH activity was higher in GR roots. The roots of the GS plants exhibited an increase in PDC and ADH activities after treatment with 0.84 kg ha^{-1} and a trend to decline to the control values when the highest dose of glyphosate was applied. Resistant plants exhibited induction of ADH activity only at the highest treatment. Fermentative induction after treatment with glyphosate cannot be easily explained, and the fermentative response can

likely be considered a physiological effect induced under stress (Orcaray et al. 2012).

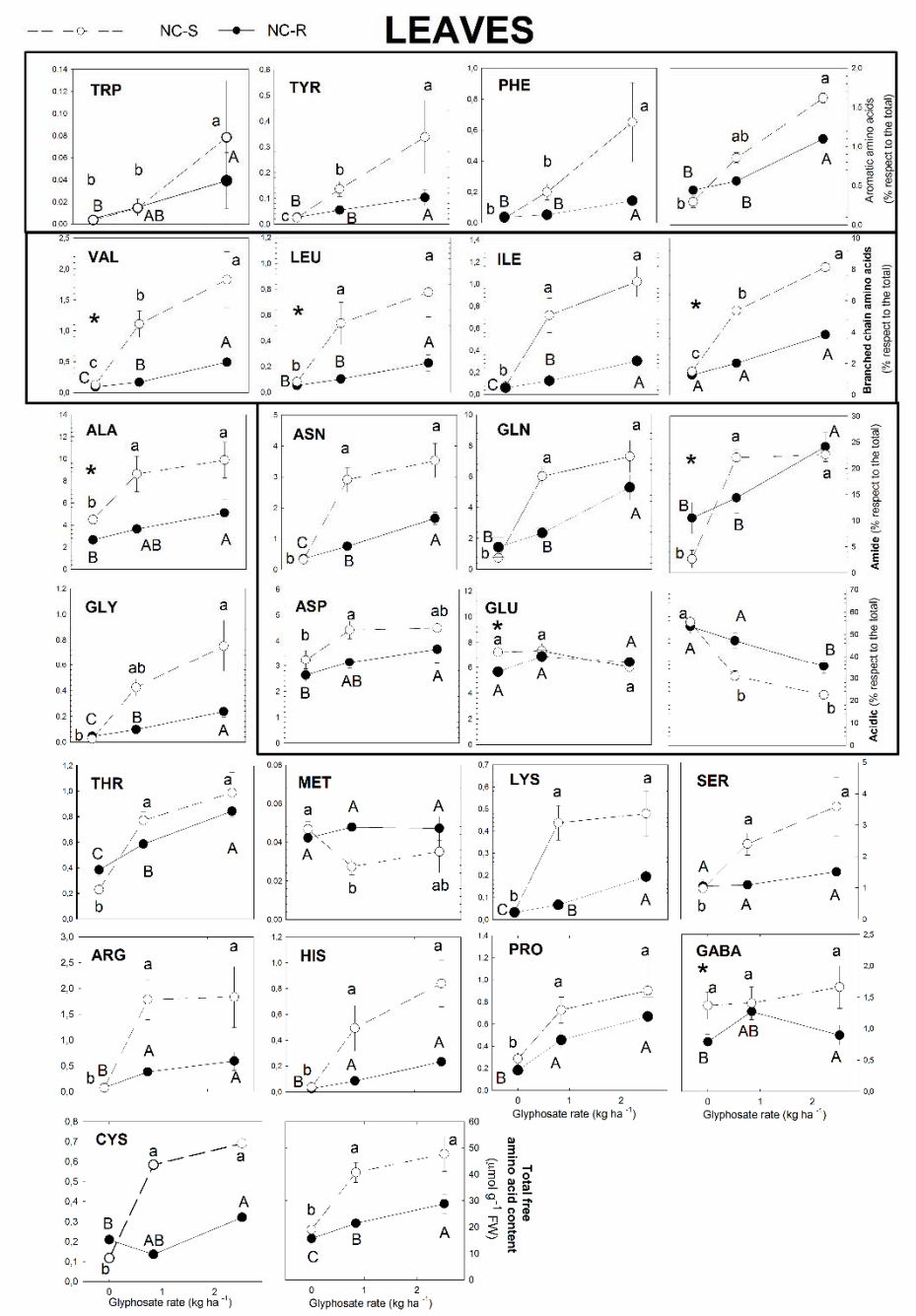
Collectively, these results indicate that *A. palmeri* exhibits the physiological markers typical of the toxic consequences of glyphosate and reported previously in other species (with some deviations): total free amino acid accumulation, carbohydrate accumulation, and ethanol fermentation induction (only at the lowest dose applied). Although both populations exhibited these symptoms, the pattern was not similar for all effects detected. Carbohydrate accumulation and ethanol fermentation were detected in GS and GR plants to a similar extent, although a trend to decline to control values was detected in GS plants treated with 2.52 kg ha^{-1}, most likely due to the severity of the dose. By contrast, individual and total free amino acid accumulation were less pronounced in GR plants than in GS plants. Free amino acid accumulation induced by glyphosate was alleviated in the resistant plants due to the reduced susceptibility to herbicide phytotoxicity, and thus this physiological marker was more directly related to the severity of the treatment and lethality. One important exception in the free amino acid content is that AAA content remained constant after glyphosate treatment, suggesting tight control of EPSPS activity *in vivo*. Different physiological patterns of sensitive and resistant biotypes after glyphosate treatment have recently been described by metabolic profiling (Maroli et al. 2015). As proposed in that study, resistance to glyphosate in GR plants, although primarily conferred by the EPSPS gene amplification, may be complemented by other physiological responses, such as an antioxidative protective mechanism (detected in Maroli et al. 2015) or the maintenance of a constant AAA content level detected in our study.

In conclusion, this study shows a complex regulation of EPSPS activity by mechanisms at transcriptional/translational and protein levels. In both populations, the herbicide induced increase of the EPSPS protein, indicating a regulation in gene expression that can be useful in the new weed management strategy based on RNA interference technology (branded BioDirect) to overcome glyphosate resistance in weeds (Shaner and Beckie 2014). There was no inherent differences in AAA content between the biotypes in the absence of glyphosate, despite the massive amount of EPSPS enzyme

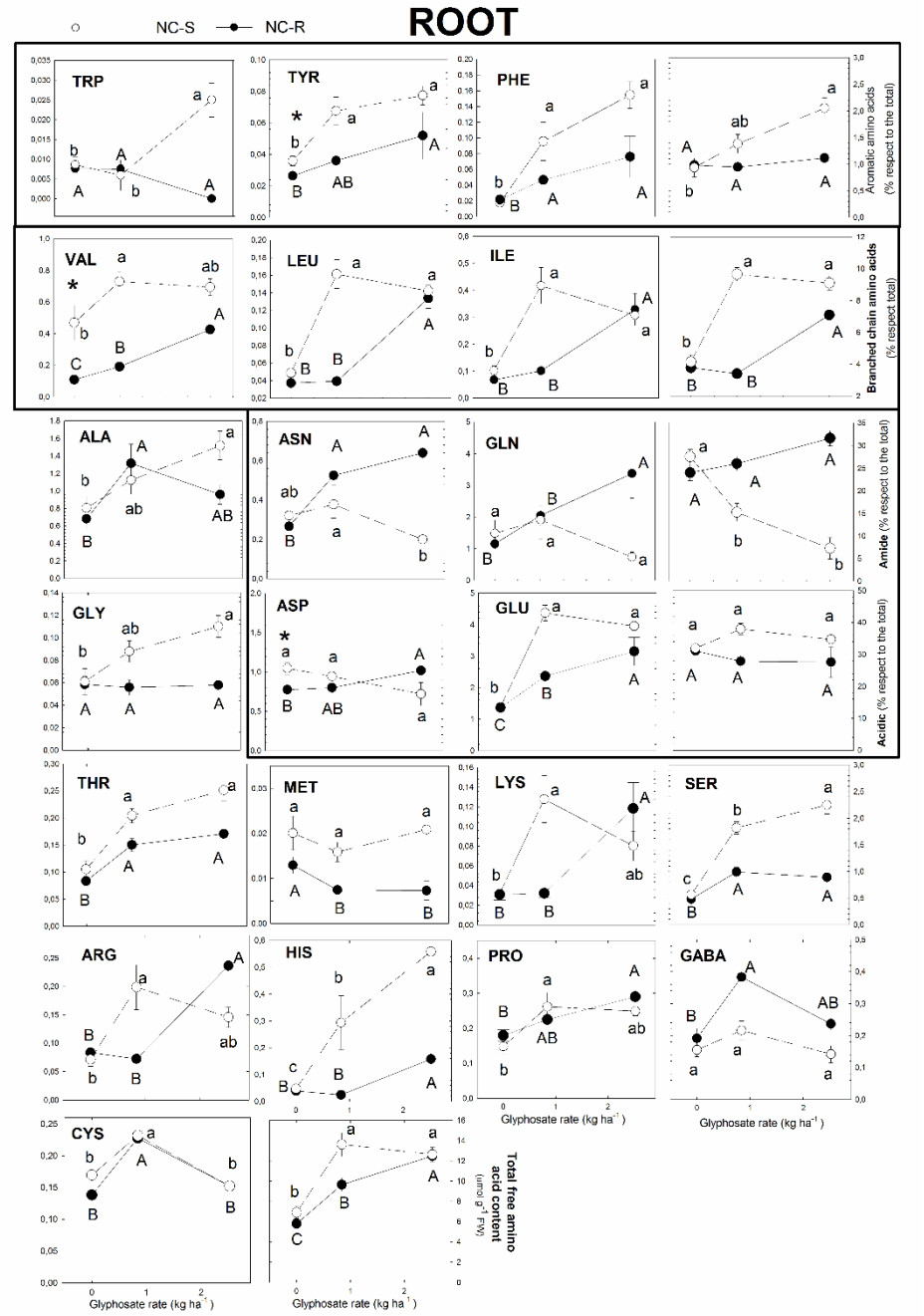
detected in GR plants. Moreover, AAA content was maintained constant in resistant plants, even with the glyphosate-induced increase of EPSPS enzyme.

These results indicate a regulation at the level of EPSPS protein, the signal of which remains unknown but cannot be AAA content. On the other hand, it has been possible to describe new insights of the physiological manifestations of the evolved glyphosate resistance. The physiological markers that have been reported before after glyphosate treatment were detected in susceptible and resistant plants: carbohydrate accumulation, induction of ethanol fermentation, and free amino acid accumulation. Resistant plants accumulate less amino acids than susceptible plants, and the effect of glyphosate on AAA content was almost abolished in resistant plants, suggesting that a constant free amino acid pool and AAA content are key parameters in complementing the resistance in GR population.

SUPPORTING INFORMATION OF CHAPTER 1



Supplemental Figure 1.1: effect of glyphosate on free amino acid content in leaves of glyphosate-resistant (GR) and -susceptible (GS) *A. palmeri* populations (3 days after application)



Supplemental Figure 1.2: effect of glyphosate on free amino acid content in roots of glyphosate-resistant (GR) and -susceptible (GS) *A. palmeri* populations (3 days after application)..

CHAPTER *2:* Effects of EPSPS copy number variation (CNV) and glyphosate application on the aromatic and branched chain amino acid synthesis pathways in *Amaranthus palmeri*

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Effects of EPSPS Copy Number Variation (CNV) and Glyphosate Application on the Aromatic and Branched Chain Amino Acid Synthesis Pathways in *Amaranthus palmeri*

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ABSTRACT

A key enzyme of the shikimate pathway, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), is the known target of the widely used herbicide glyphosate. Glyphosate resistance in *Amaranthus palmeri*, one of the most troublesome weeds in agriculture, has evolved through increased *EPSPS* gene copy number. The aim of this work was to study the pleiotropic effects of (i) *EPSPS* increased transcript abundance due to gene copy number variation (CNV) and of (ii) glyphosate application on the aromatic amino acid (AAA) and branched chain amino acid (BCAA) synthesis pathways. Hydroponically grown glyphosate sensitive (GS) and glyphosate resistant (GR) plants were treated with glyphosate 3 days after treatment. In absence of glyphosate treatment, high *EPSPS* gene copy number had only a subtle effect on transcriptional regulation of AAA and BCAA pathway genes. In contrast, glyphosate treatment provoked a general accumulation of the transcripts corresponding to genes of the AAA pathway leading to synthesis of chorismate in both GS and GR. After chorismate, anthranilate synthase transcript abundance was higher while chorismate mutase transcription showed a small decrease in GR and remained stable in GS, suggesting a regulatory branch point in the pathway that favors synthesis toward tryptophan over phenylalanine and tyrosine after glyphosate treatment. This was confirmed by studying enzyme activities *in vitro* and amino acid analysis. Importantly, this upregulation was glyphosate dose dependent and was observed similarly in both GS and GR populations. Glyphosate treatment also had a slight effect on the expression of BCAA genes but no general effect on the pathway could be observed. Taken together, our observations suggest that the high CNV of *EPSPS* in *A. palmeri* GR populations has no major pleiotropic effect on the expression of AAA biosynthetic genes, even in response to glyphosate treatment. This finding supports the idea that the fitness cost associated with *EPSPS* CNV in *A. palmeri* may be limited.

KEYWORDS: *glyphosate, aromatic amino acid pathway, branched chain amino acid pathway, mRNA relative expression, EPSPS, CM, AS, Amaranthus palmeri*

2.1 INTRODUCTION

The shikimate pathway uses carbon from primary metabolism to form chorismate, a precursor of the essential aromatic amino acids (AAAs) phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) (Tzin and Galili 2010b). These AAAs are not only essential components of protein synthesis but also serve as precursors for a wide range of secondary metabolites with multiple biological functions in plants, including plant stress tolerance (Dyer et al. 1989; Keith et al. 1991; Gorlach et al. 1995; Janzik et al. 2005; Maeda and Dudareva 2012). The AAA synthesis pathway can be subdivided into two steps: (i) the pre-chorismate (shikimate) pathway which provides the precursor chorismate used for synthesis of all AAAs and (ii) the post-chorismate pathway which can lead to either synthesis of Phe and Tyr, or Trp, via different routes (Fig. 2.1) (Maeda and Dudareva 2012). Synthesis of chorismate is catalyzed by seven enzymes acting sequentially (Fig. 2.1): 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), and chorismate synthase (CS). After formation of chorismate, synthesis of Trp is catalyzed by anthranilate synthase (AS) while synthesis of Phe and Tyr is catalyzed by chorismate mutase (CM) (Tohge et al. 2013b).

Due to its importance for plant biology, the synthesis of AAA is a tightly regulated process controlled by many inputs (Bentley and Haslam 1990; Tzin and Galili 2010b; Tohge et al. 2013b; Galili et al. 2016). Four points appear as checkpoints: the entrance of the pathway with the enzyme DAHPS (Sato et al. 2006), an exit of major importance with the phenylalanine ammonialyase (PAL) (Hahlbrock and Scheel 1989), the branch point in the post-chorismate pathway (Maeda and Dudareva 2012) and the enzyme EPSPS. The enzyme EPSPS is the target of the herbicide glyphosate (Steinrücken and Amrhein 1980) and therefore a key step in the shikimate pathway.

The intensive and continuous use of glyphosate has led to the emergence of glyphosate resistant (GR) weed populations (Powles 2008). The global issue of herbicide resistance for weed management is a serious challenge for global food security (Délye et al. 2013). One of the most damaging

glyphosate-resistant weed species is *Amaranthus palmeri* S. Wats (Culpepper et al. 2006; Powles and Yu 2010). Glyphosate resistance is conferred by gene amplification of EPSPS, which leads to a massive production of the enzyme EPSPS (Gaines et al. 2010). The recommended field dose is not sufficient to inhibit EPSPS activity, and plants survive. Copy number variation (CNV) of *EPSPS* is now reported to confer glyphosate resistance in several weed species including *Lolium multiflorum* (Salas et al. 2012) and *Kochia scoparia* (Wiersma et al. 2015) and particularly in *Amaranthus species* such as *Amaranthus tuberculatus* (Lorentz et al. 2014) and *Amaranthus spinosus* (Nandula et al. 2014).

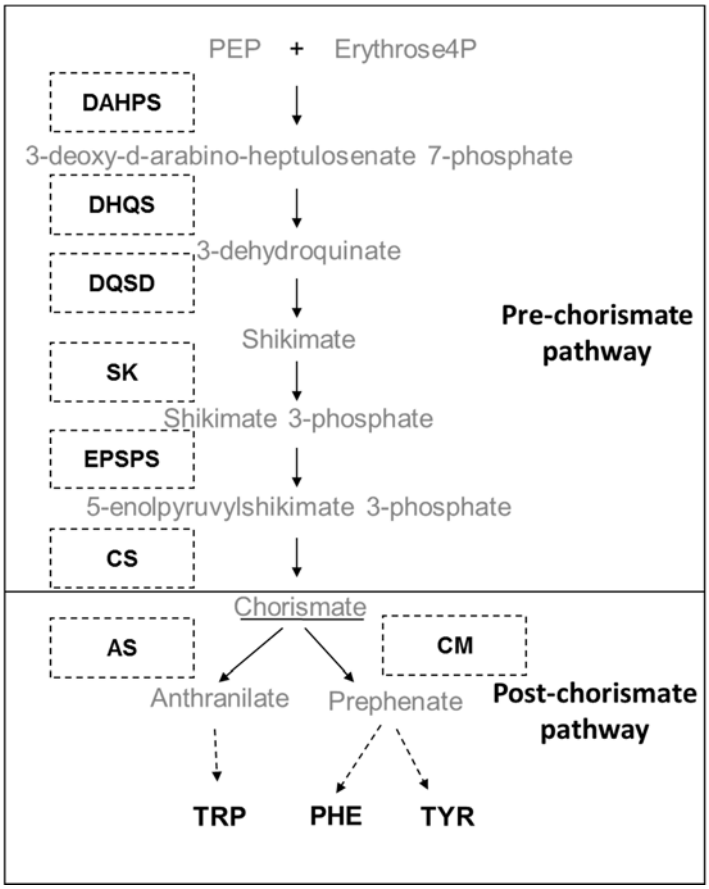


Figure 2.1 Biosynthetic pathway of aromatic amino acids (AAAs). Consecutive enzymatic steps of pre-chorismate pathway: D-arabino-heptulosonate 7-phosphate synthase (DAHPS), dehydroquinatesynthase (DHQS), 3-dehydroquinate dehydratase/shikimate dehydrogenase (DQSD), shikimate kinase (SK), 5-enolpyruvylshikimate 3-phosphatesynthase (EPSPS), and chorismate synthase (CS); and post-chorismate pathway: anthranilate synthase (AS) chorismate mutase (CM) leading to the synthesis of tyrosine (TYR), phenylalanine (PHE), and tryptophan (TRP).

To date, how the AAA pathway is regulated and how glyphosate may affect this regulation is not clearly understood. In particular, it is unknown whether there are pleiotropic effects associated with *EPSPS* CNV, particularly at the AAA synthesis pathway. Notably, no fitness cost has been associated with massive increase of EPSPS activity in GR populations (Giacomini et al. 2014; Vila-Aiub et al. 2014). However, the gene amplification resistance mechanism found in *A. palmeri* offers us the opportunity to study the regulation of the shikimate pathway, the effect of *EPSPS* overexpression due to extra *EPSPS* gene copies, and the effect of glyphosate application. In addition to the feedback regulation of AAA biosynthetic pathway, the hypothesis of the existence of cross regulation of amino acid metabolic pathways at the transcriptional level has been revised (Pratelli and Pilot 2014). There was a close correlation between AAA and branched chain amino acids (BCAAs) (Noctor et al. 2002).

In this study, the main objective was to evaluate the impact of *EPSPS* overexpression by gene amplification and of glyphosate treatment on the regulation of the AAA pathway and free AAA content. To this aim, the response of glyphosate sensitive (GS) and GR populations of *A. palmeri* to glyphosate were evaluated at the molecular and biochemical levels. Additionally, *m*RNA relative expression of the main enzymes from the BCAA pathway was developed to test whether there is any variation in their levels because of the overexpression of *EPSPS* or glyphosate treatment.

2.2 MATERIALS AND METHODS

2.2.1 Plant material and herbicide application

Seeds of *A. palmeri* GS and GR biotypes were originally collected from North Carolina (United States) (Chandi et al. 2012; Fernández-Escalada et al. 2016). The resistance mechanism of the GR biotype is *EPSPS* gene amplification (Chandi et al. 2012), with 47.5 more gene copies in GR than in GS plants (Fernández-Escalada et al. 2016). Plants were germinated and grown in aerated hydroponic culture under controlled conditions according to procedures described in Fernández-Escalada et al. (2016). Three week-old plants [after reaching the growth stage defined as BBCH 14 (Hess et al. 1997)] were treated with glyphosate (commercial formula, Glyfos, 360 g a.e. L^{-1}, isopropylamine salt, BayerGarden, Valencia, Spain) at both recommended field rate (1 X= 0.84 $kgha^{-1}$) and three times that rate (3 X= 2.52 kg ha^{-1}), according to Culpepper et al. (2006). Glyphosate treatment was performed using an aerograph (Junior Start model; Definik; Sagola). Control plants were treated with water. At 3 days after treatment, leaves were collected, frozen, and ground to a fine power as previously described (Fernández-Escalada et al. 2016). The experiment was conducted twice.

