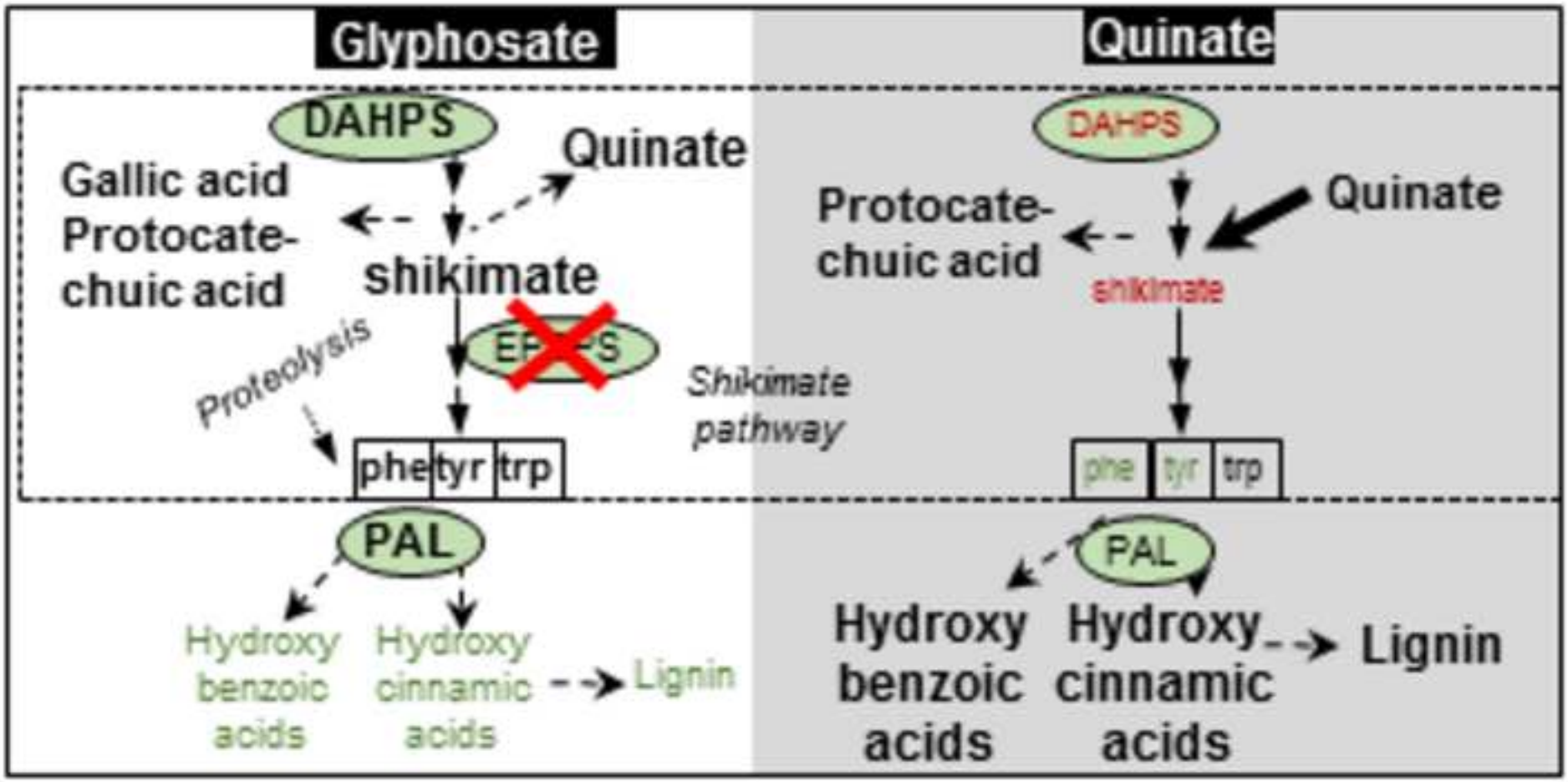
*Graphical Abstracts



HIGHLIGHTS

- This study focuses on effects of glyphosate on the pathway that specifically inhibits: the shikimate pathway.
- Different metabolites were accumulated through the shikimate and phenylpropanoids pathways after glyphosate or quinate treatment.
- The accumulation of metabolites upstream EPSPS was confirmed to be the main important change of the shikimate pathway after glyphosate.
- In contrast, the exogenous quinate supply induced accumulation of phenypropanoids and lignin.
- DAHPS enzyme amount was increased after glyphosate and decreased by quinate suggesting a regulation of the gene expression by the availability of the metabolites of shikimate pathway.
- The role of quinate in the toxicity of glyphosate is not mediated by a common pattern of the shikimate pathway

The pattern of shikimate pathway and phenylpropanoids after inhibition by glyphosate or quinate feeding in pea roots

Ana Zabalza, Luis Orcaray, Manuel Fernández-Escalada, Ainhoa Zulet-González, Mercedes Royuela*

Dpto. Ciencias Medio Natural, Universidad Pública de Navarra, Campus Arrosadia s/n, 31006 Pamplona, Spain

* Corresponding author

E-Mail address: royuela@unavarra.es (M- Royuela)

ABSTRACT

The shikimate pathway is a metabolic route for the biosynthesis of aromatic amino acids (AAAs) (i.e. phenylalanine, tyrosine, and tryptophan). One of the enzymes of the shikimate pathway (5-enolpyruvylshikimate-3-phosphate synthase, EPSPS) is the target of the widely used herbicide glyphosate. By other hand, quinate is a compound synthesized in plants through a side branch of the shikimate pathway, and is considered a reserve compound of the pathway although its physiological role has not been completely clarified. Glyphosate provokes quinate accumulation, and exogenous quinate application to plants shows a potential role of quinate in the toxicity of the herbicide glyphosate. We hypothesized that the role of quinate accumulation in the toxicity of the glyphosate would be mediated by a deregulation of the shikimate pathway. In order to gain new insights in the mode of action of the glyphosate, in this study the effect of the glyphosate and of the exogenous quinate was evaluated in roots of pea plants by analyzing the time course of a full metabolic map of several metabolites of shikimate and phenylpropanoid pathways.Glyphosate application induced an increase of the 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS, first enzyme of the shikimate pathway) protein and accumulation of metabolites upstream of the enzyme EPSPS. No common effects on the metabolites and regulation of shikimate pathway were detected between quinate and glyphosate treatments, supporting that the importance of quinate in the mode of action of glyphosate is not mediated by a common alteration of the regulation of the shikimate pathway. Contrary to glyphosate, the exogenous quinate supplied was probably incorporated into the main trunk from the branch pathway and accumulated in the final products, such as lignin, concomitant with a decrease in the amount of DAHPS protein.