2.2.2 Quantitative reverse transcription-PCR

RNA was extracted from leaf tissues using the Macherey-Nagel NucleoSpinR RNA Plant kit following manufacturer's instructions. Total RNA concentration was measured with Gen5.1.11 (Biotek Instruments, Inc., Winooski, VT, United States) and RNA quality was assessed using RNA gel electrophoresis. The gels were visualized using a Gel Doc 2000 system (BIORAD Laboratories, Inc., Hercules, CA, United States).

cDNA synthesis was performed using BIORAD iScript^{TM}cDNA Synthesis Kit with 1 mg of total RNA following manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was performed using a Thermocycler BIORAD CFX Connect TM Real-Time System. The reaction kit used for qPCR was PerfeCTa SYBRR GreenSuperMix (Quantabio, Beverly, MA, United States). Each reaction was performed using 1 mL of cDNA template. The

following thermal profile was used for all PCRs: denaturation at 95ºC for 2 min, 40 cycles of 95ºC for 15 s and 52–61ºC 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 Supplementary Table 2.1. 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 five biological replicates. Primer efficiency (E) for each primer is presented in Supplementary Table 2.1 and was calculated according to E = 10[-1/slope] (Pfaffl 2001). Relative transcript level was calculated as $E_{GOI}^{CP_{GOI}\ \text{control-}CP_{GOI}\text{treated}}/E_{REF}^{CPREF\ \text{control-}CPREF\ \text{treated}}$ (Pfaffl 2001), where GOI = gene of interest, REF = reference gene (beta tubulin was used as normalization gene), and CP = crossing point, the cycle at which fluorescence from amplification exceeded the background fluorescence. Relative transcript level was calculated for all genes of the AAA synthesis pathway, corresponding to eight enzymes and four genes of the BCAA synthesis pathway.

2.2.3 EPSPS, DAHPS, and PAL immunoblotting

Protein extraction was performed using 0.1 g of ground leaf tissue in 0.2 mL of extraction buffer (MOPS 100 mM, EDTA 5 mM, Triton-X 100 1%, glycerin 10%, KCl 50 mM, benzamidine 1 mM, iodoacetamide 100 mM, PVP 5% and PMSF 1 mM). Proteins were separated by 12.5% SDS-PAGE and immunoblots were produced according to standard techniques. The protein amount loaded per well for each antibody used is specified in the fig. legends. EPSPS and DAHPS antibody dilutions were 1:2000 (Fernández-Escalada et al. 2016) and 1:1000 (Orcaray et al. 2011), respectively. PAL antibody was produced by a custom peptide facility (Biogenes, Berlin, Germany) using a short, conjugated peptide as an antigen (C-GATSHRRTKQGGA). The antibody was raised in rabbits using standard protocols from the manufacturer, and the primary antibody dilution was 1:500. An anti-rabbit AP conjugated antibody (Sigma Chemical, Co., St. Louis, MO, United States) was used as a secondary antibody at a dilution of 1:20,000. Bands were identified using a BCIP/NBT kit which was Amplified alkaline phosphatase immunoblot assay kit (BIORAD 170, BIORAD Laboratories, Inc., Hercules, CA, United States). Immunoblots were scanned using a GS-800 densitometer, and protein bands were quantified using

QuantityOne software (BIORAD Laboratories, Inc., Hercules, CA, United States). In the case of EPSPS protein, membrane signals were normalized according to total soluble protein loading quantity. In the case of DAHPS and PAL, absolute signals were used.

2.2.4 Enzymatic activities

5-Enolpyruvylshikimate-3-phosphate synthase activity was performed using the procedure described in Gaines et al. (2010). PAL activity was carried out according to Orcaray et al. (2011) with the following modifications. Samples were immediately centrifuged after extraction (12,000 *g*, 5 min). The reaction was started by the addition of 25 mM L-phenylalanine (Maroli et al. 2015). Controls (without L-phenylalanine) were prepared to determine endogenous levels of transcinnamic acid (t-CA). Incubation was performed for 1 h at 37 ºC (Sarma et al. 1998; Wang et al. 2007).

Protein extraction for CM and AS activity assays was developed as described in Singh and Widholm (1974) with addition of 1 mM PMSF (Goers and Jensen 1984). Samples were desalted using PD-10 columns (Ishimoto et al. 2010).

CM enzymatic activity was measured as described in Goers and Jensen (1984). Control for each sample was carried out using enzymatic extracts previously inactivated with 1 N HCl. AS activity was quantified as described in Ishimoto et al. (2010). Controls were performed using boiled enzymatic extract (Matsukawa et al. 2002).

2.2.5 Shikimate determination

For shikimate content determination, three leaf disks (4 mm diameter) were excised from the youngest leaf of each plant. Leaf disks were placed in a screw-top 2 mL epitube, frozen, and stored at -80 ºC until analysis. Shikimate was extracted as described in Koger et al. (2005b). After addition of 100 mL of 0.25 N HCl per disk to each vial, samples were incubated at 22 ºC for 1.5 h and mixed by vortexing. Shikimate content was quantified spectrophotometrically (Cromartie and Polge 2000).

2.2.6 Aromatic amino acid content determination

Ground leaf (0.1 g) was homogenized in 1 M HCl for amino acid extraction. Protein precipitation was performed after incubation on ice and centrifugation (Orcaray et al. 2010). After derivatization with fluorescein isothiocyanate, AAA content was measured by capillary electrophoresis coupled to a laser induced fluorescence detector, as described in Zulet et al. (2013b). Analyses were performed at 20 ºC and at a voltage of + 30 kV. For tryptophan determination, the voltage was reduced to + 20 kV in order to improve separation.

2.2.7 Statistical analysis

Transcript level analyses were performed using five biological replicates. For immunoblot, enzyme activity, shikimate and AAA quantification, four biological replicates were used. One-way ANOVA with a multiple-comparison adjustment for least significant difference (LSD) at $p<0.05$ was used. Statistical analyses were performed using SPSS Statistics 24.0 (IBM, Corp., Armonk, NY, United States).

2.3 RESULTS

The number of *EPSPS* copies in the studied GR biotype was 47.5 fold when compared to the corresponding GS biotype (Fernández-Escalada et al. 2016). In the absence of glyphosate, protein level was increased by 25 fold (Figs. 2.2A, B) and EPSPS activity was 26 fold higher (Fig. 2.2C). In response to glyphosate, only a mild increase of the abundance of EPSPS protein was observed in the GR biotype at the highest glyphosate dose (Figs. 2. 2A, B). EPSPS activity was not affected by glyphosate in the GR biotype, regardless of the dose, while it was slightly decreased in the GS biotype with the highest dose applied (Fig. 2.2C). While shikimate content was almost negligible in untreated plants of both populations, it was accumulated after glyphosate treatment in GS and in GR only at the highest glyphosate dose. Shikimate accumulated significantly more in GS than in GR at each glyphosate dose (Fig. 2.2D), confirming the inhibition of EPSPS by glyphosate observed in GS (Fig. 2.2C).

To study the impact of the high *EPSPS* copy number on the regulation of the AAA biosynthetic pathway, transcript levels for seven enzymes were analyzed by qRT-PCR. In absence of glyphosate treatment, *EPSPS* transcript level was increased by 55 fold in GR (Fig. 2.3A), confirming the results of Fernández-Escalada et al. (2016). For other enzymes, particularly *CS* and *CM*, only marginal changes were observed (1.68 and 2.33 fold, respectively) (Fig. 2.3A).

Glyphosate provoked an induction of the expression of all the genes of the shikimate pathway, with the exception of *CM* (Fig. 2.3B). The change in gene expression was dose dependent. The same effect was observed in both GS and GR populations. *CM* showed the opposite behavior, with no change (GS) or a slight decrease (GR) in *CM* transcript accumulation after treatment with glyphosate (Fig. 2.3B). The most responsive gene was *AS* with upregulation over 15 fold in GR with the highest dose (Fig. 2.3B). This may suggest a preferential flux to the Trp biosynthesis branch rather than to the Phe and Tyr branch in response to glyphosate treatment.

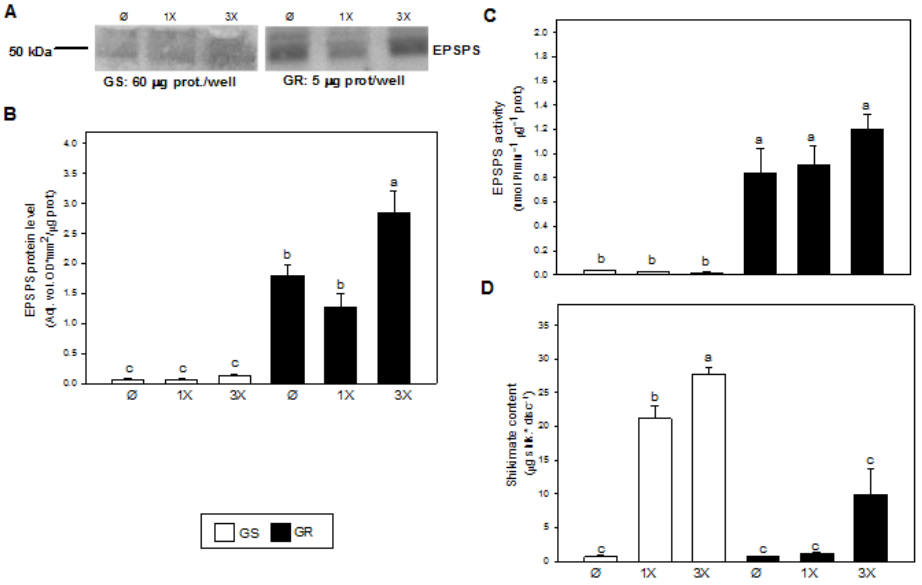


Figure 2.2 Characterization of resistance in *Amaranthus palmeri* populations. Glyphosate sensitive (white bars; GS) and glyphosate resistant (black bars; GR) populations were untreated (Ø) or treated with glyphosate and measured3 days after treatment with one (1X) or three times (3X) field dose. (**A**) Representative immunoblots for EPSPS. Total soluble proteins (60 mg for GS or 5 mg for GR) were fractioned by 12.5% SDS-PAGE and blotted. (**B**) Normalization of the intensity of the EPSPS bands expressed as optical density for unit of area per mg of protein (Mean ± SE; n = 3). (**C**) EPSPS *in vitro* enzymatic activity measured spectrophotometrically in semicrude leaf extracts (Mean ± SE; n = 4). (**D**) Shikimate content was measured spectrophotometrically after extraction from leaf disks of treated plants (Mean ± SE; n = 4). Different letters indicate significant differences between treatments and/or populations (p-value ≤ 0.05, LSD test).

To pursue this hypothesis, the activity of CM and AS enzymes was studied. In the absence of glyphosate, AS (Fig. 2.4A) and CM (Fig. 2.4B) activities were similar in both biotypes. Changes in the activity of AS and CM confirmed the trend observed at the transcript level, suggesting a preferential synthesis toward Trp after glyphosate treatment. *AS* expression induction was concomitant with an increase in the enzyme activity while CM activity was unchanged.

A

Enzyme	DAHPS	DHQS	DQSD	SK	EPSPS	CS	CM	AS
Relative expression in untreated plants R/S	0.86 ± 0.19	0.92 ± 0.12	1.48 ± 0.68	1.32 ± 0.19	55.02 ± 11.18	1.68 ± 0.17	2.33 ± 0.10	1.01 ± 0.21

B

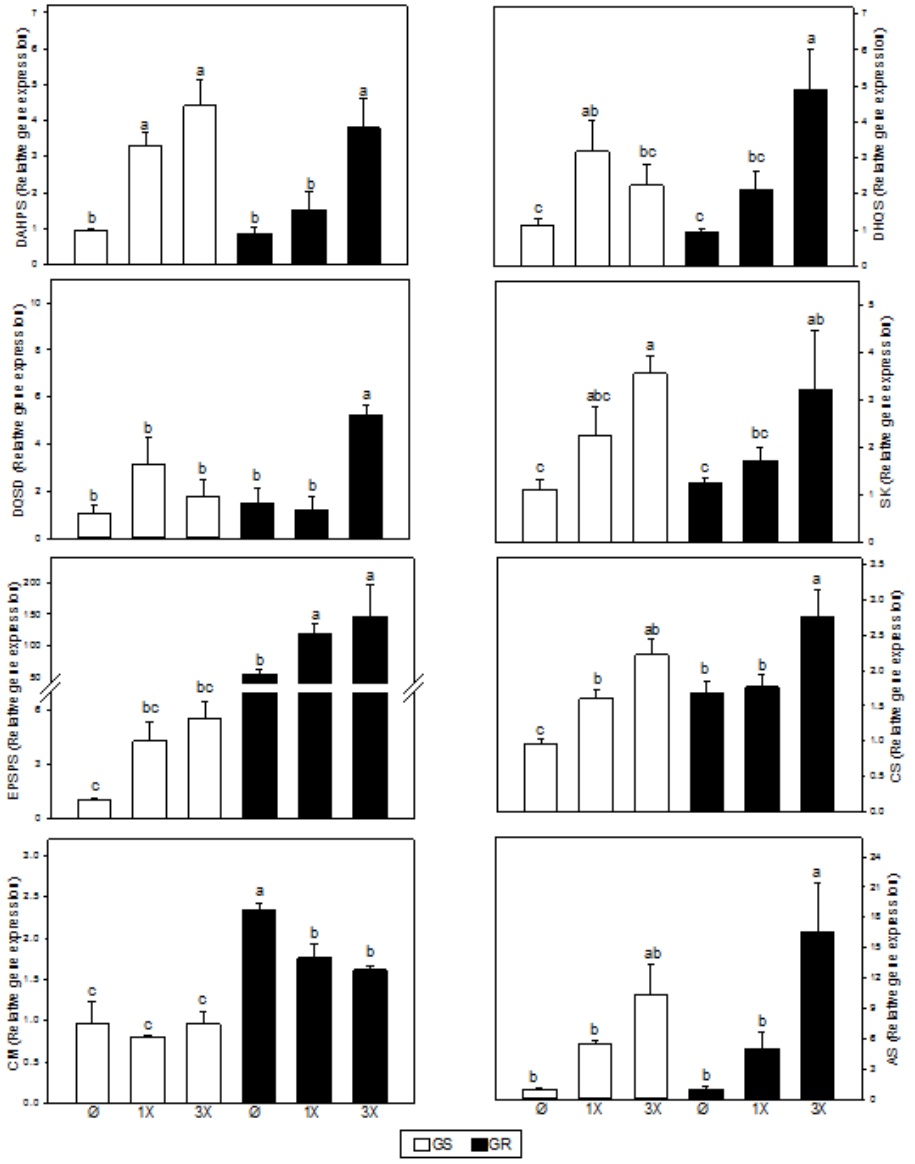
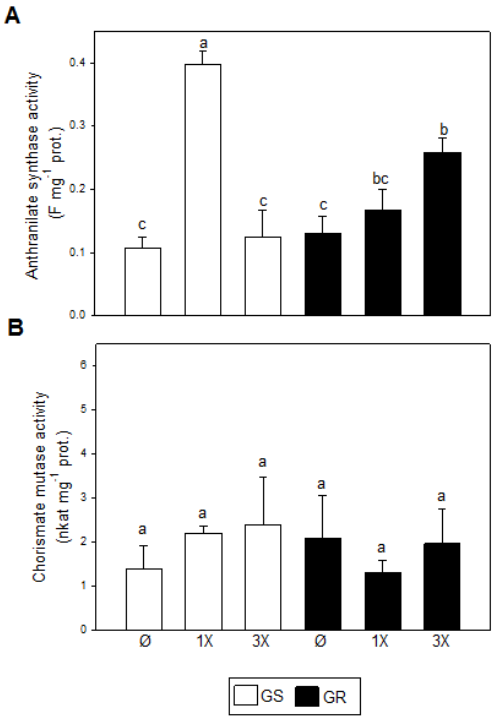


Figure 2.3 Transcript abundance of genes in the aromatic amino acid (AAA) biosynthetic pathway. Glyphosate sensitive (white bars; GS) and glyphosate resistant (black bars; GR) populations were untreated (Ø) or 3 days after treatment with glyphosate at one (1X) or three times (3X) field dose. Enzyme abbreviations as described in Figure 1. (A) Ratio of GR to GS relative transcript abundance measured with qRT-PCR normalized using the normalization gene beta tubulin.(B) Relative transcript abundance normalized using the normalization gene beta tubulin, relative to GS untreated plants (Mean ± SE; n = 5). Different letters indicate significant differences between treatments and/or populations (p-value≤0.05, LSD test).

Next, AAA levels were measured (Fig. 2.5). Before treatment with glyphosate, levels of Trp (Fig. 2.5A), Tyr (Fig. 2.5B), and Phe (Fig. 2.5C) were similar in both GS and GR biotypes. This result confirms that the striking change in *EPSPS* expression due to CNV does not have a major effect on AAA levels.

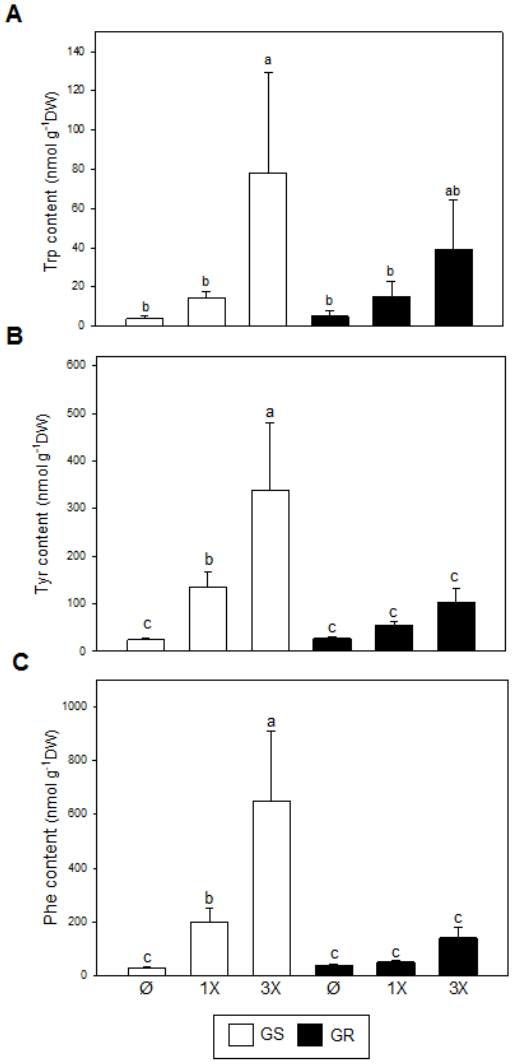


Figures 2.4 Anthranilate synthase (AS) and chorismate mutase (CM) enzymatic activities. Glyphosate sensitive (white bars; GS) and glyphosate resistant (black bars; GR) populations were untreated (Ø) or 3 days after treatment with glyphosate at one (1X) or three times (3X) field dose. (**A**) AS was measured in desalted leaf extracts by measuring the fluorescence of the produced anthranilate. (**B**) CM was measured in desalted leaf extracts by measuring prephenate production spectrophotometrically (Mean ± SE; n = 4). Different letters indicate significant differences between treatments and/or populations (p-value ≤0.05, LSD test).

After glyphosate treatment, the level of all AAA increased (Figs. 2.5A–C). However, significant changes were detected only in GS. In GR, the highest increase was detected for Trp.

Previous studies with the same populations and the same time of study and concentration of glyphosate provoked a threefold increase of total free amino acid content and a 12 fold increase of BCAA content (Fernández-Escalada et al. 2016). The higher effect of glyphosate on BCAA content than on other amino acid types suggests a possible effect of the herbicide on the BCAA biosynthetic pathway. Based on this, the expression pattern of four

enzymes of BCAA biosynthetic pathway was also measured: acetohydroxyacid synthase (AHAS), ketol-acid reductoisomerase (AHAIR), dihydroxyacid dehydratase (DHAD) and branched chain amino acid transaminase (TA) (Fig. 2.6).



Figures 2.5 Aromatic amino acid content. Glyphosate sensitive (white bars; GS) and glyphosate resistant (black bars; GR) populations were untreated (Ø) or 3 days after treatment with glyphosate at one (1X) or three times (3X) field dose. Tryptophan (Trp; **A**), tyrosine (Tyr; **B**), and phenylalanine (Phe; **C**) were measured by capillary electrophoresis in leaf acidic extracts (Mean ± SE; n = 4). Different letters indicate significant differences between treatments and/or populations (p-value ≤ 0.05, LSD test).

Transcript abundance of the BCAA biosynthetic pathway was not different between the untreated plants of both populations, suggesting that *EPSPS* overexpression does not affect BCAA pathway expression. After

glyphosate treatment, *AHAS*, *DHAD*, and TA showed no change at either dose in GS or in GR. *AHAIR* transcript abundance was increased in GS at the highest glyphosate dose, while it did not change in GR after glyphosate treatment.

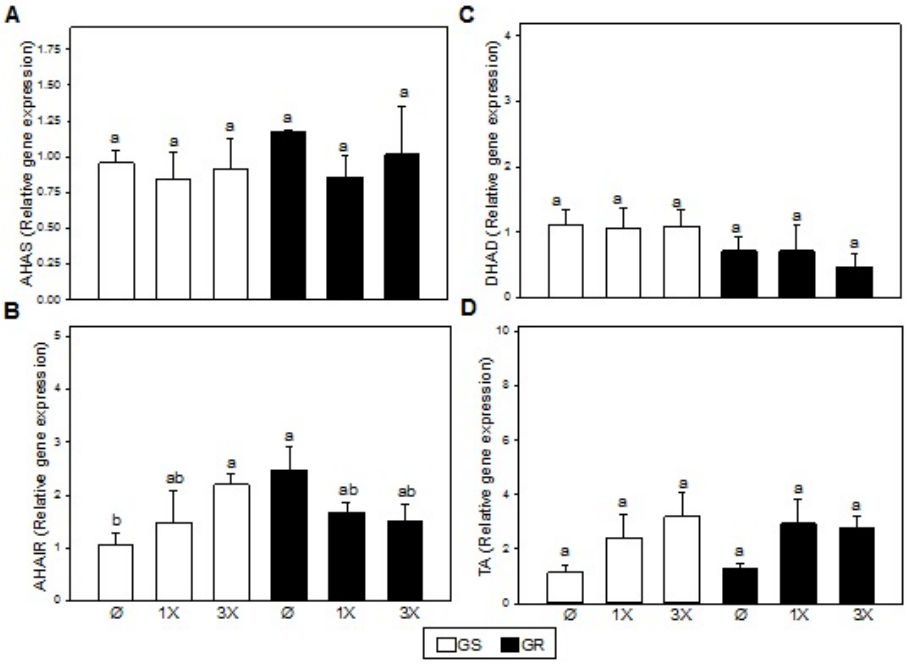


Figure 2.6 Transcript abundance of genes in the branched chain amino acid (BCAA) biosynthetic pathway. Glyphosate sensitive (white bars; GS) and glyphosate resistant (black bars; GR) populations were untreated (Ø) or 3 days after treatment with glyphosate at one (1X) or three times (3X) field dose. Relative expression of acetohydroxyacid synthase (AHAS; A) ketol-acid reductoisomerase (AHAIR; B), dihydroxyacid dehydratase (DHAD; C) and branched-chain amino acid transaminase (TA; D) normalized with the normalization gene beta tubulin, and relative to untreated GS plants (Mean ± SE; n = 5). Different letters indicate significant differences between treatments and/or populations (p-value ≤ 0.05, LSD test).

2.4 DISCUSSION

2.4.1 Characterization of resistance in *A. palmeri* populations

In the GR population of *A. palmeri* an *EPSPS* gene amplification (Fernández-Escalada et al. 2016) results in a massive increase of the accumulation of corresponding transcript (Fig. 2.3A) and of the protein level and activity (Figs. 2B, C). Our data validate results previously reported in other populations of *A. palmeri* (Gaines et al. 2010, 2011; Ribeiro et al. 2014), and other weedy plant species such as *A. tuberculatus* (Lorentz et al. 2014; Chatham et al. 2015), *Loliumperenne ssp*. *Multiflorum* (Salas et al. 2012), *Eleusine indica* (Chen et al. 2015), and *Kochia scoparia* (Wiersma et al. 2015). Additionally our data confirmed the accumulation of shikimate following treatment with glyphosate, mostly in the GS population (Fig. 2.2D). Shikimate is a known stress marker which accumulates following EPSPS inhibition in GS populations (Dyer et al. 1988; Baerson et al. 2002a; Zhu et al. 2008; Doğramacı et al. 2015; Whitaker et al. 2013; Fernández-Escalada et al. 2016; Dillon et al. 2017).

2.4.2 Gene amplification of EPSPS in *A. palmeri* GR populations has no major pleiotropic effect on the expression of AAA biosynthetic genes

Despite all these traits that characterize a GR population at molecular and biochemical levels, our work revealed that gene amplification of *EPSPS* had no major effect on the overall AAA pathway (Figs. 2.2–2.5). In particular, in untreated plants, the level of free AAA content was similar in GR and GS populations (Fig. 2.5). Similar AAA content in glyphosate resistant/sensitive biotypes has been previously described (Maroli et al. 2015). This 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).

The entrance of the primary metabolism to AAA pathway is through DAHPS enzyme (Tohge et al. 2013b). Plants control the carbon flux into the pathway by controlling *DAHPS* transcription and protein abundance (Herrmann and Weaver 1999). However, it was previously unknown whether GR populations with increased *EPSPS* expression would have altered *DAHPS*

regulation. Higher levels of DAHPS activity were described in GR populations compared to sensitive populations in *Nicotiana tabacum L.* (Dyer et al. 1988) and *Convolvulus arvensis* (Westwood and Weller 1997). In *Lolium rigidum* GR populations with higher *EPSPS* expression, levels of *DAHPS* transcripts were similar to sensitive population (Baerson et al. 2002a). In this study, while *DAHPS m*RNA relative expression was similar in both populations (Fig. 2.3B), the DAHPS protein level in GR was more than twofold higher than in GS (Supplementary Figs. 2.1A, B). It could implicate a translational regulation (or at least post-transcriptional mechanism) that controls DAHPS, and this may be related to *EPSPS* gene overexpression.

2.4.3 In sensitive and resistant plants glyphosate treatment provokes increased transcript abundance leading to synthesis of chorismate, and after this regulatory point, tryptophan

Our study shows that glyphosate treatment provoked an accumulation of the transcripts encoding virtually all the enzymes of the shikimate pathway, including *EPSPS*, in a dose-dependent manner (Fig. 2.3B). This trend is specific for enzymes of the AAA pathway and was not observed for the enzymes of the BCAA pathway (Fig. 6). Although increases in some enzymes of the shikimate pathway such as EPSPS (Baerson et al. 2002a; Yuan et al. 2002; Chen et al. 2015; Mao et al. 2016) and DAHPS (Baerson et al. 2002a) have been previously described, this is the first report suggesting a potential coordinated transcriptional regulation of the shikimate pathway after glyphosate treatment. Because this regulation is observed in both GS and GR populations (Fig. 2.3B), it suggests that this gene upregulation does not occur in response to the level of inhibition of EPSPS activity. Instead, it can be hypothesized that glyphosate itself, or indirectly, may affect plant amino acid metabolism, in addition to its known impact on EPSPS. Future research is needed to determine if glyphosate has unreported effects on plants and what signal causes this general gene induction of the pre-chorismate pathway.

This general upregulation of the expression of genes participating in the pre chorismate pathway is accompanied with an increase of the accumulation

of free AAAs, which is more pronounced in the GS population (Fig. 2.5). Although already reported (Vivancos et al. 2011; Maroli et al. 2015; Fernández-Escalada et al. 2016), this might appear counterintuitive at first glance because glyphosate is inhibiting the entry of carbon in this biosynthetic pathway, and therefore is expected to prevent synthesis of AAA. It is possible that the accumulation of free AAA comes from an increase in protein turnover in the plant following glyphosate treatment (Zabalza et al. 2006; Zulet et al. 2013a; Fernández-Escalada et al. 2016). Isotopic studies in *A. palmeri* revealed that both *de novo* synthesis of amino acids and protein turnover contribute to AAA accumulation in response to glyphosate (Maroli et al. 2016). While gene expression induction after glyphosate was similar in GR and GS populations (Fig. 2.3B), the accumulation of AAA was mainly observed in GS plants (Fig. 2.5). That observation may suggest that AAA accumulation following glyphosate treatment is rather related to the level of stress experienced by the plant.

After chorismate, AS increase in transcript abundance was higher than any other enzyme in the pathway in response to glyphosate treatment (Fig. 2.3B). AS expression was induced while CM expression was repressed, suggesting a regulatory branch point in the pathway (Fig. 2.1) for a preferential flux of carbon toward Trp biosynthesis over Phe and Tyr biosynthesis. This potential stream toward Trp was confirmed by studying AS and CM enzyme activities *in vitro* (Fig. 2.4). Data obtained in *Arabidopsis thaliana* (Sasaki-Sekimoto et al. 2005) and other plant species (Galili et al. 2016) also support this hypothesis.

However, measurements of free AAA in treated plants did not reveal any specific accumulation of Trp. Instead all three AAA were accumulated to a similar extent in GS plants (Fig. 2.5). Yet, a slight difference was detected in the GR plants, which may suggest that under “mild” stress (3x dose in GR), synthesis of Trp is prioritized over the synthesis of Phe and Tyr. It is possible that this regulation is related to the inhibition of DAHPS by arogenate (Siehl 1997), an intermediate product of the CM pathway. DAHPS may be key to the regulation of shikimate synthesis because it represents the entry point in this pathway (Maeda and Dudareva 2012). Interestingly, *DAHPS* gene expression was induced by glyphosate in both populations (Fig. 2.3B) while the increase in

DAHPS protein was only detected in GS population (Supplementary Figs. 1A,B). This might indicate that other layers of regulation (post-transcriptional) might fine-tune the regulation of this pathway. PAL protein level and enzyme activity have also been studied, because it represents the most important output from the AAA pathway (Hahlbrock and Scheel 1989). No differences were found between populations for PAL protein abundance or activity level in untreated and treated plants (Supplementary Figs. 1C–E). While other studies with other species show important effects of glyphosate on PAL (Hoagland et al. 1979; Zabalza et al. 2017), our results show that PAL abundance and enzyme activity are not affected in *A. palmeri*.

The results obtained after glyphosate treatment suggest that a stress-induced response to glyphosate increases the enzyme expression in the AAA pathway, which may require a substantial increase in energy consumption (Benevenuto et al. 2017). Trying to increase the carbon flux, which could further increase shikimate accumulation upon glyphosate treatment, could lead to the loss of feedback control in the pathway (Marchiosi et al. 2009). Reduction in AAA levels does not appear to elicit the increased expression of AAA pathway genes, because the AAA concentrations increase with glyphosate dose. Further research is needed to understand the signal(s) that upregulates the AAA pathway following glyphosate treatment.

2.4.4 No cross regulation between AAA and BCAA pathway was detected

In general, the free amino acid pool increases after glyphosate treatment (Orcaray et al. 2010; Vivancos et al. 2011; Zulet et al. 2013a; Liu et al. 2015) but the higher relative increase is in BCAA levels (Orcaray et al. 2010). The higher effect of glyphosate on BCAA than on other amino acid types suggests a possible effect of the herbicide on the BCAA biosynthetic pathway. The expression pattern of the BCAA biosynthetic pathway was measured (Fig. 2.6) and no clear patterns for expression changes of the BCAA enzymes in plants treated with glyphosate were identified (Fig. 2.6), while an induction of expression of AAA enzymes was detected (Fig. 2.3B). Although some authors (Guyer et al. 1995; Noctor et al. 2002; Pratelli and Pilot 2014) have proposed

cross regulation between the levels of AAA and BCAA, and close correlation was observed between the AAA pathway and the BCAA pathway (Noctor et al. 2002), no cross regulation at the transcriptional level was found in this study.

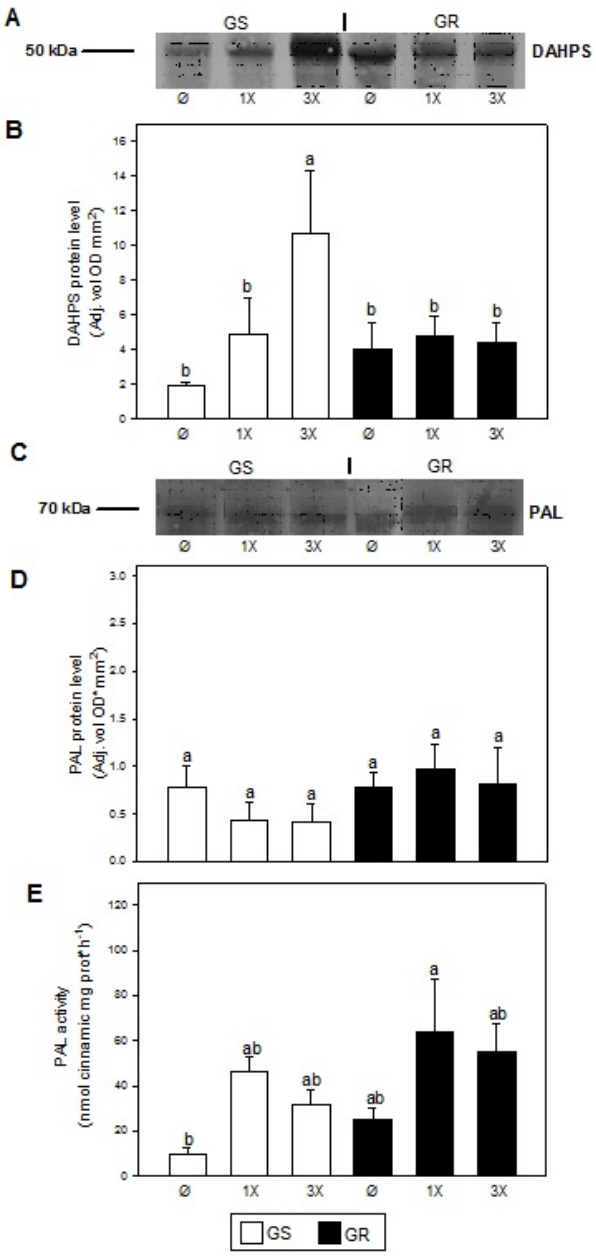
2.6 CONCLUSIONS

No differences were found (other than *EPSPS*) in transcriptional regulation of the shikimate pathway between *A. palmeri* GR and GS untreated plant, which implies that pleiotropic effects due to shikimate pathway perturbation are not apparent. Transcriptional induction of the AAA pathway was detected following glyphosate treatment in both GR and GS plants, suggesting a potential coordinated transcriptional regulation. AAA content was not the signal causing this response, because AAA accumulation was detected only in GS plants and further research will be needed to determine the signal. Glyphosate treatment resulted in an upregulation of the Trp biosynthesis branch instead of the Phe and Tyr branch, indicating that this branch point may be a regulatory point in the pathway. With respect to cross regulation between the AAA and BCAA pathways, no differences in BCAA transcriptional regulation was observed due to either *EPSPS* gene amplification or to glyphosate treatment.

SUPPORTING INFORMATION OF CHAPTER 2

GENE	FORWARD	REVERSE	Annealing temperature	Efficiency
Aromatic amino acid biosynthetic pathway				
DAHPS	cctcataggatgataagggc	ctttgcatggcagcataacc	55 ºC	96 %
DHQS	gcattgttggctagggatcc	aacctcggccttgttttcac	61 ºC	91 %
DQSD	ggtgtactcaagcaaggagc	tgtggactcttactatggcc	57 ºC	84 %
SK	gattctgaagcacaaagcagc	cagttgttttcccagagccc	55 ºC	91 %
EPSPS	aatgctaaaggaggccttcc	tcaatctccacgtctccaag	61 ºC	93 %
CS	cttgatagaaggaggcctgg	gtttctttcctaggagtagtg	57 ºC	90 %
CM	gaatacattatggcaagtatgt	gtcataagtcgctccttgtc	52ºC	97 %
AS	tttggagggaaggttgtgcg	ctggtgagctttttccatgc	57 ºC	88 %
Branched chain amino acid biosynthetic pathway				
AHAS	cttcctcgacatgaacaagg	attagtagcacctggacccg	57 ºC	84 %
AHAIR	atggctcagattgagatcttg	ccacggcttcaatcacactc	52 ºC	90 %
DHAD	taccatggcatcagctatcg	ggtgttgacgagctgtaagg	55 ºC	96 %
TA	gtgaagatgatcttcgtcggc	tcacaatcagacttgaaagatg	52 ºC	99 %
Normalization gene				
Beta tubulin	gatgccaagaacatgatgtg	tccacaaagtaggaagagttc	55 ºC	90 %

Supplemental Table 2.1 Primers (5'-3') used in the quantitative RT-PCRs.



Supplemental Figure 2.1: Income and outcome of the aromatic amino acid pathway. Sensitive (white bars; GS) and glyphosate-resistant (black bars; GR) populations were untreated (Ø) or treated with glyphosate for 3 days with one (1X) or three times (3X) field dose. **(A)** Representative immunoblot for of D-arabino-heptulosonate 7-phosphate synthase (DAHPS). Total soluble proteins (40 µg) were fractioned by 12.5% SDS-PAGE and blotted. **(B)** DAHPS band intensity expressed as optical density for unit of area (Mean ± SE; n = 3). **(C)** Representative immunoblots for phenylalanine ammonia-lyase (PAL). Total soluble proteins (90 µg) were fractioned by 12.5% SDS-PAGE and blotted. **(D)** PAL band intensity bands expressed as optical density for unit of area (Mean ± SE; n = 3). **(E)** PAL activity measured spectrophotometrically as cinnamic acid production rate in leaf extracts (Mean ± SE; n = 4). Different letters indicate significant differences between treatments and/or populations (p value ≤0.05, LSD test).

CHAPTER 3: Glyphosate and imazamox mixtures provoke physiological antagonism in *Amaranthus palmeri* sensitive and resistant to glyphosate

ABSTRACT

BACKGROUND: The herbicides glyphosate and imazamox inhibit the biosynthetic pathway of aromatic amino acids (AAAs) and branched-chain amino acids (BCAAs), respectively. Common physiological effects of both herbicides have been reported, and mixtures of both herbicides are being used to improve weed control. The aim of this study was to evaluate if there was a synergistic, antagonistic or additive physiological effect in the mixture of glyphosate and imazamox and if the effect was different in glyphosate-sensitive and -resistant populations of the troublesome weed *Amaranthus palmeri*.

RESULTS: Both herbicides applied individually induced the previously known physiological effects in both populations: shikimate, amino acid and carbohydrate accumulation. The herbicide mixture induced the same accumulations as the individual herbicides. Both populations exhibited similar effects to mixtures with the exception of the transcript levels of the AAA pathway, which were detected as an additive interaction in the sensitive population and as an antagonistic one in the resistant population.

CONCLUSIONS: The study of the physiological effects of the mixture of both herbicides in the two populations of *Amaranthus palmeri* provided evidence of a general physiological antagonism. At the transcriptional level, no cross regulation exists between AAA and BCAA inhibitors.

Keywords: glyphosate, aromatic amino acid pathway, branched-chain amino acid pathway, mRNA relative expression, EPSPS, AHAS, Amaranthus palmeri

3.1 INTRODUCTION

The aromatic amino acid (AAA) biosynthesis pathway transforms the inputs of carbon into the essential amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) (Tzin and Galili 2010b) with several successive enzymatic reactions. These AAAs are used by the plant to form proteins; in addition, they are precursors for secondary metabolites, and some of them are involved in plant stress tolerance (Janzik et al. 2005; Maeda and Dudareva 2012).The target of the herbicide glyphosate is the 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) enzyme (Steinrücken and Amrhein 1980) of the AAA pathway, which makes this step greatly important from an agronomic management standpoint.

The branched chain amino acid (BCAA) biosynthesis pathway leads to the formation of valine (Val), leucine (Leu) and isoleucine (Ile) (Galili et al. 2016). Acetohydroxy acid synthase (AHAS) has a key position in the pathway, since the enzyme catalyzes not only the synthesis of acetolactate, the valine and leucine precursor from pyruvate, but also that of acetohydroxybutyrate, the isoleucine precursor from alpha-ketobutyrate and pyruvate (Binder 2010). Due to its relevance in the BCAA biosynthesis pathway, AHAS has been widely used as target point for herbicides (Tan et al. 2006). There are five different chemical classes of AHAS-inhibitors: sulfonylureas, imidazolinones, triazolopyrimidines, sulfonylaminocarbonyl triazolinones and pyrimidinyl-oxy-benzoates (Powles and Yu 2010).

Although the target enzymes of the herbicides in the BCAA and AAA biosynthesis pathways are known, it is still unclear how exactly the inactivation of AHAS or EPSPS results in plant death. Previous findings showed that both AHAS and EPSPS inhibitors cause growth arrest followed by a slow plant death of the herbicide-treated plants although they act upon different pathways (Gruys and Sikorski 1999; Wittenbach and Abell 1999). Both types of herbicides provoke an accumulation of free amino acids (Orcaray et al. 2010; Maroli et al. 2015; Zulet et al. 2015; Fernández-Escalada et al. 2016; Zabalza et al. 2017), a decrease in the soluble protein content (Zulet et al. 2013a; Maroli et al. 2015, 2016) and accumulation of carbohydrates (Orcaray et al. 2012; Maroli et al. 2015; Zulet et al. 2015; Fernández-Escalada et al. 2016). Although they target

different enzymes, these common physiological effects suggest that these herbicides kill plants by similar mechanisms.

In addition to the similar physiological effects provoked by EPSPS and AHAS-inhibitors, other studies suggest a cross relationship between AAA and BCAA biosynthesis pathways. The hypothesis of the existence of cross regulation of amino acid metabolic pathways has been proposed (Guyer et al. 1995; Mohapatra et al. 2010; Pratelli and Pilot 2014). The contents of many minor amino acids vary in concert with different amino acid biosynthetic families (Noctor et al. 2002), and the closest correlation in these variations occurs between AAA and BCAA (Noctor et al. 2002; Orcaray et al. 2010). Moreover, there was some specific interactions between AHAS-inhibitors involved in Leu synthesis and the levels of Tyr and Phe (Wittenbach et al. 1994).

The repeated use of glyphosate and AHAS-inhibitors selects for the corresponding resistances in weed populations (Powles 2008). Now there are 159 weed species with at least one population with resistance to AHAS-inhibitors (Heap 2018; Délye et al. 2016). The most important cause of resistance is the mutations in the AHAS protein (Powles and Yu 2010). To date, 41 weed species with at least one population with resistance to glyphosate have been reported. One of the most problematic weed species resistant to glyphosate is *Amaranthus palmeri* S. Wats. (Culpepper et al. 2006; Powles and Yu 2010), whose mechanism of resistance to glyphosate is the amplification of the *EPSPS* gene (Gaines et al. 2010; Chandi et al. 2012). The recommended field dose is not sufficient to inhibit EPSPS activity when this gene is overexpressed; consequently, the EPSPS enzyme accumulates and the plants survive.

One of the most used practices to control glyphosate-resistant weeds is to mix glyphosate with AHAS-inhibitors (Hydrick and Shaw 1994; VanLieshout et al. 1996; Lich et al. 1997; Starke and Oliver 1998; Johnson, W.G. et al. 1999; Li et al. 2002; Nelson and Renner 2002; Shaw and Arnold 2002). Herbicide mixtures can interact in three different ways: antagonistically, additively, and synergistically (Barrett 1993). Previous studies evaluating the effects of AHAS-inhibitors and glyphosate mixtures application showed no conclusive results, so more studies are needed to better understand what kind of effects such mixtures could produce and to optimize their composition. The

use of effective mixtures is important for reducing selection pressure on individual target sites, which causes the selection of favorable mutations to resist the herbicide and the appearance of resistances. In the case of *A. palmeri*, it is of particular importance because populations of this species have developed multiple resistances meaning that they are not only resistant to glyphosate but also to AHAS-inhibitors (Gaedert et al. 2017; Küpper et al. 2017). The close relationship between AAA and BCAA biosynthetic pathways and the common physiological effects provoked by EPSPS and AHAS-inhibitors (as they have been introduced above) may cause noteworthy synergistic effects by the joint application of glyphosate and AHAS-inhibitors, which makes it interesting to study the physiological effects of the herbicide mixture in plants, although at the same time, the similar response of plants to both families of herbicides could question the efficacy of these mixtures.