Keywords:

Aromatic amino acids,
DAHPS,
EPSPS,
phenylpropanoid metabolism,
hydroxybenzoic acids,
hydroxycinnamic acids

1. Introduction

The biosynthesis of aromatic amino acids (AAAs) proceeds by way of the shikimate pathway: from phosphoenol pyruvate and erythrose-4-phosphate to chorismate (a common precursor of all the AAAs) and the specific terminal pathways that use chorismate as a substrate to synthesize phenylalanine (Phe)/tyrosine (Tyr) on one hand and tryptophan (Trp) on the other [1]. Plants synthesize a large number of specialized metabolites originating from the three AAAs (in particular, from Phe) and from several intermediates of the shikimate pathway leading to side branches such as quinate or dehydroquinate. In land plants, very high fluxes are noted with estimates of the amount of fixed carbon passing through the pathway varying between 20 and 50% [2]. The high flow through the shikimate pathway and its complexity in plants are related to the facts that the AAAs in plants are not only used as protein building blocks but also for many secondary metabolites, such as phenylpropanoids, with diverse physiological roles [3,4]. Although the regulation of the synthesis of AAAs from chorismate has been studied extensively in plants [4], the regulation of flux through the shikimate pathway itself in plants is much less understood.

One of the enzymes of the shikimate pathway: 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), is the only known target of the widely used herbicide glyphosate [5]. Despite its widespread use in global crop production, the precise mechanisms by which glyphosate kills plants remain unclear. In general, after the target of an inhibitor has been affected, death can occur due to (1) accumulation or increased availability of the substrates of the inhibited enzymatic pathway, (2) lack of end products generated by the inhibited pathway and/or, (3) several side reactions triggered after the inhibition of the target because the deregulation caused by the inhibition of this pathway can lead to effects on different metabolic pathways. Although it is not fully understood how plants actually die after the EPSPS many aspects of the herbicide toxicity have been described. Glyphosate affects several plant physiological processes, which could also be associated with glyphosate-herbicidal effects [6]. Although glyphosate provokes a slow plant death of the herbicide-treated plants [7], carbon and nitrogen metabolism are affected as soon as 1 or 3 days after glyphosate treatment; i.e., total free amino acid content increases, soluble protein content decreases [8] and carbohydrate content accumulates [9].

Several shikimate pathway intermediates are substrates for branch points leading to secondary metabolic processes. Among these, quinate can be formed in a single step reaction from the main shikimate pathway. Quinate is widely distributed and abundant in higher plants, particularly woody species, and may accumulate to high levels (up to 10% of the leaf dry weight) in some plants [10]. Interestingly, most of the plant which accumulates quinate do not contain a significant amount of shikimate, and the converse is also true. Both compounds exhibit a similar pattern of accumulation during an annual cycle, with a peak in spring and a decrease in summer. This biphasic pattern suggests a reserve role because these compounds could be first accumulated and then used as carbon sources for the synthesis of a wide range of phenolic compounds, such as lignins [11].

Although quinate is considered a reserve compound of the shikimate pathway; its physiological role has not been completely clarified. Indeed, quinate accumulation in leaves detected after glyphosate treatment [12] raised the question of whether quinate could mimic the action of the herbicide. In this sense, the phytotoxic and metabolic effects of exogenous quinate were studied after application through the nutrient solution or leaf spraying [13]. Both treatments affected plant growth, and mimicked some physiological effects of glyphosate. Those results indicated that quinate plays an important role in the mode of action of glyphosate. It was hypothesized that quinate may not have a target by itself, but it would mimic the mode of action of glyphosate by entering the shikimate pathway and deregulating different processes related with this pathway. Nevertheless, that hypothesis remains to be proven.

In order to gain new insights in the mode of action of the herbicide glyphosate the current study compared the effect of the glyphosate and of the exogenous quinate on several metabolites and key enzymes of the shikimate and phenylpropanoid pathway. We hypothesized that the role of quinate accumulation in the toxicity of the herbicide would be mediated by a deregulation of the shikimate pathway. Therefore, we expected to observe a similar effect of both treatments on the shikimate pathway regulation.

2. Materials and methods

2.1. Plant material and treatment application

Seeds of pea *Pisum sativum* L. cv. Snap Sugar Boys (surface sterilized) were grown in vermiculite for 3 days at 26 ºC in darkness prior to transfer to hydroponic tanks filled with nutrient solution and placed in a growth chamber [14]. Nutrient solution (2.7 l $tank^{-1}$) was aerated continuously (700 ml $tank^{-1}$ min^{-1}) and renewed every 3 days. Glyphosate or quinate was applied when the plants were 12 days old in independent experimental sets. At 12 days of age, the plants were divided into two groups: one to assess the effect of the herbicide and the other to perform the experiment with quinate.

Plants were grown under treatment for 3 weeks. Root samples were taken at 0, 1, 3, 7, 10, and 15 days after the onset of the treatment. In some cases (indicated), the study only included harvest at day 7 or 15. At harvest, samples were obtained and immediately frozen in liquid nitrogen and stored at –80 ºC for analytical determinations. Some material was dried for 48 h at 75-80ºC to obtain the fresh weight/dry weight ratio.

2.1.1. Glyphosate treatment

In preliminary studies, a concentration of glyphosate to pea roots in a hydroponic system was chosen to produce a slow, robust and synchronous death of this crop plant within 20 days [9]. In the group of plants used to assess the effect of the herbicide, half of the plants were treated with glyphosate applied to the nutrient solution as a commercial formulation (isopropylamine salt, Glyfos, BayerGarden, Valencia, Spain) at a final concentration of 0.23 mM (53 mg active ingredient l^{-1}) and the other half was not treated and served as the control treatment.

2.1.2. Exogenous quinate application

Preliminary studies were also conducted to determine a quinate dose (Fluka Chem Co, WI, USA), which produced similar effects on growth arrest and lethality to those described following glyphosate treatment to the nutrient solution [12]. Based on these results, 4 mM applied to the nutrient solution (768 mg l^{-1}) was finally selected as a comparable concentration for this study [13]. In this group, half of the plants were treated with 4 mM quinate and the other half was not treated.

2.2. Analytical determinations

2.2.1. Free amino acid determination

The extraction of amino acids was performed in HCl. After protein precipitation, amino acid concentrations were measured in the supernatant using capillary

electrophoresis equipped with a laser-induced fluorescence detector, as previously described [12].

2.2.2. Quinate

Quinate content in pea roots was extracted in trichloroacetic acid (TCA) and measured using ion chromatography as previously described [12].

2.2.3. Shikimate, HBA and HCA determination

The determination of the content of shikimate, hydroxybenzoic acids (4-hydroxybenzoic, gentisic, vanillic and syringic acids) and hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic and sinapic acids) was performed by high-performance liquid chromatography (HPLC) as described previously [12].

2.2.4. Anthocyanin content

Fresh tissues were homogenized in acidic methanol (0.1 N HCl) and the homogenates were centrifuged for 20 minutes at 20000 *g*. Anthocyanin content was quantitated by measuring the difference in absorbence at 525 nm and 585nm [15].

2.2.5. Lignin content

The lignin content was determined in pea roots according to previously described methods [16].

2.2.6. PAL assay

The PAL activity was determined in pea roots, using methods previously described [16].

2.2.7 DAHPS immunoblotting

DAHPS immunoblots were produced according to standard techniques. DAHPS immunoblotting was performed as described previously [16].

2.3 Statistical analysis

An unpaired Student's t-test was used to determine the significance between each treatment and each control plant (untreated plants) on the given day of glyphosate or quinate treatment. Each mean value was calculated using samples from single plants as biological replicates. Significant differences ($p<0.05$) are discussed.

3. Results and Discussion

The application of glyphosate or quinate to the nutrient solution of pea plants caused a rapid inhibition of plant growth [12]. Growth arrest persisted during the experimental time course, and plant death took approximately 20 days. Measurements of the plant status were performed only for 15 days from the onset of the treatment.

Although several short- term biochemical studies have been published recently [17–20], the originality of this study involves the wide time span used, covering a full period from the initial phases (when plants did not look affected) until when they were visually injured. The measurements performed in this work at the different phases of the treatment (days 1 and 3) and the continuation for 15 days allowed evaluation of the pattern of the shikimate pathway from the onset of the treatment until severe injury.

Physiological effects on the shikimate pathway have been reported in leaves of glyphosate-treated plants before, so a different organ of study (root) was used in this study. Indeed, the most prominent physiological changes were monitored in the same organ where the treatment (glyphosate or quinate) was applied. Moreover, the availability of the previously published effects of glyphosate on carbon metabolism in the same plant material [9] would help in the elucidation of the full picture of the altered carbon/nitrogen metabolism in glyphosate-treated plants

3.1. Glyphosate treatment

Figure 1 summarizes the content of specific metabolites at different time points in pea roots after glyphosate treatment. As early as 3 days from the onset of treatment accumulation of shikimate and protocatechuic were detected. Gallic acid accumulation was detected from day 7 and all accumulations increased over time. Shikimate is typically the main compound that accumulates after glyphosate, and a rapid accumulation of gallic and protocatechuic acid is also a characteristic effect of the herbicide [21–23]. This large increase in EPSP precursors was also detected in the leaves of pea plants when glyphosate was applied through the nutrient solution [12].

Interestingly, an important difference in the pattern of EPSP precursors was detected between leaves and roots of pea plants. No accumulation of quinate, a compound synthesized in a lateral branch of the shikimate pathway, was detected in roots although glyphosate was absorbed through this organ, while glyphosate induced an increase in the content of quinate in leaves of the same plants [12] and in other species [24,25].

Indeed, the organ where quinate is accumulated after glyphosate treatment seems to be species-dependent , as recently quinate accumulation has been reported in roots of *Lolium perenne* treated with glyphosate [26]. Two explanations can be proposed to the lack of quinate accumulation in roots of glyphosate-treated pea plants. On one hand, quinate accumulation can be only evident in leaves and not in roots because the flux through the shikimate pathways is higher in photosynthetic tissues. On the other hand, it can be proposed that quinate accumulation would not be detectable in roots due to a favoured translocation of the accumulated quinate upwards to the shoots.

The effect of glyphosate on the amount of the first enzyme of the pathway, (3-deoxy-D-arabino-heptulosonate-7-phosphate synthase; DAHPS; E.C.2.5.2.54) was evaluated (Fig. 2). As previously reported [25], the herbicide induced an increase in the content of DAHPS protein. The inhibition of the shikimate pathway at the EPSPS level by glyphosate has a dual-action on the unregulation of the flow of carbon through the pathway. Besides the direct blockage of the EPSPS activity, the lack of metabolites downstream chorismate (such as arogenate) implicates that they can no act as alosteric inhibitors of the DAHPS activity [27,28].Therefore, carbon entrance at the DAHPS level is not feed-back regulated leading to an intensification of the accumulation of compounds upstream of the blocked point, EPSPS. The higher amount of DAHPS protein detected after glyphosate treatment (Fig. 2) suggests that exists regulation of DAHPS expression by accumulation of a certain metabolite before EPSPS or by a lack a certain metabolite after EPSPS. So, the higher activity of DAPHS after glyphosate because of the lack of alosteric inhibitors of DAHPS (metabolites downstream EPSP) is possible because an increase in the DAHPS protein content. It can be suggested that this higher DAHPS enzyme amount would implicate an absolutely uncontrolled carbon entry into the blocked shikimate pathway, thus exacerbating the accumulation of metabolites upstream EPSPS.