The main objective in this study was to evaluate if there was a synergistic, antagonistic or additive physiological effect between glyphosate and the AHAS-inhibitor imazamox and if the effect was different if the treated plant was resistant to glyphosate. To this end, the effects of both herbicides applied individually and their mixtures on known physiological markers were evaluated (shikimate, free amino acid and carbohydrate) on two populations of *A. Palmeri* that were sensitive (GS) and resistant (GR) to glyphosate. Additionally, in order to clarify the global regulatory mechanisms of the AAA pathway and if there is a cross regulation between AAA and BCAA biosynthetic pathways, the relative expression of the genes of AAA and BCAA pathways based on *m*RNA levels and the protein content of key enzymes in the AAA pathway were tested.

3.2 MATERIALS AND METHODS

3.2.1 Plant material and treatment application

Seeds from the two biotypes of *A. palmeri*, i.e., sensitive (GS) and resistant (GR) to glyphosate, were originally collected from North Carolina (USA) (Chandi et al. 2012; Fernández-Escalada et al. 2016). The resistance mechanism of the GR biotype is *EPSPS* gene amplification (Chandi et al. 2012), with 47.5 more gene copies in GR than in GS plants (Fernández-Escalada et al. 2016). Germination and plant growth were performed according to procedures described in Fernández-Escalada et al. (2016), and all treatments were applied to three week-old plants. Glyphosate, (commercial formula, Glyfos, BayerGarden, Valencia, Spain) was applied at both 0.25 times recommended field rate (0.25G=0.21 kg ha^{-1}) and recommended field rate (1G=0.84 kg ha^{-1}), according to Culpepper et al. (2006). The AHAS inhibitor imazamox (commercial formula, 4% P/V (Pulsar 40®, BASF, Barcelona, Spain)) was applied at 1.5 mg active ingredient L^{-1} (Gil-Monreal et al. 2017) after conducting preliminary dose-responses studies to select imazamox dose. The mixtures of imazamox with the two doses of glyphosate were also applied (0.25G+I and 1G+I). Glyphosate treatment was performed using an aerograph (Definik; Sagola, Vitoria-Gasteiz, Spain) connected to a compressor (Werther one, Breverrato) with the following settings: 60 W; 10 L m^{-1}; 2.5 bar at a rate of 500 L ha^{-1}. Imazamox was added to the nutrient solution. Another set of plants sprayed with water was used as the control reference. The leaves of both populations of *A. palmeri* were collected 3 days after treatment, frozen, and ground to a fine powder as previously described (Fernández-Escalada et al. 2016).

3.2.2 Quantitative reverse transcription-PCR

The relative transcript level was measured for all genes of the AAA synthesis pathway, corresponding to eight enzymes; and four genes of the BCAA synthesis pathway. RNA extraction and the subsequent cDNA extraction were performed as described in Fernández-Escalada et al. (2017). Gene expression was measured in two different experiments and the mean of both experiments was calculated as described in Fernández-Escalada et al. (2017). Quantitative

RT-PCR (qRT-PCR) was conducted by using techniques and primers detailed in Fernández-Escalada et al. (2017). Relative transcript levels were calculated as $E_{GOI}^{CP_{GOI}\ control-CP_{GOI}\ treated}$ /$E_{REF}^{CP_{REF}\ control-CP_{REF}\ treated}$ (Fernández-Escalada et al. 2017), where the control of GS was used to calculate all GS values and the control of GR was used to calculate all GR values.

3.2.3 EPSPS and DAHPS immunoblotting

Protein extraction and immunoblotting were performed according to standard techniques and as described previously (Fernández-Escalada et al. 2017). In the case of EPSPS, the protein amount loaded per well in each population was different and is specified in the figure legends. Membrane signals were normalized according to total soluble protein loaded.

3.2.4 Shikimate content determination

Three leaf disks (4 mm diameter) were excised from the youngest leaf of each plant for shikimate content determination. Leaf disks were placed in 2 mL Eppendorf tubes and stored at −80 °C until analysis. Shikimate was extracted as described in Shanner et al. (2005). Shikimate content was quantified spectrophotometrically (Cromartie and Polge 2000).

3.2.5 Amino acid content determination

Ground leaf samples (0.1 g) were homogenized in 1 M HCl for amino acid extraction. Protein precipitation was performed after incubation on ice and centrifugation (Orcaray et al. 2010). After derivatization with fluorescein isothiocyanate, the amino acid content was measured by capillary electrophoresis coupled to a laser-induced fluorescence detector, as described in Orcaray et al. (2010). Analyses were performed at 20 ºC and at a voltage of +30 kV.

3.2.6 Carbohydrate content determination

The total soluble sugar (glucose, fructose, and sucrose) content (TSS content) was determined in ethanol-soluble extracts, and the ethanol-insoluble residue was extracted for starch analysis as in Zabalza et al. 2004. The starch and TSS contents were determined by ion chromatography (930 Compact IC Flex, Metrohm AG Ionenstrasse CH-9100 Herisau, Switzerland), following the manufacturer's instructions (Gomensoro scientific instrumentation, Madrid, Spain). The sample dilutions used for soluble carbohydrates and starch were 1:10 and 1:50, respectively. To prepare the samples, the eluent used was 300 mM NaOH/1 mM sodic acetate in mili q water solution. The applied current was 200-500 mA, with pressure of 1000-1200 psi and temperature between 30 and 35 ºC.

3.2.7 Statistical analysis

Statistical analyses were performed using IBM SPSS statistics 24.0 (IBM, Corp., Armonk, NY, United States). All analyses were performed using 4 biological replicates from two independent experiments. For all parameters tested, the difference between untreated plants of each population was evaluated using Student's *t*-test, which found no significant differences; thus, this result is not mentioned in the text. One-way ANOVA with a multiple-comparison adjustment for least significant difference (LSD) at $p \leq 0.05$ was used to determine significant differences in the results of each population. For each population, significant differences are highlighted in the figures using different letters.

3.3 RESULTS

Glyphosate treatment resulted in a dose-related increase of shikimate content in the GS population at levels 5-fold greater than in the GR population (Fig. 1), confirming the resistance to glyphosate of GR plants. Imazamox applied individually did not modify shikimate content in any of the populations, as could be expected because this metabolite is not directly related to the BCAA biosynthetic pathway. In the GR population, mixtures of glyphosate and imazamox did not exacerbate shikimate accumulation more than glyphosate applied individually, which means a slight antagonism had occurred (Fig. 1). Interestingly, significantly less shikimate accumulation was detected in the GS population when 0.25G glyphosate treatment was applied with imazamox, which also means a significant level of antagonism was present.

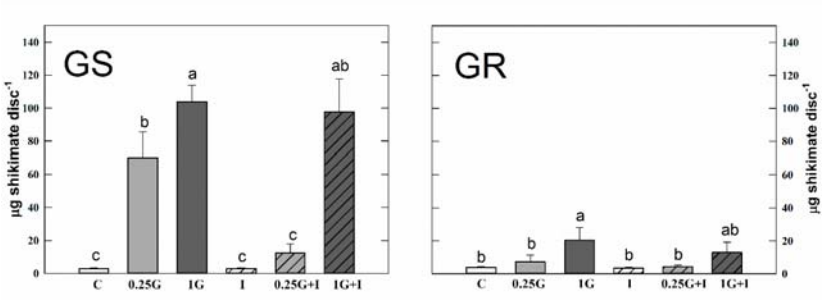


Figure 3.1 Shikimate content in the leaves of glyphosate sensitive (GS) and resistant (GR) *Amaranthus palmeri* plants 3 days after treatment. Untreated plants were sprayed with water (Control; C). Plants were treated with 0.21 kg ha^{-1} of glyphosate (0.25G), 0.84 kg ha^{-1} of glyphosate (1G), 1.5 mg L^{-1} of imazamox (I) or their mixtures (0.25G+I and 1G+I). (Mean ± SE; n=4). Different letters indicate significant differences between treatments (p-value ≤0.05, LSD test).

A general dose-dependent induction in the expression of all the genes of the AAA pathway (Fig. 2 and 3B) with the exception of *CM* (Fig. 3A) in the GS and GR populations was caused by glyphosate treatment. There was a high level of induction in the expression of the post-chorismate *AS* enzyme (Fig. 3B) and a repression of *CM* in the other post-chorismate branch of the AAA pathway (Fig. 3A). Imazamox applied individually did not induce any general pattern in the relative expression of the genes of the AAA pathway in any of the populations (Figs. 2 and 3) with few exceptions. Interestingly, an opposite pattern in the relative gene expression in response to mixtures was detected between the populations.

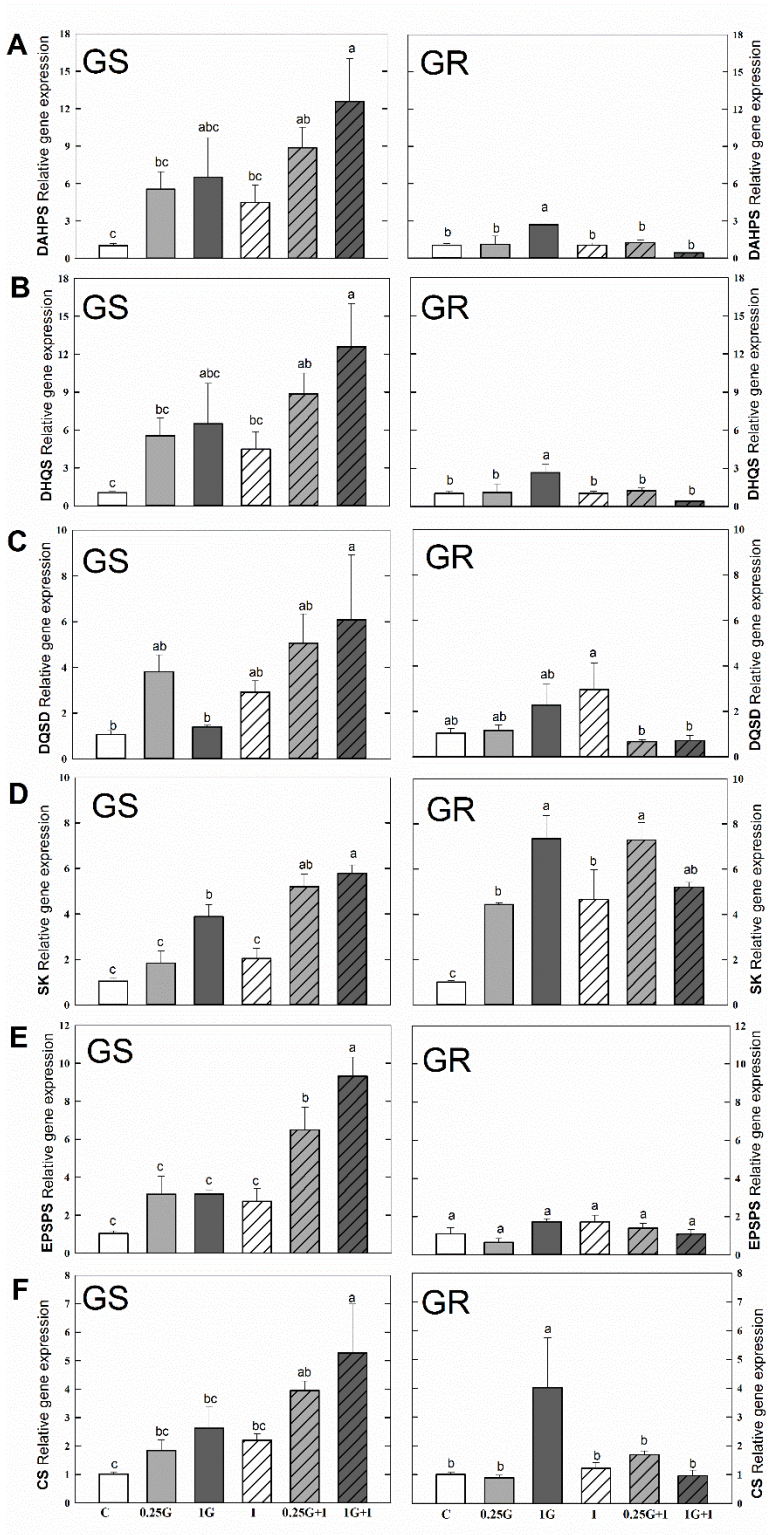


Figure 3.2 Transcript abundance in genes in pre-chorismate aromatic amino acid (AAA) pathway enzymes. Relative transcript abundance was normalized using the normalization gene *beta tubulin* and each population to its own control in *Amaranthus palmeri* plants 3 days after herbicide treatment in sensitive (GS) and resistant (GR) populations of: A) D-arabino-heptulosonate 7-phosphate synthase (DAHPS), B) dehydroquinate synthase (DHQS), C) 3-dehydroquinate dehydratase/shikimate dehydrogenase (DQ/SD), D) shikimate kinase (SK), E) 5-enolypyruvylshikimate 3-phosphate synthase (EPSPS) and F) chorismate synthase (CS). Untreated plants were sprayed with water (Control; C). Plants were treated with 0.21 kg ha^{-1} of glyphosate (0.25G), 0.84 kg ha^{-1} of glyphosate (1G), 1.5 mg L^{-1} of imazamox (I) or their mixtures (0.25G+I and 1G+I). (Mean ± SE; n=4). Different letters indicate significant differences between treatments (p-value ≤0.05, LSD test).

There was a general induction in the GS population after mixtures treatments; in contrast, in the GR population, there was no induction in the AAA pathway enzyme transcripts with mixtures, showing even a slight repression in *DAHPS, DHQS, DQSD*, and *CM* (Figs. 2 and 3).

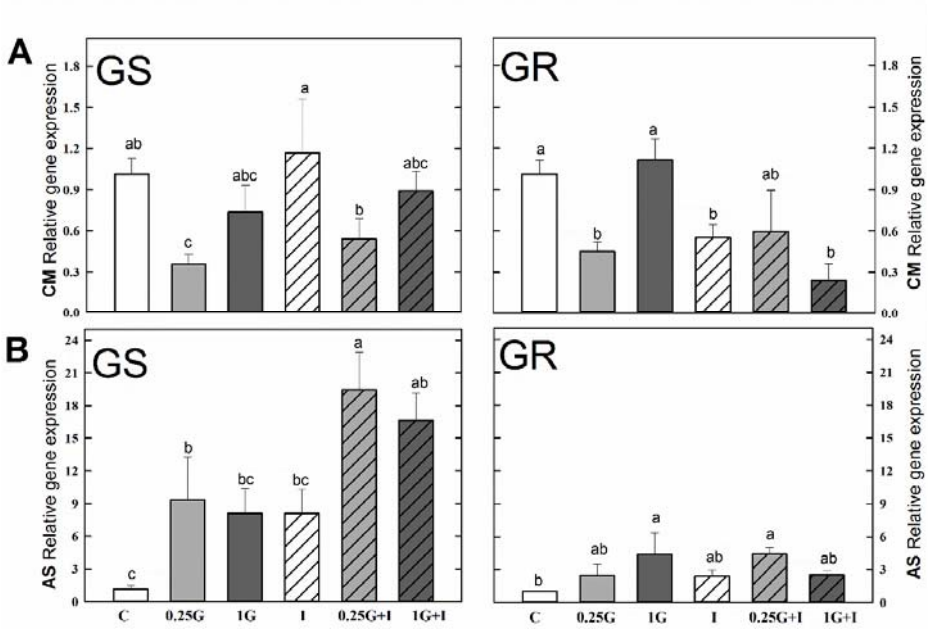


Figure 3.3 Transcript abundance in genes in post-chorismate aromatic amino acid (AAA) pathway enzymes. Relative transcript abundance was normalized using the normalization gene *beta tubulin* and each population to its own control in *Amaranthus palmeri* plants 3 days after herbicide treatment in sensitive (GS) and resistant (GR) populations of: A) chorismate mutase (CM) and B) anthranilate synthase (AS). Untreated plants were sprayed with water (Control; C). Plants were treated with 0.21 kg ha^{-1} of glyphosate (0.25G), 0.84 kg ha^{-1} of glyphosate (1G), 1.5 mg L^{-1} of imazamox (I) or their mixtures (0.25G+I and 1G+I). (Mean ± SE; n=4). Different letters indicate significant differences between treatments (p-value ≤0.05, LSD test).

The expression pattern of the genes involved in the BCAA pathway was also evaluated (Fig. 4). When glyphosate was applied individually, there were significantly higher levels of expression compared to the control with the 1G treatment in *AHAS* and 0.25G in *AHAIR* in the GR population (Fig. 4A-B). No

significant changes in gene expression were detected after the inhibition of one of the enzymes of the BCAA pathway, AHAS, by imazamox applied individually. The herbicide mixtures did not induce any remarkable change in the relative expression of the enzymes in this pathway (Fig. 4).

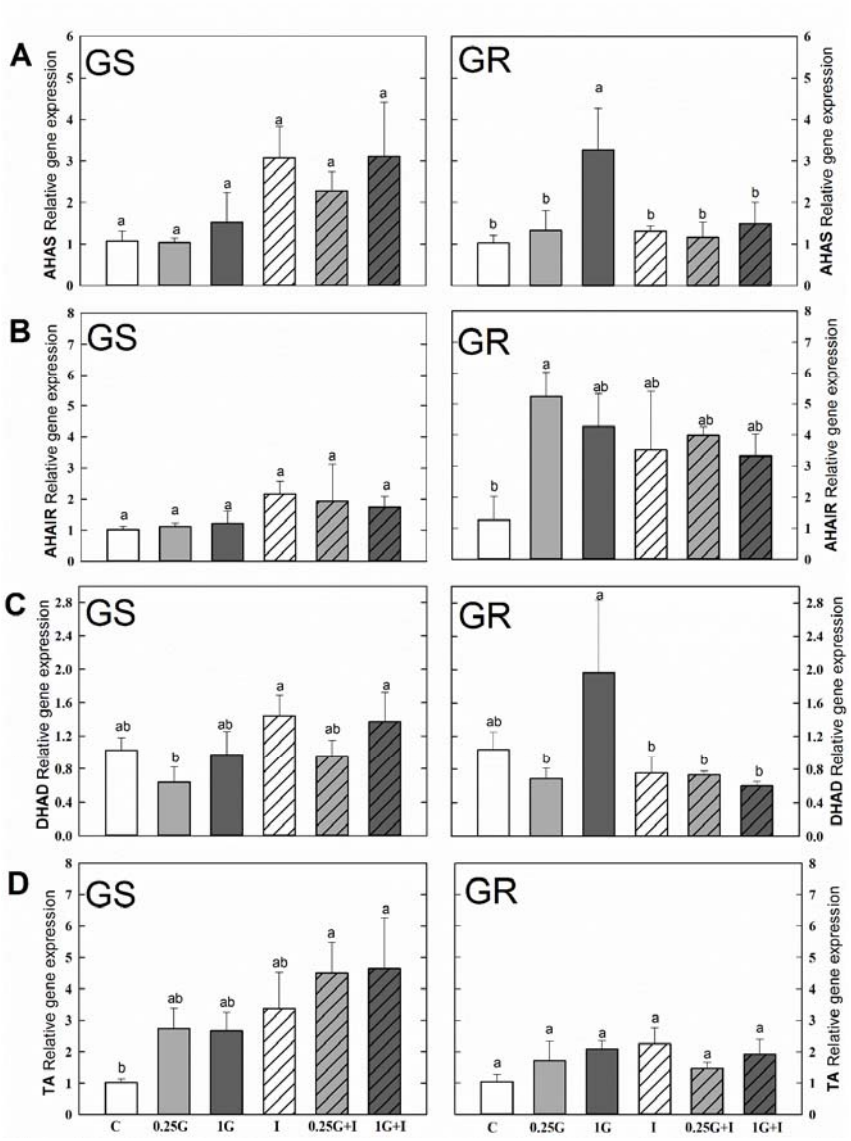


Figure 3.4 Transcript abundance in genes in the branched chain amino acid (BCAA) pathway enzymes. Relative transcript abundance was normalized using the normalization gene *beta tubulin* and each population to its own control in *Amaranthus palmeri* plants 3 days after herbicide treatment in sensitive (GS) and resistant (GR) populations of: A) acetohydroxy acid synthase (AHAS), B) acetohydroxy acid isomer reductase (AHAIR) C) dihydroxy acid dehydratase (DHAD) and D) BCAA transaminase (TA). Untreated plants were sprayed with water (Control; C). Plants were treated with 0.21 kg ha^{-1} of glyphosate (0.25G), 0.84 kg ha^{-1} of glyphosate (1G), 1.5 mg L^{-1} of imazamox (I) or their mixtures (0.25G+I and 1G+I). (Mean ± SE; n=4). Different letters indicate significant differences between treatments (p-value ≤0.05, LSD test).

The protein contents of two important proteins of AAA pathway (DAHPS and EPSPS) were measured. In the GS population, only in the 1G+I treatment were we able to detect a significant increase of the DAHPS protein with respect to control; and in the GR population, there were no significant differences between treatments (Fig. 5A). Both populations showed no effect in response to imazamox applied individually, and there were no significant differences between glyphosate treatments and their mixtures with imazamox (Fig. 5A). The EPSPS protein content in non-treated plants was 10-fold higher in the *A. palmeri* GR population than in the GS population (Fig. 5B).