The inhibition of the shikimate pathway by glyphosate results in the disruption of AAA biosynthesis. The content of each AAA increased but the time course of the accumulation pattern of each AAA was different. Tyr accumulation was significant from the beginning of glyphosate treatment while Phe and Trp levels were significantly higher from days 7 and 3 respectively. Other studies have demonstrated no clear pattern in the AAA contents in relation to exposure of glyphosate, with different patterns for each AAA depending on the species and dose studied [17–20,29–31]. Although various authors assume that AAA production at a level insufficient to maintain necessary

protein synthesis is the main effect and that this mechanism is consistent with the slow development of symptoms [5], a decrease in the specific content of each AAA was not detected after glyphosate treatment. On the contrary, the specific content of each individual AAA was increased (Fig. 1) evidencing that no shortage of any individual AAA was detected in the mode of action of the glyphosate at any moment of the experiment. Although the biosynthetic pathways is inhibited at the EPSPS point by glyphosate, an increase in the individual AAA concentration is possible. The absolute amount of each AAA cannot be interpreted only in terms of synthesis because a general increase of free amino acid content after glyphosate has been reported in these plants [8] and others [12,31–34] and related to proteolysis [8], as the concomitant soluble protein decrease has also been described [8,20].

The L-phenylalanine ammonia lyase (PAL; EC 4.3.15) enzyme catalyzes the deamination of Phe to yield cinnamic acid. Cinnamic acid is converted in *p*-coumaric acid , which is the common precursor of phenolic derivatives, such as hydroxycinnamic acids and hydroxybenzoic acids (HBA and HCA, respectively) and anthocianyns, a class of flavonoids . PAL activity was determined spectrophotometrically. Glyphosate elicited PAL activity in roots of pea plants, as has been reported in other species. [35–38]. The increase in PAL activity after glyphosate treatment can be related to the higher availability of Phe, substrate of the enzymatic activity.

The content of four hydroxybenzoic acids (HBA) and four hydroxycinnamic acids (HCA) was evaluated to detect the pattern of phenylpropanoid metabolites that are synthesized after PAL activity in glyphosate treated plants. Most of them were not affected by glyphosate in most of the cases, with the exception of the decrease of sinapic acid and the accumulation of caffeic acid (Fig. 1). The content of anthocyanins was increased after glyphosate treatment.

Lignin, which represents a significant proportion of all the carbon fixed by plants, is a complex heteropolymer derived from the HCA metabolism. While other studies have reported that glyphosate-treated plants produce less lignin [6,37], our results showed no significant changes in the lignin content in glyphosate-treated roots. Lignin is synthesized from the lignans derived from Phe and lignin accumulation is accompanied by the activation of enzymes that mediate lignin biosynthesis, such as PAL. Surprisingly, roots of glyphosate treated pea plants showed a striking PAL activity induction while lignin content was not significantly affected. This discrepancy might be due to any PAL regulation mechanism operating *in vivo* and not detected in the

255 determination of the *in vitro* activity spectrophotometrically or to lack of direct
256 correlation between PAL and lignin content. Indeed, PAL induction has been reported
257 as a general response after stress situations such as wounding and pathogen attack [39].
258 The study of the pattern of the shikimate pathway suggests that the toxicity of the
259 glyphosate is more related to substrate accumulation or to induction of side reactions
260 than to lack of the final products of the pathway. This is supported by 1) no lack of any
261 AAA was detected at any moment of the present study (indeed, they were accumulated (
262 Fig. 1) and have been reported to be accumulated in other species [19,20] and 2)
263 massive levels of EPSP precursors were detected (Fig. 1) and have been reported [22].
264 Moreover, toxic effects of shikimate accumulation have been proposed [24].

265 *3.2. Exogenous quinate application*

266 The pattern of the content of metabolites located downstream and upstream of
267 EPSPS (Fig. 1) after glyphosate treatment was compared with the pattern of the content
268 of the same metabolites after applying exogenous quinate (Fig. 3). Quinate can be
269 formed in a single step reaction from dehydroquinate catalyzed by quinate
270 dehydrogenase and from shikimate catalyzed by quinate hydrolyase. Quinate can re-
271 enter the shikimate pathway upon conversion to dehydroquinate or to shikimate by the
272 above enzymes [10,40,41].
273 Application of quinate to the roots increased the concentration of quinate (Fig. 3),
274 which demonstrated that quinate is absorbed. The other chorismate precursors evaluated
275 did not show a common pattern: shikimate content decreased and protocatechuic acid
276 increased respectively. It was not possible to detect gallic acid after quinate supply.
277 The DAHPS activity from microorganisms is generally regulated by allosteric
278 feedback inhibition by the different AAAs. In contrast, plant DAHPS enzymes are not
279 regulated by feedback inhibition loops; however, some *in vitro* exceptions have been
280 reported [4,42]. To date, only three enzymes in this pathway, namely, chorismate
281 mutase (of Phe and Tyr synthesis), tryptophan synthase (of Trp biosynthesis) and
282 arogenate dehydratase (of Phe biosynthesis), were experimentally shown to be
283 allosterically regulated [43]. In the roots of pea plants, the effect of quinate on DHAPS
284 protein amount was different at the two time-points of study: the band corresponding to
285 DAHPS protein was not detected in quinate-fed plants at day 7 and was similar in
286 untreated and quinate-treated roots at day 15. (Fig. 2). To confirm this result, it was

checked in the leaves of the same plants, where a decrease in the DAHPS band intensity in the quinate-supplied plant was detected both at day 7 and day 15 (Supplementary Figure 1). Recently, an increase in the content of Phe, Trp, and some phenylpropanoids was detected in transgenic *Arabidopsis* expressing a feedback insensitive bacterial DAHPS, suggesting that this enzyme is a key regulatory enzyme in the shikimate pathway [44,45]. DAHPS activity determines the carbon entering through the shikimate pathway and it is not feedback regulated by AAA in plants [4]. The results shown in this study suggest that besides a potential allosteric DAHPS activity regulation, DAHPS expression is regulated by the availability of some metabolites located in the pathway before or after EPSP, as DAHPS gene expression is greater than before by EPSPS blockage it is decreased after feeding the pathway with quinate.

Interestingly, only Trp was accumulated when exogenous quinate was supplied, whereas Phe and Tyr remained unaffected (Fig. 3). Most of the HCA evaluated, two out of the four HBA evaluated and anthocyanins showed higher contents after 7 or 15 days of quinate supply. The increase in lignin content after quinate feeding was significant at the end of the period of study.