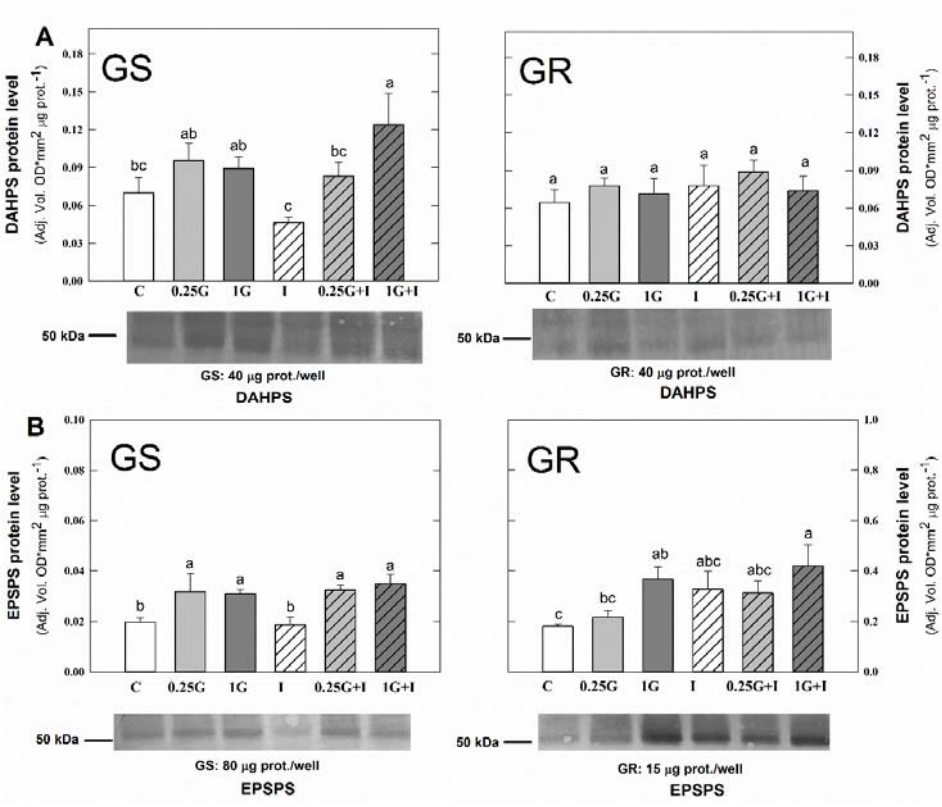


Figure 3.5 DAHPS and EPSPS protein levels. Protein level in the leaves of A) DAHPS glyphosate sensitive (GS) and resistant (GR) and B) protein level in the leaves of EPSPS glyphosate sensitive (GS) and D) resistant (GR) of *Amaranthus palmeri* plants 3 days after herbicide treatment. Total soluble protein (40 µg per well in A, 80 µg per well in B (GS) and 15 µg per well in B (GR)) were fractioned by 12.5% SDS-PAGE and blotted. In all cases results are accompanied with a representative picture of the immunoblot. Untreated plants were sprayed with water (Control; C). Plants were treated with 0.21 kg ha^{-1} of glyphosate (0.25G), 0.84 kg ha^{-1} of glyphosate (1G), 1.5 mg L^{-1} of imazamox (I) or their mixtures (0.25G+I and 1G+I). (Mean ± SE; n=4). Different letters indicate significant differences between treatments (p-value ≤0.05, LSD test).

There were significant increases in EPSPS protein levels in the GS population after both glyphosate treatments and after the 1G glyphosate treatment dose in the GR population (Fig. 5B). Imazamox applied individually did not change the EPSPS content in either of the populations. Mixtures caused no effects in the EPSPS content in the GS population with respect to glyphosate applied individually, and slight non-significant increases in the GR population were observed (Fig. 5B).

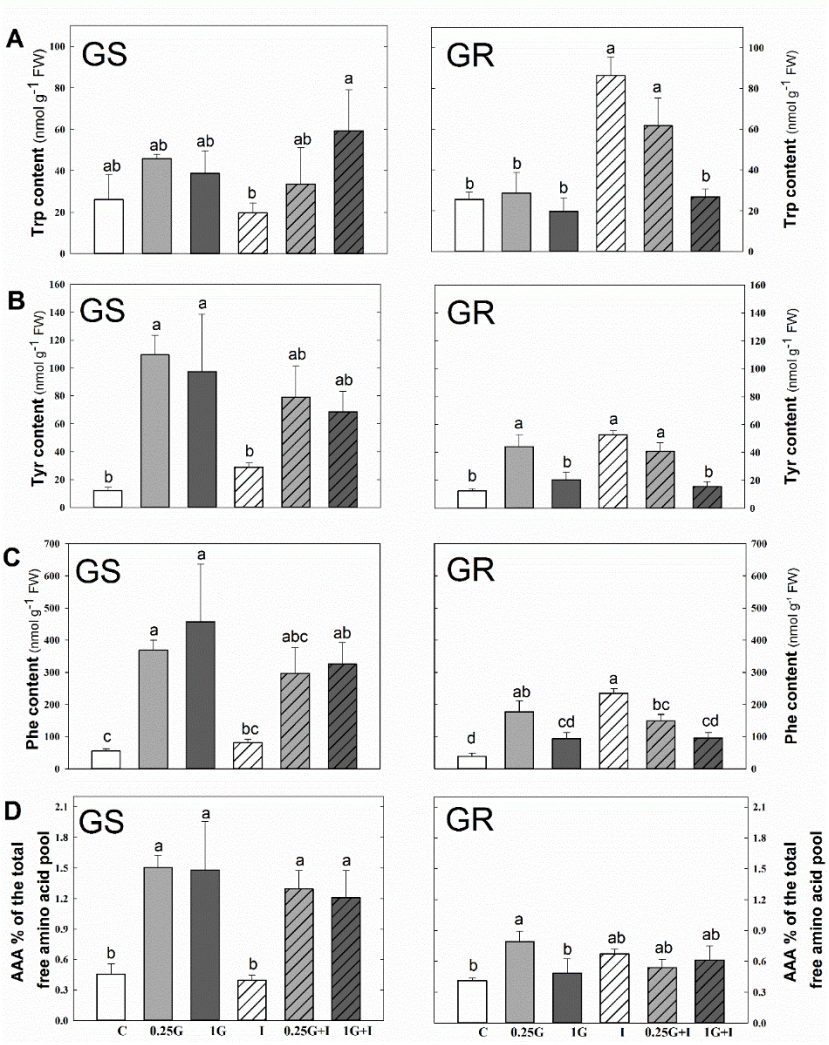


Figure 3.6 Aromatic amino acid content. Content of A) tryptophan, B) tyrosine, C) phenylalanine, and D) aromatic amino acid percentage of the total free amino acid pool, in the leaves of glyphosate sensitive (GS) and resistant (GR) *Amaranthus palmeri* plants 3 days after herbicide treatment measured by capillary electrophoresis in leaf acidic extracts. Untreated plants were sprayed with water (Control; C). Plants were treated with 0.21 kg ha^{-1} of glyphosate (0.25G), 0.84 kg ha^{-1} of glyphosate (1G), 1.5 mg L^{-1} of imazamox (I) or their mixtures (0.25G+I and 1G+I). (Mean ± SE; n=4). Different letters indicate significant differences between treatments (p-value ≤0.05, LSD test).

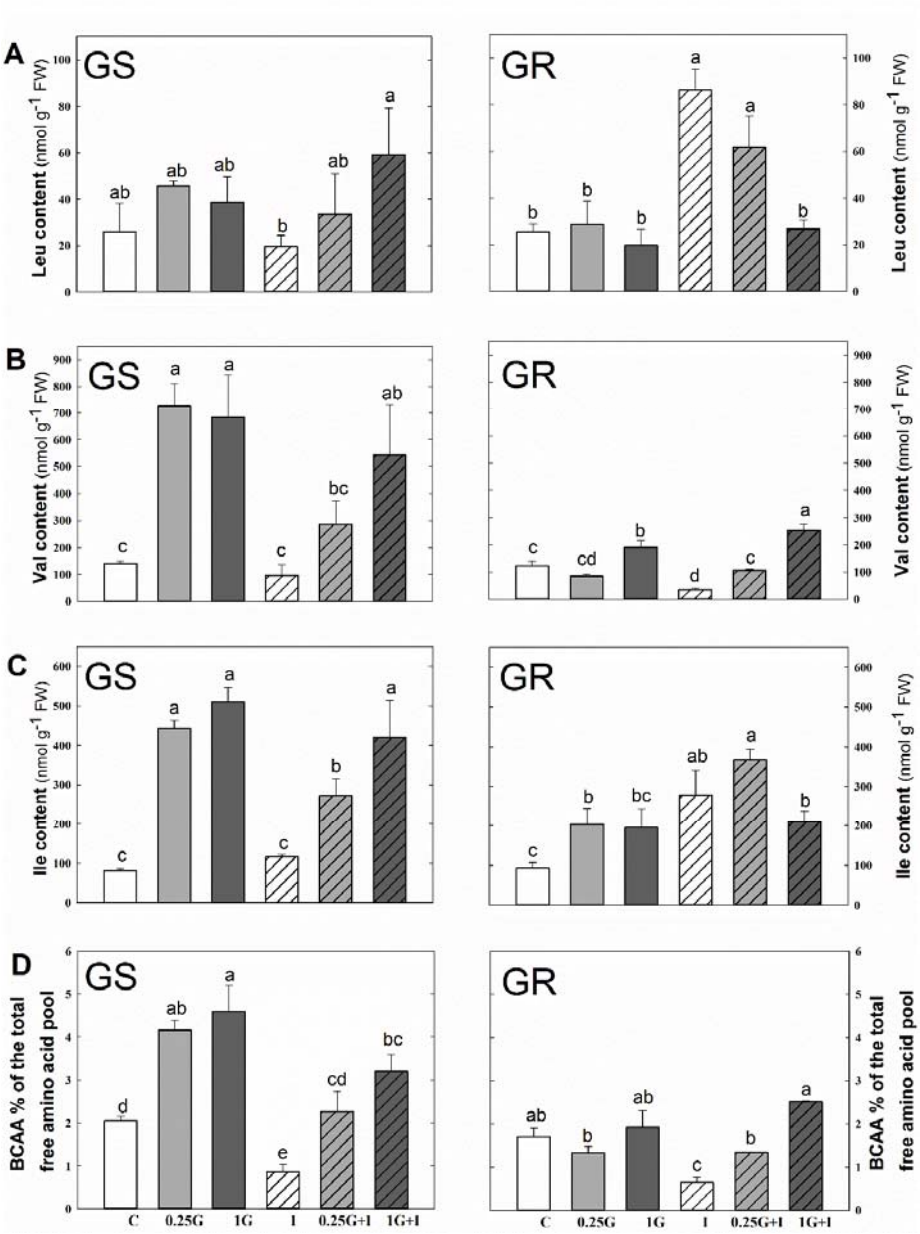


Figure 3.7 Branched chain amino acid content. Content of A) leucine (Leu), B) valine (Val), C) phenylalanine (Ile), and D) branched chain amino acid percentage of the total free amino acid pool, in the leaves of glyphosate sensitive (GS) and resistant (GR) *Amaranthus palmeri* plants 3 days after herbicide treatment measured by capillary electrophoresis in leaf acidic extracts. Untreated plants were sprayed with water (Control; C). Plants were treated with 0.21 kg ha^{-1} of glyphosate (0.25G), 0.84 kg ha^{-1} of glyphosate (1G), 1.5 mg L^{-1} of imazamox (I) or their mixtures (0.25G+I and 1G+I). (Mean ± SE; n=4). Different letters indicate significant differences between treatments (p-value ≤0.05, LSD test).

The free amino acid profiles of GS and GR plants were also evaluated. The AAA content in GS population increased significantly with both doses of glyphosate compared to the control, as shown in the Tyr and Phe contents (Fig. 6A-C). Although the content of Tyr and Phe increased after 0.25G treatment in GR, the effect of glyphosate on this population was milder than its effect on the GS population. We only detected a significant increase in the contents of Trp, Tyr and Phe after imazamox treatment in the GR population but not in the GS (Fig. 6A-C). The general increase in the content of free amino acids could mask the specific changes in each absolute value of AAA and BCAA (Fernández-Escalada et al. 2016); therefore, the relative contents of AAA and BCAA are represented in terms of percentage of total free amino acid (Figs 6 D and 7D) in response to all treatments. In the GS population, the relative AAA content (Fig. 6D) showed the same pattern as that of each amino acid, while in GR plants, the increase in each individual AAA was not observed when viewed in terms of relative AAA content. Mixtures had a similar or less effect on the content of the three AAA than glyphosate or imazamox applied individually (Fig. 6A-C).

The specific content of the BCAAs was also evaluated. In the GS population, there was a significant increase (in terms of both absolute and relative contents) of BCAAs after both doses of glyphosate compared to control, which was not observed in the GR population (Fig. 7A-C). Imazamox did not affect the BCAA content in the GS population while in the GR, Leu and Ile content increases were detected (Fig. 7A-C). A decrease in the relative content of BCAA (as a percentage of the total free amino acid content) after imazamox treatment was detected in both populations (Fig. 7D). In the GS population, there was a general antagonistic behavior with the exception of the mixture of 1G+I on Leu, where an additive behavior can be seen.

In the GS population, both glyphosate doses produced a significant increase in total free amino acid content with respect to control (Fig. 8A), while the free amino acid accumulation detected in the GR population after any of the glyphosate doses was much lower. Imazamox applied individually induced free amino acid accumulation in both populations. In the GS population, the level of free amino acid accumulation was the same for the mixtures and for the

individual herbicide applications, while in the GR population, the level was lower for the mixtures. Acidic and amide amino acids displayed similar behavior in both populations and after both herbicides were applied individually, with a general and significant decrease in acidic amino acids and significant increases in amide amino acids (Fig. 8B-C). In all cases, the behavior was antagonistic.

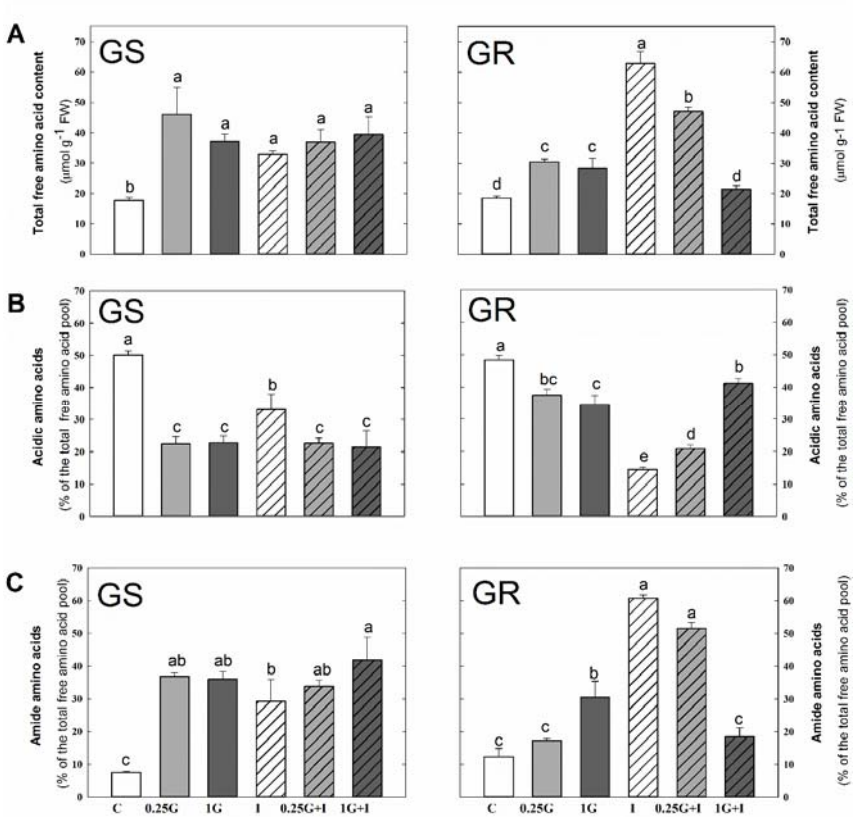


Figure 3.8 Total free, acidic and amide amino acid content. A) total free amino acid content, B) acidic amino acids (glutamic acid (Glu) + aspartate (Asp)) as percentage of the total free amino acid pool and C) amide amino acids (glutamine (Gln) + asparagine (Asn)) as percentage of the total free amino acid pool, in the leaves of glyphosate sensitive (GS) and resistant (GR) *Amaranthus palmeri* plants 3 days after herbicide treatment measured by capillary electrophoresis in leaf acidic extracts Untreated plants were sprayed with water (Control; C). Plants were treated with 0.21 kg ha^{-1} of glyphosate (0.25G), 0.84 kg ha^{-1} of glyphosate (1G), 1.5 mg L^{-1} of imazamox (I) or their mixtures (0.25G+I and 1G+I). (Mean ± SE; n=4). Different letters indicate significant differences between treatments (p-value ≤0.05, LSD test).

Total soluble sugars (the sum of glucose, fructose, and sucrose) and starch contents were measured in the leaves of both populations. We observed a general increase in TSS and starch content in the GS and GR populations after the application of both herbicides and there were no significant differences

between glyphosate and imazamox applied individually or as mixtures (Fig. 9A-B).

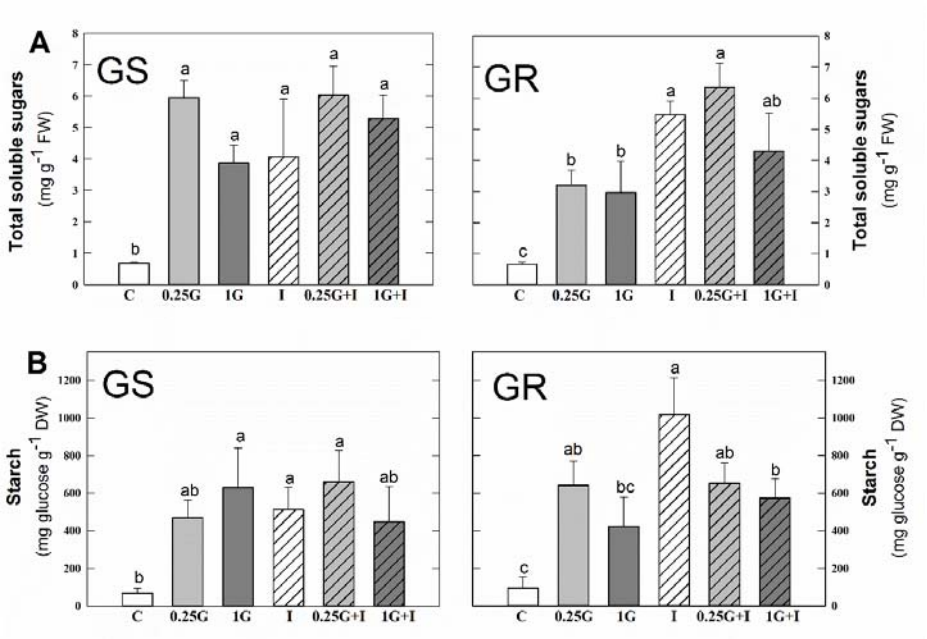


Figure 3.9 Carbohydrate content. A) total soluble sugar (fructose, glucose and sucrose) content in glyphosate sensitive (GS) and resistant (GR) populations of *Amaranthus palmeri* and B) starch content in the leaves of C) glyphosate sensitive (GS) and resistant (GR) populations of *Amaranthus palmeri* plants 3 days after herbicide treatment. Untreated plants were sprayed with water (Control; C). Plants were treated with 0.21 kg ha^{-1} of glyphosate (0.25G), 0.84 kg ha^{-1} of glyphosate (1G), 1.5 mg L^{-1} of imazamox (I) or their mixtures (0.25G+I and 1G+I). (Mean ± SE; n=4). Different letters indicate significant differences between treatments (p-value ≤0.05, LSD test).

3.4 DISCUSSION

3.4.1 Physiological effects of treatments in *A. palmeri* plants

Shikimate content is a well-known stress marker: it accumulates when the EPSPS enzyme is inhibited by glyphosate (Baerson et al. 2002a; Zhu et al. 2008; Orcaray et al. 2010; Gaines et al. 2011; Whitaker et al. 2013; Lorentz et al. 2014; Doğramacı et al. 2015; Fernández-Escalada et al. 2016, 2017; Dillon et al. 2017). We observed that it accumulated more in sensitive than in resistant plants (Fig. 1). While imazamox applied individually did not affect shikimate content, its mixture with 0.25G alleviated the shikimate accumulation induced by glyphosate applied individually, suggesting an antagonistic effect in the mixture.

An increase in the protein turnover rate after herbicide treatment (Rhodes et al. 1987) was proposed to explain the increase in the size of the free amino acid pool and the general decrease in total soluble proteins induced by amino acid biosynthesis-inhibiting herbicides. Isotopic studies in *A. palmeri* revealed that both *de novo* synthesis of amino acids and protein turnover contribute to AAA accumulation in response to glyphosate (Maroli et al. 2016).

Total free amino acid content was determined as a physiological marker of the effect of the herbicides (Fig. 8A). In the GS population, both glyphosate doses produced a significant increase in total free AA content with respect to control, as it has been reported before (Moldes et al. 2008; Orcaray et al. 2010, 2012; Vivancos et al. 2011; Zulet et al. 2013a, 2015; Liu et al. 2015; Fernández-Escalada et al. 2016). Due to the resistance to glyphosate, a smaller amount of free amino acids accumulated in the GR population after glyphosate treatment. Imazamox applied individually induced free amino acid accumulation in both populations, as has been previously reported (Zulet et al. 2013a). In the GS population, the amounts of free amino acid accumulation were the same after the application of the mixtures and after individual herbicide application; in the GR population, the mixtures yielded lower amounts of free amino acids, evidencing again an antagonistic behavior.

The contents of two other groups of amino acids with known behaviors (acidic amino acids (Glu and Asp) and amide amino acids (Gln and Asn)) (Orcaray et al. 2010; Zulet et al. 2015), was also determined after treatment

with amino acid biosynthesis inhibitors (Fig. 8B-C). The general and significant decreases in acidic amino acid contents and the significant increases in amide amino acid contents detected in both populations had been previously reported (Orcaray et al. 2010; Zulet et al. 2015). In both groups of amino acids in the GS and GR populations, there was a general antagonistic behavior in the mixtures.