It should be noted that interpretation of the physiology of plants based on pool sizes of the pathway intermediates is difficult because pool size does not reflect pool flux. Nevertheless, the accumulation of some HCA and HBA, anthocyanins and lignin at the end of the period of treatment indicates that quinate was incorporated to the main trunk of the shikimate pathway. These results support the notion that plants can use quinate as a carbon source for the biosynthesis of AAAs [46,47]. The accumulation of the secondary metabolites formed from Phe and Tyr but not the two AAAs themselves suggested a coordinated response of the shikimate pathway. Indeed, an increase in the content of phenylpropanoid metabolites has always been detected with a concomitant increase in the activity of the upstream Phe pathway and most of the transcription factors that have been shown to regulate the expression of the genes of the phenylpropanoid pathway also control, maybe indirectly, the expression of the genes of AAAs pathway [48]. Nevertheless, although an increase in PAL activity would be expected in roots of quinate supplied-plants it was not affected by the quinate (Fig. 3). Although to explain this incongruity it can be proposed that quinate is proceeding down a different metabolic pathway than the shikimate pathway or it is not entering the symplast in the root, we consider that quinate fed to pea roots is metabolized by the phenylpropanoids mechanism derived from the shikimate pathway, even though PAL

activity is not altered by exogenous quinate. Indeed the role of quinate in biosynthesis of lignin was proved by ^{14}C-quitate incorporation in Scotch pine [49].

In higher plants, quinate is a precursor for chlorogenic acid, which the ester of caffeic acid and quinate [50]. Although the accumulation of quinate appears to be restricted to specific plants, the occurrence of chlorogenic and its derivatives are more widespread. The chlorogenic acid has been related to several abiotic and biotic stresses [51,52] mainly related to its antioxidant properties [53]. The content of chlorogenic acid was not determined in this study, but, interestingly, caffeic acid was the only HCA increased in the roots of the glyphosate-treated plants (Fig. 1). Therefore, it is suggested that increased quinate availability in the leaves after glyphosate treatment [12] elicited increased concomitant caffeic acid levels in roots probably to support an increased rate of chlorogenic acid.

When quinate is applied exogenously though the nutrient solution, it enters into the shikimate pathway deregulating different steps located in this pathway. Although a common carbohydrate accumulation has been detected in leaves and roots after glyphosate [9] or quinate treatment [13], the deregulation provoked in the shikimate pathway by quinate is different from the deregulation provoked by glyphosate. It would have been very interesting to detect similar common effects on the shikimate pathway after the lethal doses of glyphosate or quinate, because it would have proved that the role of quinate in the toxicity of glyphosate was mediated by a particular pattern of the shikimate pathway . As different effects on the shikimate pathway and phenylpropanoids contents were detected after two lethal treatments, no common lethal pattern can be elucidated.

4. Conclusions

A different pattern was detected in the shikimate pathway and secondary metabolites deriving from it after inhibiting the EPSPS step or fueling exogenous carbon from the quinate branch pathway. Glyphosate application induced a non regulated carbon entrance through DAHPS and accumulation of metabolites upstream of the inhibited EPSPS while no changes in the HCA and HBA content was detected after herbicide treatment. When exogenous quinate was supplied, the the amount of DAHPS protein decreased and HCA and HBA accumulated. An increase of each AAA after glyphosate was detected, although absolute AAA contents are difficult to integrate because they are

values that result from biosynthesis, catabolism, proteolysis and use rate in protein synthesis.

In the present study, we focused on the mode of action of glyphosate. The comparison of the pattern of shikimate pathway of glyphosate-treated pea roots with quinate fed-plants has provided plants have provided insights into its mode of action. It has not been possible to establish common toxic effects of both lethal treatments on the shikimate pathway elements, indicating that both compounds alter the shikimate pathway, but do it differently. Therefore, it has not been possible to draw a causal link between the lethal effect of quinate and glyphosate.

Abbreviations

AAA	Aromatic amino acid
DAHPS	3-deoxy-D-arabino-heptulosonate-7-phosphate synthase
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
HBA	Hydroxybenzoic acids
HCA	Hydroxycinnamic acids
PAL	Phenylalanine ammonia lyase

Acknowledgments

We thank Gustavo Garijo for technical assistance. This work was financially supported by a grant from the Ministerio Español de Economía y Competitividad (AGL-2013-40567R). L.O. received a grant from the Ministerio de Educación. M. F-E and AZ-G received funding from fellowships from Universidad Pública de Navarra.

References

[1] K.M. Herrmann, L.M. Weaver, The shikimate pathway, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 473–503.

[2] T. Tohge, M. Watanabe, R. Hoefgen, A.R. Fernie, Shikimate and phenylalanine biosynthesis in the green lineage., Front. Plant Sci. 4 (2013) 62.

[3] T. Tohge, M. Watanabe, R. Hoefgen, A.R. Fernie, The evolution of phenylpropanoid metabolism in the green lineage, Crit. Rev. Biochem. Mol. Biol. 48 (2013) 123–52.

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

[5] S.O. Duke, S.B. Powles, Glyphosate: a once-in-a-century herbicide, Pest Manag. Sci. 64 (2008) 319–325.

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

[7] K. Gruys, J. Sikorski, Inhibitors of tryptophan, phenylalanine, and tyrosine biosynthesis as herbicides, in: B.K. Singh (Ed.), Plant amino acids: Biochemistry and biotechnology, Marcel Dekker, New York, 1999: pp. 357–384.

[8] A. Zulet, M. Gil-Monreal, J.G. Villamor, A. Zabalza, R. A. L. van der Hoorn, M. Royuela, Proteolytic pathways induced by herbicides that inhibit amino acid biosynthesis, PLoS One. 8 (2013) e73847

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

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

[11] A.M. Boudet, Polyphenols: From plant adaptation to useful chemical resources, in: V. Cheynier, P. Sarni-Manchado, S. Quideau (Eds.), Recent Advances in polyphenol research. Wiley-Backwell, Chichester, West Sussex, UK, 2012: pp. 41–70.

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

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

[14] A. Zabalza, E.M. González, C. Arrese-Igor, M. Royuela, Fermentative metabolism is induced by inhibiting different enzymes of the branched-chain

amino acid biosynthesis pathway in pea plants, J. Agric. Food Chem. 53 (2005) 7486–7493.

[15] M.M.N. Alla, M.E. Younis, Herbicide effects on phenolic metabolism in maize (*Zea mays* L.) and soybean (*Glycine max* L.) seedlings, J. Exp. Bot. 46 (1995) 1731–1736.

[16] L. Orcaray, M. Igal, A. Zabalza, M. Royuela, 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 (2011) 10162–10168.

[17] C.A. Moldes, J.M. Camiña, L.O. Medici, S.M. Tsai, R.A. Azevedo, Physiological effects of glyphosate over amino acid profile in conventional and transgenic soybean (*Glycine max*), Pestic. Biochem. Physiol. 102 (2012) 134–141.

[18] A.S. Maroli, V.K. Nandula, F.E. Dayan, S.O. Duke, P. Gerard, N. Tharayil, 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 (2015) 9199–9209.

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

[20] A.S. Maroli, V.K. Nandula, S.O. Duke, N. Tharayil, 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. (2016) J. Agric. Food Chem. 64 (2016) 7040-7048

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

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

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

[24] N. de María, J.M. Becerril, J.I. García-Plazaola, A. Hernandez, M.R. De Felipe, M. Fernandez-Pascual, New insights on glyphosate mode of action in nodular metabolism: Role of shikimate accumulation, J. Agric. Food Chem. 54 (2006) 2621–2628.

[25] J.E.B.P. Pinto, W.E. Dyer, S.C. Weller, K.M. Herrmann, Glyphosate induces 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase in potato (*Solanum tuberosum* L.) cells grown in suspension-culture, Plant Physiol. 87 (1988) 891–893.

[26] A.A. Serra, I. Couée, D. Renault, G. Gouesbet, C. Sulmon, Metabolic profiling of *Lolium perenne* shows functional integration of metabolic responses to diverse subtoxic conditions of chemical stress, J. Exp. Bot. 66 (2015) 1801–1816.

[27] R.A. Jensen, The shikimate arogenate pathway - link between carbohydrate-metabolism and secondary metabolism, Physiol. Plant. 66 (1986) 164–168.

[28] D.L. Siehl, in: R.M. Roe, J.D. Burton, R.J. Kuhr (Eds.), Hebicide Act. Toxicol. Biochem. Mol. Biol., IOS press, Amsterdam, 1997: pp. 37–67.

[29] P.D. Vivancos, S.P. Driscoll, C. a Bulman, L. Ying, K. Emami, A. Treumann, C. Mauve, G. Noctor, C.H. Foyer, 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 (2011) 256–268.

[30] C. Wang, W. ChingYuh, C. Wang, Effect of glyphosate on aromatic amino acid metabolism in purple nutsedge (*Cyperus rotundus*), Weed Technol. 15 (2001) 628–635.

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

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

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

[34] W.E. Cooley, C.L. Foy, Efects of SC-0224 and glyphosate on free amino-acids, soluble-protein, and protein-synthesis in inflated duckweed (*Lemna gibba*), Weed Sci. 40 (1992) 345–350.

[35] R.E. Hoagland, Effects of glyphosate on metabolism of phenolic compounds: VI. Effects of glyphosine and glyphosate metabolites on phenylalanine ammonia-lyase activity, growth and protein, chlorophyll and anthocyanin levels in soybean (*Glycine max*) seedlings, Weed Sci. 28 (1980) 393–400.

[36] R.E. Hoagland, Acifluorfen action on growth and phenolic metabolism in soybean (*Glycine max*) seedlings, Weed Sci. 37 (1989) 743–747.

[37] R. Marchiosi, M.D.L. Lucio Ferrarese, E.A. Bonini, N.G. Fernandes, A.P. Ferro, O. Ferrarese-Filho, Glyphosate-induced metabolic changes in susceptible and glyphosate-resistant soybean (*Glycine max* L.) roots, Pestic. Biochem. Physiol. 93 (2009) 28–33.

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

[39] K. Hahlbrock, D. Scheel, Biology of phenylpropanoid metabolism , Annu. Rev. Plant Physiol. Plant Mol. Biol. 40 (1989) 347–369.

[40] C. Leuschner, K.M. Herrmann, G. Schultz, The metabolism of quinate in pea roots - purification and partial characterization of a quinate hydrolyase, Plant Physiol. 108 (1995) 319–325.

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

[42] V. Tzin, G. Galili, New insights into the shikimate and aromatic amino acids biosynthesis pathways in plants, Mol. Plant. 3 (2010) 956–972.

[43] V. Tzin, G. Galili, The biosynthetic pathways for shikimate and aromatic amino acids in *Arabidopsis thaliana*, in: Arab. B., 2010: p. e0132.

[44] V. Tzin, S. Malitsky, M.M. Ben Zvi, M. Bedair, L. Sumner, A. Aharoni, G. Galili, 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 (2012) 430–439.

[45] M. Oliva, R. Ovadia, A. Perl, E. Bar, E. Lewinsohn, G. Galili, M. Oren-Shamir, Enhanced formation of aromatic amino acids increases fragrance without affecting flower longevity or pigmentation in *Petunia* × *hybrida*, Plant Biotechnol. J. 13 (2015) 125–136.

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

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

[48] R. Pratelli, G. Pilot, Regulation of amino acid metabolic enzymes and transporters in plants, J. Exp. Bot. 65 (2014) 5535–5556.

[49] V.I. Osipov, I. V. Shein, Role of quinic acid in biosynthesis of lignin in Scotch pine, Sov. Plant Physiol. 37 (1990) 395–401.

[50] L.D. Beaudoin-Eagan, T.A. Thorpe, Turnover of shikimate pathway metabolites during shoot initiation in tobacco callus cultures, Plant Cell Physiol. 25 (1984) 913–921.

[51] E.A. Maher, N.J. Bate, W.T. Ni, Y. Elkind, R.A. Dicon, C.J. Lamb, Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products, Proc. Natl. Acad. Sci. USA. 91 (1994) 7802–7806.

[52] A. Kirakosyan, P. Kaufman, S. Warber, S. Zick, K. Aaronson, S. Bolling, S. Chul Chang, Applied environmental stresses to enhance the levels of polyphenolics in leaves of hawthorn plants, Physiol. Plant. 121 (2004) 182–186.

[53] M.H. Kweon, H.J. Hwang, H.C. Sung, Identification and antioxidant activity of novel chlorogenic acid derivatives from bamboo (*Phyllostachys edulis*), J. Agric.

Food Chem. 49 (2001) 4646–4655.