AAA accumulation after glyphosate treatment was reported as a physiological marker of damage (Orcaray et al. 2010; Zulet et al. 2015; Fernández-Escalada et al. 2016, 2017), thus the higher increase of AAA in the GS population compared with the GR population (Fig. 6A-C) suggests a higher level of damage on the GS due to its higher sensitivity to the herbicide. A general increase of the AAA content after AHAS inhibitors has also been previously described (Orcaray et al. 2010; Zabalza et al. 2013) but in our study, that significant increase was detected in the GR population and not in the GS (Fig. 6A-C). As AAA accumulation after glyphosate treatment implies higher damage, the maintenance of AAA levels with glyphosate and mixtures indicates an antagonistic behavior. On the other hand, the dose of glyphosate treatment was too low to see a significant increase in Leu, Val and Ile (BCAA) contents in the GR population, but in the GS population, there was a significant increase in the BCAA level with both doses of glyphosate compared to control (Fig. 7A-C), which is consistent with previous reports (Orcaray et al. 2010; Zabalza et al. 2013) and with the AAA results (Fig. 6A-C). Interestingly, it was possible to detect a decrease in the relative content of BCAA after imazamox treatment in both populations (Fig. 7D). Previous studies reported transient decreases in the proportion of amino acids whose pathways were specifically inhibited by AHAS-inhibitors (Trenkamp et al. 2009; Orcaray et al. 2010).

Carbohydrates accumulate in response to the application of glyphosate (Orcaray et al. 2012; Maroli et al. 2015; Zulet et al. 2015; Fernández-Escalada et al. 2016) and AHAS-inhibitors (Zabalza et al. 2004, 2017; Zulet et al. 2015), which can be a physiological marker of herbicide toxicity. Total soluble sugars and starch contents were measured in the leaves of both populations (Fig. 9A-B). The carbohydrate accumulation detected in leaves after herbicide treatment (Fig. 9A-B) has been previously attributed to growth arrest. The accumulation of unused carbohydrates in sinks abolishes the sugar gradient required for long-distance transport, and carbohydrates accumulate in the leaves of treated

plants because of a decrease in sink strength (Orcaray et al. 2012). If carbohydrates are used as a physiological marker of stress, then the absence of differences between mixtures and individual herbicides could be interpreted as an antagonistic interaction.

In general, the changes detected in amino acid and carbohydrate contents in response to herbicide mixtures were similar to the changes detected after individual treatments and less than the sum of the individual effects; thus, the mixtures display a general antagonistic behavior.

3.4.2 Coordinated expression response was only detected after EPSPS inhibition and not after AHAS inhibition

A general dose-dependent induction in the expression of all the genes of the AAA pathway (Fig. 2 and 3B), with the exception of *CM* (Fig. 3A), was observed in the GS and GR populations and was caused by glyphosate treatment, as described in Fernández Escalada et al. (2017). A higher degree of induction occurred in the *AS* gene (Fig. 3B), and *CM* repression in the post-chorismate branches of the AAA pathway (Fig. 3A) indicate a priority flux of carbon towards Trp biosynthesis instead of Phe and Tyr under herbicide stress conditions (Fernández-Escalada et al. 2017). The transcriptional responses of the AAA pathway (specifically in *AS*) to a wide range of stress conditions have been proposed (Less and Galili 2008; Pratelli and Pilot 2014).

The expression pattern of the genes of the BCAA pathway was also evaluated (Fig. 4). With few exceptions, both of the populations generally showed no significant change in gene expression after glyphosate treatment, as previously reported (Fernández-Escalada et al. 2017). No significant changes in gene expression were detected after the inhibition of one of the enzymes of the pathway, AHAS, by imazamox applied individually. It was previously reported that the BCAA enzyme transcript levels increased after treatment with pyroxsulam (Délye et al. 2015) (another AHAS inhibitor) within a short period of time (less than 48 h). However, in agreement with our results, the slight change of BCAA enzyme gene expression level induced by another AHAS-inhibitor (imazapyr) provided evidence that transcriptional regulation may not be a major regulatory mechanism of the synthesis of branched-chain

amino acids (Manabe et al. 2007). Herbicide mixtures did not induce any remarkable change in the relative expression of the enzymes in this pathway (Fig. 4).

A similar regulatory program, combining transcriptional and post-translational controls in response to abiotic stresses, was proposed to the metabolic pathways of 11 amino acids, including AAA and BCAA biosynthesis (Less and Galili 2008). Specifically, it was proposed that allosteric biosynthetic enzymes respond post-translationally to changes in the level of the amino acids (Less and Galili 2008). Nevertheless, our results show an opposite transcriptional behavior between the BCAA and AAA pathways. The inactivation of the BCAA biosynthetic pathway at the level of AHAS by imazamox did not provoke any significant or common pattern in the relative expression level of the other genes. In contrast, after glyphosate treatment, a general increase in the transcript levels of the enzymes of the shikimate pathway and *AS* was detected in both populations. This different pattern cannot be explained by any specific pattern in the content of amino acids whose specific biosynthesis is inhibited because decreases, increases and no changes have all been reported.

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, supporting the lack of cross regulation between the pathways. The lack of effect of imazamox on the transcript levels of the genes of the AAA pathway confirms this result.

To evaluate if the changes in gene transcription of the enzymes of the AAA pathway (Fig. 3) were reflected by the protein levels, EPSPS and DAHPS enzyme amounts were studied. The importance of the DAHPS protein is based on its control of the entrance of carbon flux to AAA pathway (Tohge et al. 2013b). In the GS population, only in the 1G+I treatment was a significant increase of the protein detected with respect to the control and in the resistant population; there were no significant differences between treatments (Fig. 5A), as has been reported previously (Fernández-Escalada et al. 2017). The EPSPS protein content in the absence of herbicides was 10-fold higher in *A. palmeri* GR population than in GS (Fig. 5B) due to the amplification of *EPSPS*, as has been previously reported (Gaines et al. 2010, 2011; Ribeiro et al. 2014;

Fernández-Escalada et al. 2016, 2017). There were significant increases in the EPSPS protein levels in the GS population after glyphosate treatment and with 1G glyphosate in the GR population (Fig. 5B), as previously reported (Fernández-Escalada et al. 2016, 2017). The significant increases in EPSPS protein after glyphosate treatment suggest that this protein is under strong transcriptional control with a tight concordance between the increases in gene expression and in the protein content. In contrast, the induction of DAHPS gene expression after glyphosate treatment (Fig. 2A) had no clear relation with DAHPS protein level (Fig. 5A), which suggests that the presence of post-transcriptional factors added to the transcriptional regulation, as has been suggested before (Fernández-Escalada et al. 2017).

Both populations showed no effect in response to imazamox applied individually and no significant differences between glyphosate treatments and their mixtures with imazamox in DAHPS and EPSPS content (Fig. 5A), which suggest the absence of a relevant effect of imazamox on this parameter, while glyphosate elicited an effect.

3.4.3 In both populations, antagonism was the main type of interaction between glyphosate and imazamox when physiological effects were evaluated

We used physiological parameters as indicators of additivity, synergism and antagonism in the mixtures of glyphosate and AHAS-inhibitors. The mixture of glyphosate and imazamox resulted in a generalized antagonistic effect on the main indicator parameters: shikimate content (Fig. 1), amino acid levels (Figs. 6-8), and carbohydrate levels (Fig. 9). This conclusion is based on finding the mixture effects to be mostly less than the sum of the individual effects. Although some additive results have been reported before (Starke and Oliver 1998; Li et al. 2002; Nelson and Renner 2002), antagonism between AHAS-inhibitors (specifically imidazolinones) and glyphosate has been previously reported in dose-response studies (Hydrick and Shaw 1994; VanLieshout et al. 1996; Lich et al. 1997; Johnson, W.G. et al. 1999; Shaw and Arnold 2002), although their effect in physiological parameters was not tested.

Antagonism was the general behavior detected in all parameters and in both populations. Nevertheless, at the transcriptional level, in AAA pathway enzymes, we observed a clearly different pattern in the GS population compared to the GR population when they were treated with mixtures. The GS population showed an additively slight synergic effect and the GR population showed a strong antagonism that even brought down transcript levels below control levels in the mixtures. This particular transcriptional pattern detected in GR plants could be related to *EPSPS* gene amplification and overexpression. Further investigations to clarify the molecular mechanism underlying this specific transcriptional response of GR would be very useful.

Considering the general pattern, it can be concluded that glyphosate and imazamox applied together are physiologically antagonistic; the evidence for this is that toxicity markers are affected in the mixture to the same extent or even less than with one herbicide acting individually. This behavior means that when the herbicides are applied in the field the recommended doses cannot be lowered and that both herbicides have to be applied at the recommended rate to be effective on all desired target plants.

3.5 CONCLUSIONS

The study of physiological effects as parameters to evaluate the interaction between glyphosate and imazamox when mixed together shows a general physiological antagonism. This type of interaction was detected in shikimate, protein, amino acid and carbohydrate content, and it was independent of the EPSPS copy number, as it was detected in both populations. In the case of the transcriptional activation of the AAA pathway induced by glyphosate, interesting and contrary interactions were detected for both populations; the effect was additive in the GS population and antagonistic in the GR population.

We confirmed that no cross regulation exists between AAA and BCAA pathways, in spite of similar patterns in the content of free amino acids and carbohydrates after imazamox or glyphosate treatment.

GENERAL DISCUSSION

Glyphosate inhibits the biosynthesis of AAAs by the specific inhibition of EPSPS, an enzyme that converts shikimate-3-phosphate and phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate in plastids. The toxic effect of this herbicide goes beyond the interaction with the target site and provokes a physiological roadblock that leads to plant death. The precise physiological processes by which glyphosate kills plants have not been completely elucidated. The main aim of this study was to continue unravelling the precise physiological mechanisms that are related to glyphosate toxicity.

The intensive use of glyphosate has resulted in the evolution of resistance to this herbicide. *Amaranthus palmeri* is a troublesome weed that has become a major glyphosate-resistant weed and whose mechanism of resistance is EPSPS gene amplification, as first reported by Gaines et al. (2010). The availability of a biotype with overexpression of the EPSPS enzyme provided an opportunity to analyse how overexpression of EPSPS affects AAA synthesis and other physiological effects by comparison with a sensitive population. In addition, comparison of the different effects of glyphosate or glyphosate mixed with other herbicides on both populations will facilitate the comprehensive elucidation of a possible different mode of action in sensitive and resistant plants. In this study, two types of comparisons have been used, one taking into account comparison between untreated plants of sensitive or resistant populations and another focusing on the variation of different parameters in the plant after glyphosate treatment in each population. This structure was maintained throughout the three chapters of this work, although in the last chapter imazamox and its mixtures with glyphosate were included as well.

In the **first chapter**, it was confirmed that GR population originally collected in North Carolina was resistant to glyphosate and *EPSPS* gene amplification as mechanism of resistance as described in Chandi et al. (2012) for that population was confirmed.

In vivo shikimate assay (Fig. 1.2) and glyphosate dose-response study confirmed the resistance of GR population (Fig. 1.1A). Accumulation of shikimate content (Fig. 1.1B) was the parameter used to establish the doses employed in the following physiological characterizations: 0.84 and 2.52 kg ha $^{-1}$. The 0.84 kg ha^{-1} dose was selected because at this dose the highest

shikimate accumulation in GS was observed, and thus the physiological status is expected to be strongly affected. The 2.52 kg ha^{-1} dose was selected because it was the highest dose used and did not result in shikimate accumulation in GR plants.

It was confirmed that the glyphosate resistance level was related to higher *EPSPS* genomic copy number (Fig. 1.3A), as the *EPSPS* copy number of GR plants was 47.5 fold the copy number of GS plants, as was reported before for other populations or species (Gaines et al. 2010; Ribeiro et al. 2014; Vila-Aiub et al. 2014; Salas et al. 2015), moreover, it was confirmed that the levels of EPSPS protein (Fig. 1.3B) and EPSPS activity (Fig. 1.3C) were higher in GR than in GS individuals as in previous reports (Gaines et al. 2010, 2011; Ribeiro et al. 2014). Comparing GS and GR plants under control conditions, amino acid content was similar in the two populations (Fig. 1.4A,C), thus, the levels of free amino acid contents were not affected by the overexpression of EPSPS supporting the idea that no fitness costs were associated with EPSPS overexpression (Vila-Aiub et al. 2014).

Protein levels were higher in glyphosate treated plant than in untreated plants in both populations. EPSPS activity only increased after glyphosate treatment in GR population, which was probably a reflection of the less EPSPS transcript levels of GS population that prevented from increasing the EPSPS protein content. The relative low levels of protein were not enough for GS individuals to overcome the glyphosate treatment effects when was applied, while in GR it was possible, which is the cause of resistance in this *A. palmeri* population (Gaines et al. 2010).

In addition, other physiological responses that are usually affected by glyphosate treatment, such as free amino acid content, carbohydrate content and ethanol fermentation, could be different between populations and could be affected in a different way by the herbicide in sensitive and resistant plants, so they were studied to describe new insights of the physiological manifestations of the evolved glyphosate resistance.

Free amino acid levels were increased by glyphosate treatment in the leaves and roots of GS and GR plants in a dose-dependent manner (Fig. 1.4B and D) althought the detected increase in leaves was much lower in GR. Total free amino acid accumulation after glyphosate treatment was reported

previously in several species, including pea (Orcaray et al. 2010; Zulet et al. 2013a), *Arabidopsis thaliana* (Zulet et al. 2015), maize (Liu et al. 2015), and soybean (Moldes et al. 2008; Vivancos et al. 2011).

AAA content was greatly increased in the leaves and roots of GS plants after glyphosate treatment, as previously described (Orcaray et al. 2010; Zulet et al. 2015), but in GR plants, only a mild increase was detected in leaves. (Fig. 1.5).

Sugar accumulation was clearly produced in GS and GR population with recommended field dose as previously reported (Orcaray et al. 2012; Zulet et al. 2015; Maroli et al. 2015), although in sensitive plants it was a tendency to decline with the highest glyphosate dose. It was propossed that the severity of this treatment on sensitive plants makes it difficult to maintain carbohydrate accumulation, corresponding to a possible decline in carbon assimilation (Zabalza et al. 2004). Ethanol fermentation was assessed in roots of both populations (Fig. 1.7). In all cases there were an induction of activity PDC and ADH with the lowest dose of glyphosate individuals it was a tendency to decline with the highest glyphosate dose. So, these two physiological markers that have been reported after glyphosate treatment were detected in sensitive and resistant plants in this study.

The first chapter showed a complex regulation of EPSPS activity by mechanisms at transcriptional/translational and protein levels as EPSPS protein amount was incrased in both populations by the herbicide while AAA content was incresed only in GS population. These results indicate a regulation at the level of EPSPS protein, the signal of which remains unknown. In order to obtain new insights in the specific effects of glyphosate on AAA biosyntetic pathway a full transcriptional study of the pathway was performed, as it is presented in chapter two.

In the **second chapter**, the main objective was to evaluate the impact of EPSPS overexpression and of glyphosate on the regulation of the AAA pathway and free AAA content. Protein and activity of some important enzymes of AAA pathway were measured. These enzymes are the key points of the pathway, the entrance (DAHPS enzyme) and the enzyme inhibited by glyphosate.

In GR population of *A. palmeri* an *EPSPS* gene amplification described in chapter 1, resulted in a massive increase of the corresponding transcript (Fig. 2.3A) and of the protein content and activity (Figs. 2B,C, GD.1). Our data validate results previously reported in other populations of *A. palmeri* (Gaines et al. 2010, 2011; Ribeiro et al. 2014), and other weedy plant species such as *A. tuberculatus* (Lorentz et al. 2014; Chatham et al. 2015), *Lolium perenne ssp*. m*ultiflorum* (Salas et al. 2012), *Eleusine indica* (Chen et al. 2015), and *Kochia scoparia* (Wiersma et al. 2015).

Gene amplification of *EPSPS* had no major effect on the overall AAA pathway (Figs. 2.2–2.5). In untreated plants, free AAA contents were similar in GR and GS populations (Fig. 2.5) as previously described (Maroli et al. 2015). This 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).

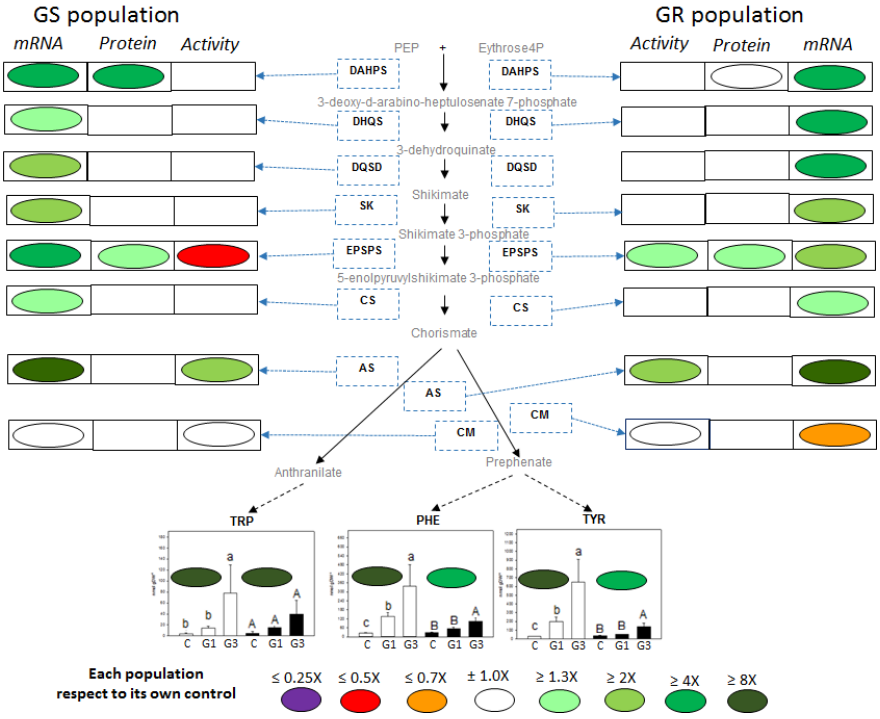


Figure GD.1 Overview of the effects of three times glyphosate field dose (2.52 kg ha^{-1}) on *m*RNA, protein, activity and aromatic amino acid content of the enzymes of aromatic amino acid synthesis pathway in sensitive population (GS) (left) and in resistant population (GR) (right). The color of the circles shows the value of the ratio of the parameter after referring glyphosate-treated value to control (untreated) values. Sensitive population is represented by white bars and resistant population with black bars in amino acid content grephics, and treatments are control (C), 0.84 kg ha^{-1} of glyphosate (G1) and 2.52 kg ha^{-1} of glyphosate (G3).

Glyphosate treatment provoked an accumulation of the transcripts encoding almost all the enzymes of the shikimate pathway, including *EPSPS*, in a dose-dependent manner (Fig. 2.3B). Although increases in some enzymes of the shikimate pathway such as EPSPS (Baerson et al. 2002a; Yuan et al. 2002; Chen et al. 2015; Mao et al. 2016) and DAHPS (Baerson et al. 2002a) have been previously described, this was the first study suggesting a potential coordinated transcriptional regulation of the shikimate pathway after glyphosate treatment (Fig. GD.1). Because this regulation is observed in both GS and GR populations (Fig. 2.3B and GD.1), it suggests that this gene upregulation does not occur in response to the level of inhibition of EPSPS activity. Instead, it can be hypothesized that glyphosate itself, or indirectly, may affect plant amino acid metabolism, in addition to its known impact on EPSPS.

Glyphosate provoked a general upregulation of the expression of genes participating in the pre chorismate pathway but downstream chorismate, AS increased in transcript abundance more than any other enzyme in the pathway (Fig. 2.3B and GD.1). AS expression was induced while CM expression was repressed, suggesting a regulatory branch point in the pathway (Fig. 2.1) for a preferential flux of carbon toward Trp biosynthesis over Phe and Tyr biosynthesis. This potential stream toward Trp was confirmed by studying AS and CM enzyme activities *in vitro* (Fig. 2.4). Data obtained in *Arabidopsis thaliana* (Sasaki-Sekimoto et al. 2005) and other plant species (Galili et al. 2016) also support this hypothesis.

The general upregulation of the expression of genes participating in the pre chorismate pathway is accompanied with an increase of the accumulation of free AAAs (Fig.GD.1), which is more pronounced in the GS population (Fig. 2.5) as it was already described in chapter 1 and reported in other studies (Vivancos et al. 2011; Maroli et al. 2015). It is possible that the accumulation of free AAA came from an increase in protein turnover in the plant following glyphosate treatment (Zabalza et al. 2006; Zulet et al. 2013a; Fernández-Escalada et al. 2016). Isotopic studies in *A. palmeri* revealed that both *de novo* synthesis of amino acids and protein turnover contribute to AAA accumulation in response to glyphosate (Maroli et al. 2016). Reduction in AAA levels does not appear to elicit the increased expression of AAA pathway genes (Fig. 2.3), because the AAA concentrations increase with glyphosate dose (Fig. 2.5).

Further research is needed to understand the signal(s) that upregulates the AAA pathway following glyphosate treatment.

Previous researches showed that an interaction between the AAA pathway and other amino acid synthesis pathways was probable (Noctor et al. 2002), and the nearest pathway could be the BCAA pathway (Fernández-Escalada et al. 2016; Maroli et al. 2015; Orcaray et al. 2010). The effect of glyphosate on the transcript levels of the genes of the BCAA pathway was evaluated in both populations (Fig. 3.4 and GD1). Transcript abundance was similar in the untreated plants of both populations, suggesting that EPSPS overexpression does not affect BCAA pathway expression. Moreover glyphosate did not affect BCAA pathway expression (Fig. 3.4) no-cross regulation at transcriptional level was observed.