FIGURE CAPTIONS

Fig. 1 A metabolic map describing the time course pattern of specific metabolites of phenylpropanoid metabolism, including the aromatic amino acid biosynthesis pathway in roots of pea plants. Detected metabolites in this study are presented in bold, and the time course of controls (white circles) and glyphosate treated plants (closed circles) is presented in the nearest graph to each specific name. Enzymatic activities are embedded in boxes. The dashed black arrows represent several consecutive enzymatic steps.
Abbreviations: DHAPS, 3-deoxy-D-arabino-heptulosonate-7-phosphate-synthase; EPSPS, 5-enolpyruvoylshikimate-3-phosphate-synthase; CM, chorismate mutase; AS, anthralinate synthase; TYR, tyrosine; PHE, phenylalanine; TRP, tryptophan; PAL, phenylalanine ammonia lyase; HBA, hydroxybenzoic acids; HCA, hydroxycinnamic acids.
Axis-units: Major ticks in the X-axis denote time: 0-5-10-15 days after treatment. Metabolites are shown as μg DW^{-1}, except for SHI (mg DW^{-1}); Anthocyanins (difference of $mAbs_{525}$-mA_{585nm} per g^{-1} fresh weight in 1 ml extract) and Lignin (relative units). PAL activity is expressed as nmol cinnamic acid mg^{-1}prot h^{-1}.

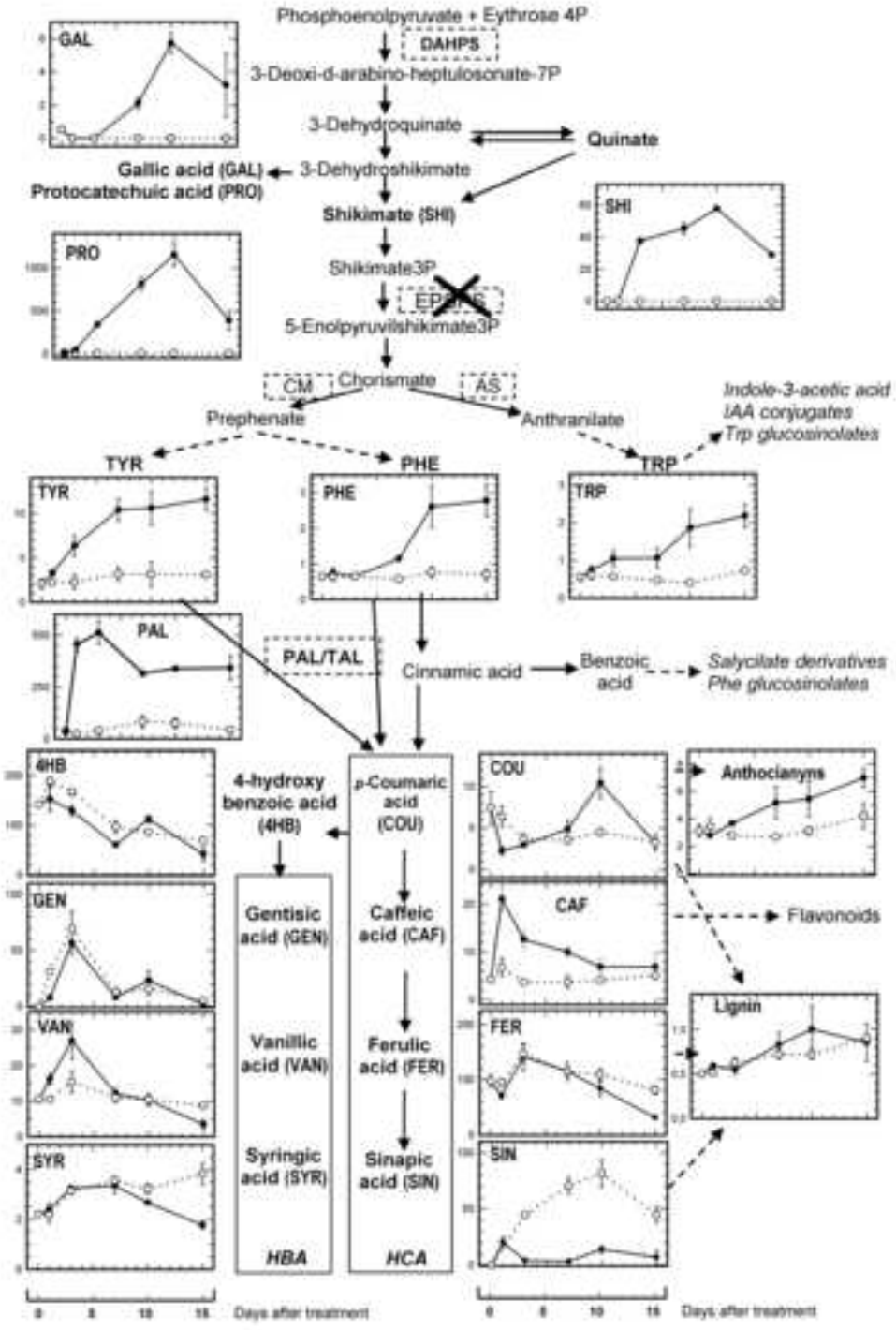
Fig. 2. Immunoblots of 3-deoxy-D-arabino-heptulosonate-7-phosphate-synthase in roots of control (C) pea plants or those treated with glyphosate (GLP) or quinate (QUI); 3 days, 7 days, 10 days, or 15 days after treatment. Each lane contains 40 μg of protein.

Fig. 3. A metabolic map describing the time course pattern of specific metabolites of phenylpropanoid metabolism, including the aromatic amino acid biosynthesis pathway in roots of pea plants. Detected metabolites in this study are presented in bold, and the time course of control (white triangles) and quinate treated plants (closed triangles) are shown in the nearest graph to each specific name. Enzymatic activities are embedded in boxes. The dashed black arrows represent several consecutive enzymatic steps.
Abbreviations: DHAPS, 3-deoxy-D-arabino-heptulosonate-7-phosphate-synthase; EPSPS, 5-enolpyruvoylshikimate-3-phosphate-synthase; CM, chorismate mutase; AS, anthralinate synthase; TYR, tyrosine; PHE, phenylalanine; TRP, tryptophan; PAL, phenylalanine ammonia lyase; HBA, hydroxybenzoic acids; HCA, hydroxycinnamic acids.