AHAS inhibitors are herbicides that inhibit the BCAA biosynthesis pathway. Previous findings showed that both AHAS and EPSPS inhibitors provoke similar physiological effects on treated plants: an accumulation of free amino acids (Orcaray et al. 2010; Maroli et al. 2015; Zulet et al. 2015; Fernández-Escalada et al. 2016; Zabalza et al. 2017), a decrease in the soluble protein content (Zulet et al. 2013a; Maroli et al. 2015, 2016), and accumulation of carbohydrates (Orcaray et al. 2010; Maroli et al. 2015; Zulet et al. 2015; Fernández-Escalada et al. 2016). This suggests that, although they target different enzymes, they kill plants by similar mechanisms. Beside this, one of the most used practice to control glyphosate-resistant weeds is to mix glyphosate with AHAS-inhibitors. The close relationship between AAA and BCAA biosynthetic pathways and the common physiological effects provoked by EPSPS and AHAS-inhibitors may cause noteworthy synergistic effects by the joint application of glyphosate and AHAS-inhibitors, which makes interesting the physiological study of the herbicide mixture in plants, although at the same time, the similar response of plants to both families of herbicides could question the efficacy of these mixtures.

In the **third chapter**, the physiological and transcriptional effects of herbicide mixtures of glyphosate and imazamox in *A. palmeri* resistant and sensitive plants were studied. The AHAS inhibitor imazamox was selected for investigation. The plants were treated with two doses of glyphosate applied

individually; imazamox and two mixtures of both herbicides with the same doses as the individually applied herbicide were used.

In both the GS and GR populations, a general dose-dependent induction of all the genes of the AAA pathway (Figs. 3.2, 3.3B and GD. 2), with the exception of *CM* (Figs. 3A and GD. 2), was caused by glyphosate treatment, as described in the experiments of chapter 2 (Fig. 2.3B and GD.1) and published in Fernández-Escalada et al. (2017). A higher induction occurred for the *AS* gene (Figs. 3.3B and GD. 2), and a *CM* repression in the post-chorismate pathway of the AAA pathway (Figs. 3.3A and GD. 2) indicated a priority flux of carbon towards the branch of Trp biosynthesis instead of Phe and Tyr under herbicide stress conditions (Fernández-Escalada et al. 2017).

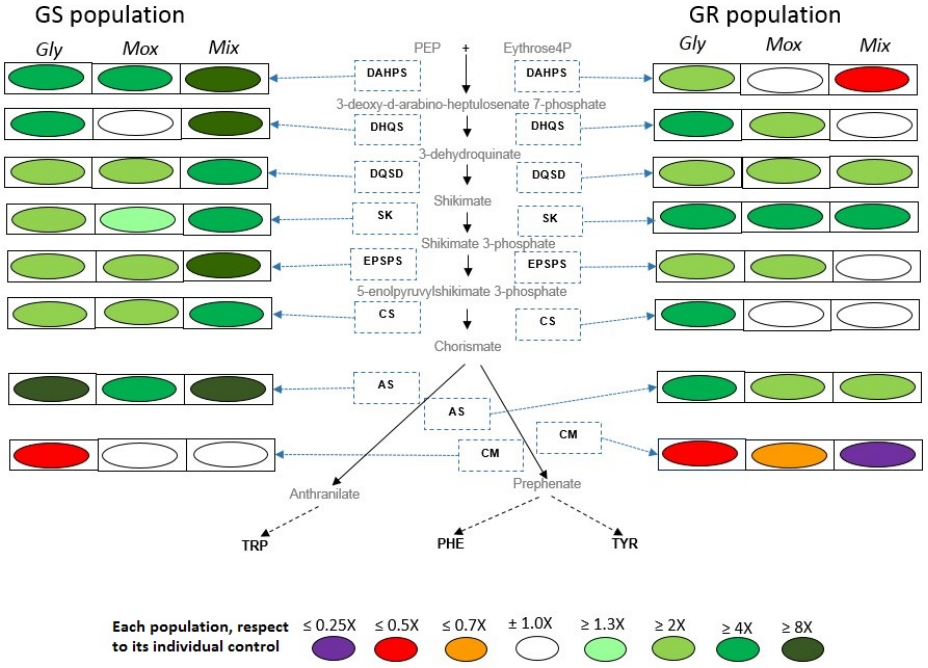


Figure GD.2 Overview of the effects of glyphosate field dose (0.84 kg ha^{-1}), imazamox and a mixture with glyphosate and imazamox doses, on *m*RNA in sensitive population (GS) (left) and in resistant population (GR) (right). The color of the circles shows the value of the the ratio of the parameter after referring glyphosate-treated value to control (untreated) values.

Imazamox applied individually did not induce a general significant change in the AAA pathway expression (Figs. 3.3 and GD. 2). At the transcriptional level in the AAA pathway enzymes, a clear differential pattern in the GS population compared to the GR population was observed when they were treated with mixtures (Fig. GD.2). The GS population showed a slight

additive synergic effect, and the GR population showed a strong antagonism that reduced transcript levels to below those of the control in the mixtures. The particular transcriptional pattern detected in GR plants could be related to *EPSPS* gene amplification and overexpression. Further investigation to clarify the molecular mechanism underlying this specific transcriptional response of GR would be useful.

The expression pattern was also evaluated in genes of the BCAA pathway (Figs. 3.4). With few exceptions, generally, neither of the populations exhibited a significant gene expression change after glyphosate treatment, as was previously reported (Fernández-Escalada et al. 2017). After imazamox treatment, the slight change of BCAA enzyme gene expression showed that transcriptional regulation may not be a major regulatory mechanism in the synthesis of BCAAs (Manabe et al. 2007). As shown in the experiments of chapter 2 (Fig. 2.6) and published in Fernández-Escalada et al. (2017), glyphosate did not provoke any significant change in the transcriptional levels of genes in the BCAA pathway, supporting a lack of cross-regulation between the pathways. The lack of effect of imazamox on gene transcripts in the AAA pathway (Figs. 3.3 and GD. 2) confirmed this result.

Glyphosate applied individually induced the previously described effects, including accumulation of shikimate, AAAs, total free amino acid content and carbohydrates (Fig. 3.1, 3.6, 3.7, 3.8 and 3.9), as was described in the other chapters (Fig. 1.1, 1.4, 1.5, 1.6, 2.2 and 2.5).

Similar physiological responses were detected when imazamox was applied individually as were seen when glyphosate was applied, with the exception of shikimate accumulation. Specific AAA and total free amino acid content increased (Fig. 3.6 and 3.8), and carbohydrates accumulated in the leaves of treated plants (Fig. 3.9), as was described previously for other species (Zabalza et al. 2004; Zulet et al. 2013, 2015).

In the last chapter, physiological parameters were used as indicators of additivity, synergism or antagonism in the mixtures of glyphosate and AHAS inhibitors. The mixture of glyphosate and imazamox resulted in a general antagonistic effect in the main indicator parameters (shikimate content (Fig. 3.1), amino acid levels (Figures 3.6-3.8), and carbohydrate levels (Fig. 3.9)) because the detected effects upon application of the mixtures were mostly

lower than the addition of the individual effects. Although some additive results have been previously reported (Starke and Oliver 1998; Li et al. 2002; Nelson and Renner 2002), antagonism between AHAS inhibitors (imidazolinones) and glyphosate has been previously reported in dose-response studies, (Hydrick and Shaw 1994; VanLieshout et al. 1996; Lich et al. 1997; Johnson, W.G. et al. 1999; Shaw and Arnold 2002), although their effect on physiological parameters had not been tested. Considering this general pattern, it can be established that when the herbicides are applied in the field, the recommended doses cannot be lowered and that both herbicides must be applied at the recommended rate to be effective on all desired target plants.

In this study, the high CNV of EPSPS in *A. palmeri* has no major pleiotropic effect on the expression of the AAA and BCAA biosynthetic genes, the free amino acid profile or the carbohydrate content. Moreover, new insights of the physiological response of *A. palmeri* to glyphosate treatment in GS and GR populations are presented. Glyphosate provoked similar increases of EPSPS protein content and of transcripts corresponding to genes in the AAA pathway in both GS and GR, the signal of which remains unknown but cannot be AAA content, as this was increased only in GS. The expression of BCAA genes was not significantly affected by glyphosate, and expression of AAA genes was not significantly affected by imazamox, evidencing that no cross-regulation exists between the AAA and BCAA pathways. Finally, interactions between mixtures of two different doses of glyphosate and one of imazamox were tested in *A. palmeri*, and a general antagonism was reported.

CONCLUSIONS

- A correlation was confirmed between EPSPS CNV, EPSPS protein content and EPSPS activity with the level of resistance of *A. palmeri* GR plants.
- *A. palmeri* GR and GS untreated plants showed a similar expression of AAA and BCAA biosynthetic genes. Likewise, the free amino acid profile and carbohydrate content were similar in GS and GR plants, suggesting that the high CNV of EPSPS in the GR population has no major pleiotropic effects on the physiology of the resistant plants.
- Glyphosate provoked an increase in total free amino acids and in AAAs that was lower in the GR population than in the GS population, and shikimate accumulation was only observed in the GS population, which is related to the higher level of damage seen in the GS population.
- Transcriptional induction of the AAA pathway was detected following glyphosate treatment in both GR and GS plants, suggesting a potentially coordinated transcriptional regulation. The AAA content was not the signal causing this response, because AAA accumulation was detected only in GS plants.
- In both populations, glyphosate treatment resulted in an upregulation of the Trp biosynthesis branch compared to the Phe and Tyr branch, indicating that this branch point may be a regulatory point in the pathway.
- Despite similar patterns in the content of free amino acids and carbohydrates after imazamox or glyphosate treatments, it was confirmed that no cross-regulation exists between the AAA and BCAA pathways at the expression level.
- The study of physiological effects as parameters to evaluate the interaction between glyphosate and imazamox in their mixtures evidence a general physiological antagonism. This type of interaction was detected in the shikimate, protein, amino acid and carbohydrate content in GS and in GR populations of *A. palmeri*, suggesting that it is independent of the EPSPS copy number.

GENERAL BIBLIOGRAPHY

Ahsan N, Lee D-G, Lee K-W, Alam, I, Lee S-H, Bahk J-D, Lee B-H (2008) Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. *Plant Physiol Biochem* 46:1062–70.

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

Anderson DD, Nissen SJ, Martin AR, Roeth FW (1998) Mechanism of primisulfuron resistance in a shattercane (*Sorghum bicolor*) biotype. *Weed Sci* 46:158–162.

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* 926:319–325.

Armendáriz O, Gil-Monreal M, Zulet A, Zabalza A, Royuela M (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, Tran M, You J, Kreuger RW, Dill GM (2002a) Investigating the mechanism of glyphosate resistance in rigid ryegrass (*Lolium ridigum*). *Weed Sci* 50:721–730.

Baerson SR, Rodriguez DJ, Tran M, Feng Y, Biest NA, Dill GM (2002b) Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. *Plant Physiol* 129:1265–1275.

Barrett M (1993) Pesticide Interactions in Crop Productions, ed. *by* J. Altman, CRC Press, Boca Raton, pp.113–132 (1993).

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 USA* 95:5655–5660.

Benevenuto RF, Agapito-Tenfen SZ, Vilperte V, Wikmark OG, van Rensburg PJ, Nodari RO (2017) Molecular responses of genetically modified maize to abiotic stresses as determined through proteomic and metabolomic analyses. *Plos One* 12:1–23.

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

Binder S (2010) Branched-chain amino acid metabolism in *Arabidopsis thaliana*. *Arab B.* 8:1–14.

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

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

Bouny JM, Saglio PH (1996) Glycolytic flux and hexokinase activities in anoxic maize root tips acclimated by hypoxic pretreatment. *Plant Physiol* 111:187–194.

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.

Brazier-Hicks M, Knight KM, Sellars JD, Steel PG, Edwards R (2018) Testing a chemical series inspired by plant stress oxylipin signalling agents for herbicide safening activity. *Pest Manag Sci* 74:828-836.

Brunk DG, Rhodes D (1988) Amino acid metabolism of *Lemna minor L.1*. *Plant Physiol* 447–453.

Busi R, Vila-Aiub MM, Powles SB (2011) Genetic control of a cytochrome P450 metabolism-based herbicide resistance mechanism in *Lolium rigidum*. *Heredity (Edinb)* 106:817–824.

Chandi A, Milla-Lewis SR, Giacomini D, Westra P, Preston C, Jordan DL, York AC, Burton JD, Whitaker JR (2012) Inheritance of evolved glyphosate resistance in a North Carolina Palmer amaranth (*Amaranthus palmeri*) biotype. *Int J Agron* 2012:1–7.

Chatham LA, Wu C, Riggins CW, Hager AG, Young BJ, Roskamp GK,Tranel PJ (2015) EPSPS gene amplification is present in the majority of glyphosate-resistant Illinois waterhemp (*Amaranthus tuberculatus*) populations. *Weed Technol* 29:48–55.

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

Colombo SL, Andreo CS, Chollet R (1998) The interaction of shikimic acid and protein phosphorylation with pep carboxylase from the C4 dicot *Amaranthus viridis*. *Phytochemistry* 48:55–59.

Cromartie TH, Polge ND (2000) An improved assay for shikimic acid and its use as monitor for the activity of sulfosate. *Proc Weed Sci Soc Am* 4, 121.

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

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

Davis VM, Kruger GR, Stachler JM, Loux MM, Johnson WG (2009) Growth and seed production of horseweed (*Conyza canadensis*) populations resistant to glyphosate, ALS-Inhibiting, and multiple (glyphosate + ALS-inhibiting) herbicides. *Weed Sci* 57:494–504.

Dayan FE, Duke SO, Grossmann K (2010) Herbicides as Probes in Plant Biology. *Weed Sci* 58:340–350.

De Maria N, Becerril JM, Garcia-Plazaola JI, Hernández A, de Felipe MR, Fernández-Pascual M (2006) New insights on glyphosate mode of action in nodular metabolism: Role of shikimate accumulation. *J Agric Food Chem* 54:2621–2628.

Della-Cioppa G, Bauer SC, Klein BK, Shah DM, Fraley RT, Kishore GM (1986) Translocation of the precursor of 5-enolpyruvylshikimate-3-phosphate synthase into chloroplasts of higher plants *in vitro*. *Proc Natl Acad Sci USA* 83:6873–7.

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, Causse R, Michel S (2016) Genetic basis, evolutionary origin and spread of resistance to herbicides inhibiting acetolactate synthase in common groundsel (*Senecio vulgaris*). *Pest Manag Sci* 72:89–102.

Délye C, Duhoux A, Pernin F, Riggins CW, Tranel PJ (2015) Molecular mechanisms of herbicide resistance. *Weed Sci* 63:91–115.

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

Diebold R, Schuster J, Daschner K, Binder S (2002) The branched-chain amino acid transaminase gene family in Arabidopsis encodes plastid and mitochondrial proteins. *Plant Physiol* 129:540–550.

Dillon A, Varanasi VK, Danilova TV, Koo D-H, Nakka S, Peterson DE, Tranel PJ, Friebe B, Gill BS, Jugulam M (2017) Physical mapping of amplified copies of the 5-enolpyruvylshikimate-3-phosphate synthase gene in glyphosate-resistant *Amaranthus tuberculatus*. *Plant Physiol* 173:1226–1234.

Dinelli G, Marotti I, Bonetti A, Catizone P, Urbano JM, Barnes J (2008) Physiological and molecular basis of glyphosate resistance in *C. bonariensis* biotypes from Spain. *Weed Res* 48:257–265.

Doğramacı M, Foley ME, Horvath DP, Hernandez AG, Khetani RS, Fields CJ, Keating KM, Mikel MA; Anderson JV (2015) Glyphosate's impact on vegetative growth in leafy spurge identifies molecular processes and hormone cross-talk associated with increased branching. *BMC Genomics* 16:1–22.

Duggleby RG, McCourt JA, Guddat LW (2008) Structure and mechanism of inhibition of plant acetohydroxyacid synthase. *Plant Physiol Biochem* 46:309–324.

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

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

Dumas R, Cornillonbertrand C, Guiguetalet P, Genix P, Douce R, Job, D (1994) Interactions of plant acetohydroxy acid isomeroreductase with reaction intermediate analogs - correlation of the slow, competitive, inhibition-kinetics of enzyme-activity and herbicidal effects. *Biochem J* 301:813–820.

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

Dyer WE, Weller SC, Bressan RA, Herrmann KM (1988) Glyphosate tolerance in tobacco (*Nicotiana tabacum L.*). *Plant Physiol* 88:661–666.

Edwards R, Hannah M (2014) Focus on wedd control. *Plant Physiol* 166: 1087-1089.

Ehleringer J (1983) Ecophysiology of *Amaranthus palmeri*, a Sonoran desert summer annual. *Oecologia* 64:107–112.

Fernández-Escalada M, 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 M, Zulet-González A, Gil-Monreal M, Zabalza A, Ravet K, Gaines TA, Royuela M (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* 8:1–11.

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

Gaedert JW, Peterson DE, Horak MJ (2017) Control and cross-resistance of an acetolactate synthase inhibitor-resistant Palmer amaranth (*Amaranthus palmeri*) biotype. *Weed Technol* 11:132–137.

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

Gaines TA , Wright AA , Molin WT, Lorentz L, Riggins CW, Tranel PJ, Beffa R, Westra P, Powles SB (2013) Identification of genetic elements associated with EPSPs gene amplification. *PLoS One* 8: e65819 1-10.

Gaines TA, Zhang W, Wang D, Bukun B, Chisholm ST, Shaner DL, Nissen SJ, Patzoldt WL, Tranel PJ, Culpepper SA, Grey TL, Webster TM, Vencill WK Sammons RD, Jiang J, Preston C, Leach JE, Westra P (2010) Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. *Proc Natl Acad Sci USA* 107:1029–1034.

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.

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

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.

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

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, Müller C, Weisshaar B, Flügge U-I (2007) The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. *Plant J* 50:886–901.

Gil-Monreal M, Zabalza A, Missihoun TD, Dörmann P, Bartels D (2017) Induction of the PDH bypass and upregulation of the ALDH7B4 in plants treated with herbicides inhibiting amino acid biosynthesis. *Plant Sci* 264:16–28.

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, Hénault-Ethier L, Labrecque M, Lepage L, Lucotte M, Juneau P. (2014) Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid: An overview. *J Exp Bot* 65:4691–4703.

Gorlach J, Raesecke H-R, Rentsch D, Regenass M, Roy P, Zala M, Keel C, Boller T, Amrheim N, Schmid J (1995) Temporally distinct accumulation of transcripts encoding enzymes of the prechorismate pathway in elicitor-treated, cultured tomato cells. *Proc Natl Acad Sci* USA 92:3166–3170.

Gruys K, Sikorski J (1999) Inhibitors of tryptophan, phenylalanine, and tyrosine biosynthesis as herbicides. In: Singh BK (ed) *Plant amino acids: Biochemistry and biotechnology*. Marcel Dekker, New York, pp 357–384.

Guillet G, Poupart J, Basurco J, De Luca V (2000) Expression of tryptophan decarboxylase and tyrosine decarboxylase genes in tobacco results in altered biochemical and physiological phenotypes. *Plant Physiol* 122:933–43.

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

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. *Annu Rev Plant Physiol Plant Mol Biol* 40:347–369.

Halgand F, Wessel PM, Laprévote O, Dumas R (2002) Biochemical and mass spectrometric evidence for quaternary structure modifications of plant threonine deaminase induced by isoleucine. *Biochemistry* 41:13767–13773.

Haslam E (1993) Shikimic acid. Metabolism and metabolites. Wiley (ed), Hoboken, New Jersey, USA 387 pp.

Healy-Fried ML, Funke T, Priestman MA, Han H, Schönbrunn E (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 April 2018. 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.

Herrmann KM, Weaver LM (1999) The shikimate pathway. *Annu Rev Plant Physiol Plant Mol Biol* 50:473–503.

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

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.

Hoagland RE, Duke SO, Elmore CD (1979) Effects of glyphosate on metabolism of phenolic-compounds. III. Phenylalanine ammonia-lyase activity, free amino-acids, soluble-protein and hydroxyphenolic compounds in axes of dark-grown soybeans. *Physiol Plant* 46:357–366.

Hoagland RE, Jordan RH, Teaster ND (2013) Bioassay and characterization of several Palmer amaranth (*Amaranthus palmeri*) biotypes with varying tolerances to glyphosate. *Am J Plant Sci* 4:1029–1037.

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

Hydrick D, Shaw D (1994) Effects of tank-mix combinations of non-selective foliar and selective soil-applied herbicides on three weed species. *Weed Technol* 8:129–133.

Ishimoto M, Rahman SM, Hanafy MS, Khalafalla MM, El-Shemy HA, Nakamoto Y, Kita Y, Takanashi K, Matsuda F, Murano Y, Funabashi T, Miyagawa H, Wakasa K (2010) Evaluation of amino acid content and nutritional quality of transgenic soybean seeds with high-level tryptophan accumulation. *Mol Breed* 25:313–326.

Janzik I, Preiskowski S, Kneifel H (2005) Ozone has dramatic effects on the regulation of the prechorismate pathway in tobacco (*Nicotiana tabacum L. cv. Bel W3*). *Planta* 223:20–27.

John C, Greenway H (1976) Alcoholic fermentation and activity of some enzymes in rice roots under anaerobiosis. *Aust J Plant Physiol* 3:325–336.

Johnson, W.G., Wait JD, Holman CS (1999) Glyphosate and other soybean post herbicides. *North Cent Weed Sci Soc Res Rep* 56:342–344.

Keeley P, Carter C, Thullen R (1987) Influence of planting date on growth of Palmer amaranth (*Amaranthus palmeri*). *Weed Sci* 199–204.

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* USA 88:8821–8825.

Klingaman TE, Oliver LR (2018) Palmer amaranth (*Amaranthus palmeri*) interference in soybeans (*Glycine max*). *Weed Sci* 42:523–527.