Axis-units: Major ticks in the X-axis denote time: 0-5-10-15 days after treatment. Metabolites are shown as μg DW^{-1}, except for SHI and QUI (mg DW^{-1}); Anthocyanins (difference of $mAbs_{525}$-mA_{585nm} per g^{-1} fresh weight in 1 ml extract) and Lignin (relative units). PAL activity is expressed as nmol cinnamic acid mg^{-1}prot h^{-1}.

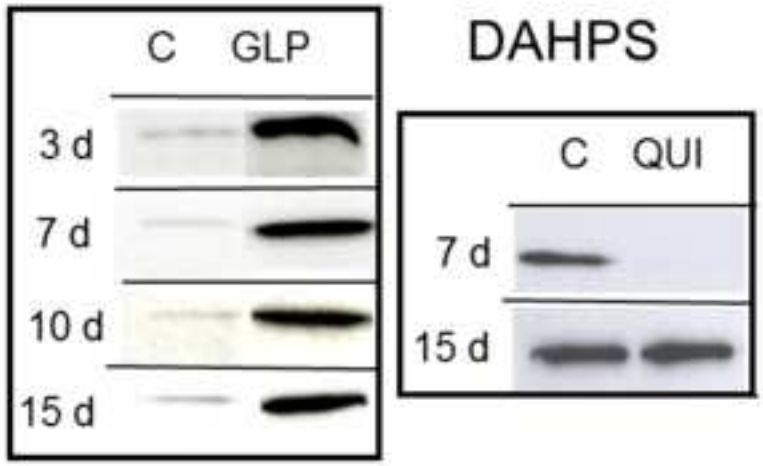
Figure(s)

Click here to download high resolution image



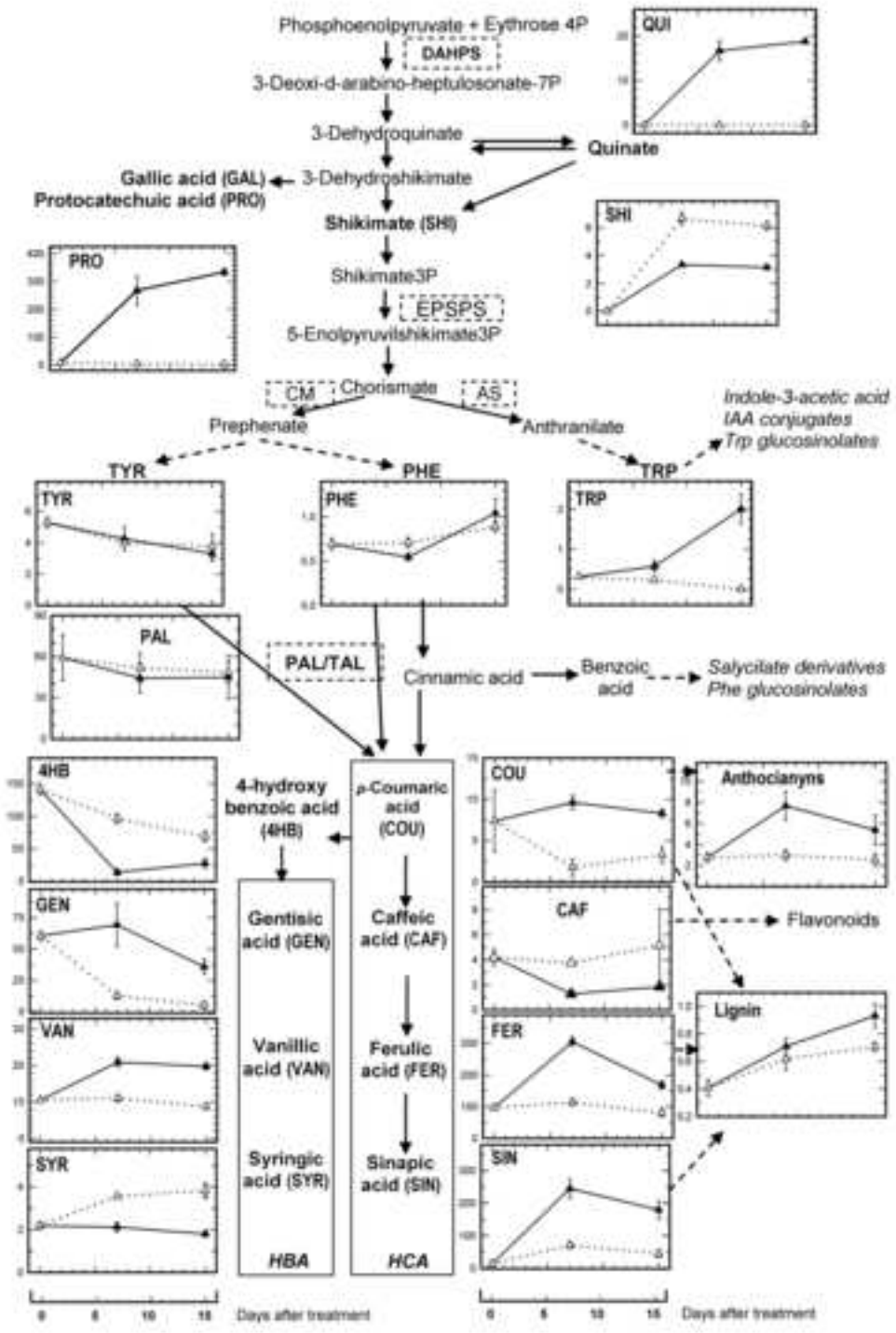
Figure(s)

Click here to download high resolution image



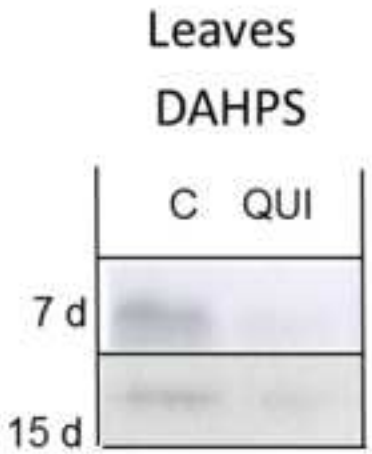
Figure(s)

Click here to download high resolution image



Figure(s)

Click here to download high resolution image



Supplementary Fig. 1
Immunoblots of DAHPS in leaves of control (C) pea plants or those treated with quinate (QUI), 7 days, or 15 days after treatment. Each lane contained 40 µg of protein.