Koger CH, Shaner DL, Henry WB, Nadler-Hassar T, Thomas WE, Wilcut JW (2005a) Assessment of two nondestructive assays for detecting glyphosate resistance in horseweed (*Conyza canadensis*). *Weed Sci* 53:559–566.

Koger CH, Shaner DL, Krutz LJ, Walker TW, Buehring N, Henry WB, Thomas WE, Wilcut JW (2005b) Rice (*Oryza sativa*) response to drift rates of glyphosate. *Pest Manag Sci* 61:1161–1167.

Küpper A, Borgato EA, Patterson EL, Netto AG, Nicolai M, de Carvalho SJP, Niessen SJ, Gaines TA, Christoffoleti PJ (2017) Multiple resistance to glyphosate and acetolactate synthase inhibitors in Palmer amaranth (*Amaranthus palmeri*) Identified in Brazil. *Weed Sci* 65:317–326.

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.

Legleiter TR, Bradley KW (2008) Glyphosate and multiple herbicide resistance in common waterhemp (*Amaranthus rudis*) populations from Missouri. *Weed Sci* 56:582–587.

Less H, Galili G (2008) Principal transcriptional programs regulating plant amino acid metabolism in response to abiotic stresses. *Plant Physiol* 147:316–330.

Li JM, Johnson WG, Smeda RJ (2002) Interactions between glyphosate and imazethapyr on four annual weeds. *Crop Prot* 21:1087–1092.

Li M, Yu Q, Han H, Vila-Aiub M Powles SB (2013) ALS herbicide resistance mutations in *Raphanus raphanistrum*: Evaluation of pleiotropic effects on vegetative growth and ALS activity. *Pest Manag Sci* 69:689–695.

Lich JM, Renner KA, Penner D (1997) Interaction of glyphosate with postemergence soybean (*Glycine max*) herbicides. *Weed Sci* 45:12–21.

Liu Y, Zhang Y, Liu Y, Lu W, Wang G (2015) Metabolic effects of glyphosate on transgenic maize expressing a *G2-EPSPS* gene from *Pseudomonas fluorescens*. *Plan Biochem Biotech* 24:233–241.

Livingston M, Fernandez-Cornejo J, Unger J, Osteen C, Schimmelpfennig D, Park T, Lambert D (2015) The economics of glyphosate resistance management in corn and soybean production. *Economic Research Service/USDA* ERR-184.

Lorentz L, Gaines T a, Nissen SJ, Westra P, Streck HJ, Dehne HW, Ruiz-Santaella JP, Roland B (2014) Characterization of glyphosate resistance in *Amaranthus tuberculatus* populations. *J Agric Food Chem* 62:8134–8142.

Lorraine-Colwill DF, Powles SB, Hawkes TR, Hollinshead PH, Warner SAJ, Preston C (2002) Investigations into the mechanism of glyphosate resistance in *Lolium rigidum*. *Pestic Biochem Physiol* 74:62–72.

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

MacDonald MJ, D'Cunha GB (2007) A modern view of phenylalanine ammonia lyase. *Biochem Cell Biol* 85:273–282.

MacRae JC (1971) Quantitative measurement of starch in very small amounts of leaf tissue. *Planta* 96:101–108.

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

Maeda H, Shasany AK, Schnepp J, Orlova I, Taguchi G, Cooper BR, Rhodes D, Pichersky E, Dudareva N (2010) RNAi suppression of *Arogenate Dehydratase1* reveals that phenylalanine is synthesized predominantly via the arogenate pathway in petunia petals. *Plant Cell* 22:832–849.

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

Maloney GS, Kochevenko A, Tieman DM, Tohge T, Krieger U, Zamir D, Taylor MG, Fernie AR, Klee HJ (2010) Characterization of the branched-chain amino acid aminotransferase enzyme family in tomato. *Plant Physiol* 153:925–936.

Manabe Y, Tinker N, Colville A, Miki B (2007) CSR1, the sole target of imidazolinone herbicide in *Arabidopsis thaliana*. *Plant Cell Physiol* 48:1340–1358.

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

Marchiosi R, Lucio Ferrarese MDL, Bonini EA, Gomes Fernandes N, Ferro AP, Ferrarese-Filho O (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, Duke S, Gerard P, Tharayil N (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, Nandula VK, Duke SO, Tharayil N (2016) Stable isotope resolved metabolomics reveals the role of anabolic and catabolic processes in glyphosate-induced amino acid accumulation in *Amaranthus palmeri* biotypes. *J Agric Food Chem* 64:7040–7048.

Massinga RA, Curry RS, Horak MJ, Jr., Boyer J (2001) Interference of Palmer amaranth in corn. *Weed Sci* 49:202–208.

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

Mazzucotelli E, Mastrangelo AM, Crosatti C, Guerra D, Stanca AM, Cattivelli L (2008) Abiotic stress response in plants: When post-transcriptional and post-translational regulations control transcription. *Plant Sci* 174:420–431.

McCourt JA, Duggleby RG (2006) Acetohydroxyacid synthase and its role in the biosynthetic pathway for branched-chain amino acids. *Amino Acids* 31:173–210.

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.

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

Moldes CA, Medici LO, Abrahao OS, Tsai SM, Azevedo RA (2008) Biochemical responses of glyphosate resistant and susceptible soybean plants exposed to glyphosate. *Acta Physiol Plant* 30:469–479.

Morgan GD, Baumann PA, Chandler JM (2001) Competitive impact of Palmer amaranth (*Amaranthus palmeri*) on cotton (*Gossypium hirsutum*) development and yield. *Weed Technol* 15:408–412.

Nandula VK (2010) Glyphosate resistance in crops and weeds: History, development, and management. Wiley (Ed), Hoboken, New Jersey, USA 344 pp.

Nandula VK, Ray JD, Ribeiro DN, Pan Z, Reddy KN (2013) Glyphosate resistance in Tall waterhemp (*Amaranthus tuberculatus*) from Mississippi is due to both altered target-site and nontarget-site mechanisms. *Weed Sci* 61:374–383.

Nandula VK, Reddy KN, Poston DH, Rimando AM, Duke SO (2008) Glyphosate tolerance mechanism in italian ryegrass (*Lolium multiflorum*) from Mississippi. *Weed Sci* 56:344–349.

Nandula VK, Wright AA, Bond JA, Ray JD, Eubank TW, Molin TW (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.

NCBI Website. https://www.ncbi.nlm.nih.gov/

Nelson KA, Renner KA (2002) Yellow nutsedge (*Cyperus esculentus*) control and tuber production with glyphosate and ALS-Inhibiting herbicides. *Weed Technol* 16:512–519.

Neve P, Busi R, Renton M, Vila-Aiub MM (2014) Expanding the eco-evolutionary context of herbicide resistance research. *Pest Manag Sci* 70: 1385–1393.

Neve P, Sadler J, Powles SB (2004) Multiple herbicide resistance in a glyphosate-resistant rigid ryegrass (*Lolium rigidum*) population. *Weed Sci* 52:920–928.

Ngo TD, Krishnan M, Boutsalis P, Gill G, Preston C (2018a) Target-site mutations conferring resistance to glyphosate in feathertop Rhodes grass (*Chloris virgata*) populations in Australia. *Pest Manag Sci* 74:1094-1100.

Ngo TD, Malone JM, Boutsalis P, Gill G, Preston C (2018b) *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, Zabalza A, Royuela M (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.

Owen MDK, Zelaya IA (2005) Herbicide-resistant crops and weed resistanceto herbicides. *Pest Manag Sci* 61:301–311.

Petersen IL, Hansen HCB, Ravn HW, Sorensen JC, Sorensen H (2007) Metabolic effects in rapeseed (*Brassica napus L.*) seedlings after root exposure to glyphosate. *Pestic Biochem Physiol* 89:220–229.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:2002–2007.

Pinto JE, 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.

Politycka B, Mielcarz B (2007) Involvement of ethylene in growth inhibition of cucumber roots by ferulic and p-coumaric acids. *Allelopath J* 19:451–460.

Powles SB (2008) Evolved glyphosate-resistant weeds around the world: lessons to be learnt. *Pest Manag Sci* 64:360–365.

Powles SB, Lorraine-Colwill DF, Dellow JJ, Preston C (1998) Evolved resistance to glyphosate in rigid ryegrass (*Lolium rigidum*) in Australia. *Weed Sci* 46:604–607.

Powles SB, Preston C (1995) Herbicide cross resistance and multiple resistance in plants. *HRAC* 1982:1–13.

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.

Rhodes D, Hogan AL, Deal L, Jamieson GC, Haworth P (1987) Amino acid metabolism of *Lemna minor L.*: II. Responses to chlorsulfuron. *Plant Physiol* 84:775–780.

Riar DS, Norsworthy JK, Srivastava V, Nandula V, Bond JA, Scott RC (2013) Physiological and molecular basis of acetolactate synthase-inhibiting herbicide resistance in barnyardgrass (*Echinochloa crus-galli*). *J Agric Food Chem* 61:278–289.

Ribeiro DN, Pan Z, Duke SO, Nandula VK, Baldwin BS, Shaw DR, Dayan FE (2014) Involvement of facultative apomixis in inheritance of EPSPS gene amplification in glyphosate-resistant *Amaranthus palmeri*. *Planta* 239:199–212.

Ritz C, Kniss AR, Streibig JC (2015) Research methods in weed science: Statistics. *Weed Sci* 63:166–187.

Rivero RM, Ruiz JM, Garcia PC, López-Lefevre LR, Sánchez E, Romero L (2001) Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. *Plant Sci* 160:315–321.

Sabbadin F, Glover R, Stafford R, Rozado-Aguirre Z, Boonham N, Adams I, Mumford R, Edwards R (2017) Transcriptome sequencing identifies novel persistent viruses inherbicide resistant wild-grasses. *Sci Rep* 7: 41987.

Salas RA, Dayan FE, Pan Z, Watson SB, Dickson JW, Scott RC, Burgos NR (2012) *EPSPS* gene amplification in glyphosate-resistant Italian ryegrass (*Lolium perenne ssp. multiflorum*) from Arkansas. *Pest Manag Sci* 68:1223–1230.

Salas RA, Scott RC, Dayan FE, Burgos NR (2015) *EPSPS* gene amplification in glyphosate-resistant Italian ryegrass (*Lolium perenne ssp. multiflorum*) populations from Arkansas (United States). *J Agric Food Chem* 63:5885–5893.

Sammons RD, Gaines TA (2014) Glyphosate resistance: State of knowledge. *Pest Manag Sci* 70:1367–1377.

Sarma AD, Sreelakshmi Y, Sharma R (1998) Differential expression and properties of phenylalanine ammonia-lyase isoforms in tomato leaves. *Phytochemistry* 49:2233–2243.

Sasaki-Sekimoto Y, Taki N, Obayashi T, Aono M, Matsumoto F, Sakuari N, Suzuki H, Hirai MY, Noji M, Saito K, Masuda T, Takamiya K, Shibata D, Ohta H (2005) Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. *Plant* J 44:653–668.

Sato K, Mase K, Nakano Y, Nishikubo N, Sugita R, Tsuboi Y, Kajita S, Zhou J, Kitano H, Katayama Y (2006) 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase is regulated for the accumulation of polysaccharide-linked hydroxycinnamoyl esters in rice (*Oryza sativa L.*) internode cell walls. *Plant Cell Rep* 25:676–688.

Schmid J, Amrhein J (1999) The shikimate pathway. In: Singh BK (ed) Plant amino acids: Biochemistry and biotechnology. Marcel Dekker, New York, pp 147–169.

Schönbrunn E, Eschenburg S, Shuttleworth WA, Schloss JV, Amrhein N, Evans JN, Kabsch W (2001) Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. *Proc Natl Acad Sci* USA 98:1376–80.

Schuster J, Knill T, Reichelt M, Gershenzon J, Binder S (2006) Branched-chain aminotransferase 4 is part of the chain elongation pathway in the biosynthesis of methionine-derived glucosinolates in arabidopsis. *Plant Cell* 18:2664–2679.

Seefeldt S, Jensen JE, Fuerst P (1995) Log-logistic analysis of herbicide dose-response relationships. *Weed Technol* 9:218–227.

Shaner DL (2009) Role of translocation as a mechanism of resistance to glyphosate. *Weed Sci* 57:118–123.

Shaner DL (2000) The impact of glyphosate-tolerant crops on the use of other herbicides and on resistance management. *Pest Manag Sci* 56:320–326.

Shaner DL (1999) Resistance to acetolactate synthase (ALS) inhibitors in the United States: History, occurrence, detection, and management. *J Weed Sci Tech* 44:405–411.

Shaner DL, Beckie HJ (2014) The future for weed control and technology. *Pest Manag Sci* 70:1329–1339.

Shaner DL, Nadler-Hassar T, Henry WB, Koger CH (2005) A rapid *in vivo* shikimate accumulation assay with excised leaf discs. *Weed Sci* 53:769–774.

Shaw DR, Arnold JC (2002) Weed control from herbicide combinations with glyphosate. *Weed Technol* 16:1–6.

Shumilin IA, Bauerle R, Kretsinger RH (2003) The high-resolution structure of 3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase reveals a twist in the plane of bound phosphoenolpyruvate. *Biochemistry* 42:3766–3776.

Siehl DL (1997) Hebicide activity: Toxicology, biochemistry and molecular biology In: Roe RM, Burton JD, Kuhr RJ (eds). IOS Amsterdam, Netherlands pp 37–67.

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, Marton I, Edelbaum O, Masci T, Prathapani NK, Shklarman E, Ovadis M, Vainstein A (2010) EOBII , a gene encoding a flower-specific regulator of phenylpropanoid volatiles' biosynthesis in petunia. *Plant Cell* 22:1961–1976.

Starke RJ, Oliver LJ (1998) Interaction of glyphosate with chlorimuron, fomesafen, imazethapyr, and sulfentrazone. *Weed Sci* 46:652–660.

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.

TAIR Website. https://www.arabidopsis.org/

Takatsuji H, Mori M, Benfey PN, Ren L, Chua NH (1992) Characterization of a zinc finger DNA-binding protein expressed specifically in Petunia petals and seedlings. *EMBO J* 11:241–249.

Tan S, Evans R, Singh B (2006) Herbicidal inhibitors of amino acid biosynthesis and herbicide-tolerant crops. *Amino Acids* 30:195–204.

The EMBOSS Website. http://emboss.open-bio.org/

Teaster ND, Hoagland RE (2014) Characterization of glyphosate resistance in cloned *Amaranthus palmeri* plants. *Weed Biol Manag* 14:1–10.

Tétard-Jones C, Edwards R (2016) Potential roles for microbial endophytes in herbicide tolerance in plants. *Pest Manag Sci* 2016, 72: 203-209.

Tétard-Jones C, Sabbadin F, Moss S, Hull R, Neve P, Edwards R (2018), Changes in the proteome of the problem weed blackgrass correlating with multiple herbicide resistance. *Plant J* (ePub ahead of print).

Tohge T, Watanabe M, Hoefgen R, Fernie AR (2013a) The evolution of phenylpropanoid metabolism in the green lineage. *Crit Rev Biochem Mol Biol* 48:123–52.

Tohge T, Watanabe M, Hoefgen R, Fernie AR (2013b) Shikimate and phenylalanine biosynthesis in the green lineage. *Front Plant Sci* 4:62.

Tranel, Patrik J. and Wright TR (2002) Resistance of weeds to ALS inhibiting herbicides: what have we learned? *Weed Sci* 50:700–712.

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, Galili G (2010a) The biosynthetic pathways for shikimate and aromatic amino acids in *Arabidopsis thaliana*. *Arab B*. 8:e0132.

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

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

Van Der Fits L, Memelink J (2000) Primary and secondary metabolism ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. *Science* 295:295–297.

Van Moerkercke A, Haring MA, Schuurink RC (2011) The transcription factor EMISSION of BENZENOIDS II activates the MYB ODORANT1 promoter at a MYB binding site specific for fragrant petunias. *Plant J* 67:917–928.

VanLieshout LA, Loux MM, Kuzmak BR (1996) Characterizing the nature of interactions between glyphosate and preemergence soybean herbicides. *Proc North Cent Weed Sci Soc* 51:24.

Verdonk JC (2005) ODORANT1 regulates fragrance biosynthesis in petunia flowers. *Plant Cell* 17:1612–1624.

Vila-Aiub MM, Balbi MC, Distéfano AJ, Fernández L, Hopp E, Yu Q, Powles SB (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, Han H, Busi R, Yu Q, Powles SB (2014) No fitness cost of glyphosate resistance endowed by massive EPSPS gene amplification in *Amaranthus palmeri*. *Planta* 239:793–801.

Vivancos PD, Driscoll SP, Bulman CA, Ying L, Emami K, Treumann A, Mauve C, Noctor G, Foyer CH. (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.

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

Wang Y, Chen J-Y, Jiang Y-M, Lu W-J (2007) Cloning and expression analysis of phenylalanine ammonia-lyase in relation to chilling tolerance in harvested banana fruit. *Postharvest Biol Technol* 44:34–41.

Warren C, Adams M (2000) Capillary electrophoresis for the determination of major amino acids and sugars in foliage: application to the nitrogen nutrition of sclerophyllous species. *J Exp Bot* 51:1147–1157.

Westwood JH, Weller SC (1997) Cellular mechanisms influence differential glyphosate sensitivity in field bindweed (*Convolvulus arvensis*) biotypes. *Weed Sci* 45:2–11.

Whitaker JR, Burton JD, York AC, Jordan D, Chandi A (2013) Physiology of glyphosate-resistant and glyphosate-susceptible Palmer amaranth (*Amaranthus palmeri*) biotypes collected from North Carolina. *Int J Agron* 2013:1–6.

White AD, Owen MDK, Cardina J (2002) Common sunflower resistance to acetolactate synthase-inhibiting herbicides. *Weed Sci* 50:432–437.

Wiersma AT, Gaines TA, Preston C, Hamilton JP, Giacomini D, Robin Buell C, Leach JE, Westra P (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, Rayner DR, Schloss JV. (1994) Herbicidal activity of an isopropylmalate dehydrogenase inhibitor. *Plant Physiol* 106:321–328.

Wittenbach V, Abell L (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.

Wittenbach VA, Koeppe MK, Lichtner FT, Zimmerman WT, Reiser RW (1994) Basis of selectivity of triflusulfuron methyl in Sugar beets (*Beta vulgaris*). *Pestic Biochem Physiol* 49:72–81.

Yu Q, Abdallah I, Han H, Owen M, Powles SB (2009) Distinct non-target site mechanisms endow resistance to glyphosate, ACCase and ALS-inhibiting herbicides in multiple herbicide-resistant *Lolium rigidum*. *Planta* 230:713–723.

Yu Q, Powles SB (2014) Resistance to AHAS inhibitor herbicides: Current understanding. *Pest Manag Sci* 70:1340–1350.

Yu WC, Zhang R, Li RZ, Guo SD (2007) Isolation and characterization of glyphosate-regulated genes in soybean seedlings. *Plant Sci* 172:497–504.

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.

Yuan JS, Tranel PJ, Stewart CN (2007) Non-target-site herbicide resistance: a family business. *Trends Plant Sci* 12:6–13.

Zabalza A, Gaston S, Ribas-Carbó M, Orcaray L, Igal M, Royuela M (2006) Nitrogen assimilation studies using 15N in soybean plants treated with imazethapyr, an inhibitor of branched-chain amino acid biosynthesis. *J Agric Food Chem* 54:8818–8823.

Zabalza A, Orcaray L, Fernández-Escalada M, Zulet-González A, Royuela M (2017) The pattern of shikimate pathway and phenylpropanoids after inhibition by glyphosate or quinate feeding in pea roots. *Pestic Biochem Physiol* 141:96:102.

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

Zabalza A, Zulet A, Gil-Monreal M, Igal M, Royuela M (2013) Branched-chain amino acid biosynthesis inhibitors: Herbicide efficacy is associated with an induced carbon-nitrogen imbalance. *J Plant Physiol* 170:814–21.

Zhang C, Pang Q, Jiang L, Wang S, Yan X, Chen S, He Y (2015) Dihydroxyacid dehydratase is important for gametophyte development and disruption causes increased susceptibility to salinity stress in Arabidopsis. *J Exp Bot* 66:879–888.

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

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

Zulet A, Gil-Monreal M, Villamor JG, Zabalza A, van der Hoorn RA, Royuela M (2013a) Proteolytic pathways induced by jerbicides 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, van Dongen JT, Royuela M (2015) Fermentation and alternative oxidase contribute to the action of amino acid biosynthesis-inhibiting herbicides. *J Plant Physiol* 175:102–112.

APPENDIX

LIST OF PUBLICATIONS

-Publication 1: **Manuel Fernández-Escalada**, Miriam Gil-Monreal, Ana Zabalza, Mercedes Royuela

Characterization of the *Amaranthus palmeri* physiological response to glyphosate in susceptible and resistant populations (2016). 64:95–106. DOI: 10.1021/acs.jafc.5b04916.

Journal: Journal of agricultural and food chemistry. Factor de impacto (2016) 3.153. Rank: Q1 (2/56)

Área temática: Agriculture, multidisciplinary

-Publication 2: **Manuel Fernández-Escalada**, Ainhoa Zulet-González, Miriam Gil-Monreal, Ana Zabalza, Karl Ravet, Todd Gaines, Mercedes Royuela

Effects of EPSPS copy number variation (CNV) and glyphosate application on the aromatic and branched chain amino acid synthesis pathways in *Amaranthus palmeri.* 8:1–11 (2017) DOI: 10.3389/fpls.2017.01970.

Journal: Frontiers in plant science. Factor de impacto (2016) 4.291. Rank Q1 (20/212)

Area temática: Plant science

In both publications Manuel Fernández Escalada is the first author showing that is part of their thesis, which is confirmed by its directors: Mercedes Royuela and Ana Zabalza