



Both foliar and residual applications of herbicides that inhibit amino acid biosynthesis induce alternative respiration and aerobic fermentation in pea roots

Journal:	<i>Plant Biology</i>
Manuscript ID:	Draft
Manuscript Type:	Research Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Armendariz, Oscar; Universidad Publica de Navarra, Miriam, Gil-Monreal; Universidad Publica de Navarra, Ciencias Medio Natural Zulet, Amaia; Universidad Publica de Navarra, Ciencias Medio Natural Zabalza, Ana; Universidad Publica de Navarra, Ciencias Medio Natural Royuela, Mercedes; Universidad Publica de Navarra, Ciencias Medio Natural
Keyword:	Imazamox, glyphosate, fermentation, alternative respiratory pathway, acetolactate synthase inhibitor, imidazolinone, 5-enolpyruvylshikimate-3-phosphate synthase

1 *Running Head:* Common toxicity of imazamox and glyphosate

2

3 **Both foliar and residual applications of herbicides that inhibit**
4 **amino acid biosynthesis induce alternative respiration and**
5 **aerobic fermentation in pea roots**

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22 **Keywords**

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Imazamox, glyphosate, fermentation, alternative respiratory pathway, acetolactate

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synthase inhibitor, imidazolinone, 5-enolpyruvylshikimate-3-phosphate synthase

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30 **Abbreviations**

31 ADH, Alcohol dehydrogenase; ALS, Acetolactate synthase; AOX, Alternative oxidase;
32 EPSPS, 5-Enolpyruvylshikimate-3-phosphate synthase; GLP, Glyphosate; IMX,
33 Imazamox; PDC, Pyruvate decarboxylase

34

35

ABSTRACT

36

37 The objective of this work was to ascertain whether there is a general pattern of
38 carbon allocation and utilization in plants following herbicide supply, independent of
39 the site of application: sprayed on the leaves or supplied to the nutrient solution. The
40 herbicides studied were the amino acid biosynthesis-inhibiting herbicides (ABIH):
41 glyphosate, an inhibitor of aromatic amino acid biosynthesis, and imazamox, an
42 inhibitor of branched-chain amino acid biosynthesis. All treated plants showed impaired
43 carbon metabolism. Carbohydrate accumulation was detected in both the leaves and
44 roots of the treated plants. The accumulation in the roots was due to a lack of utilization
45 of available sugars as growth was arrested, which elicited soluble carbohydrate
46 accumulation in the leaves due to a decrease in sink strength. Under aerobic conditions,
47 the ethanol fermentative metabolism was enhanced in the roots of the treated plants.
48 This fermentative response was not related to a change in the total respiratory rates or
49 cytochrome respiratory capacity, but an increase in the alternative oxidase capacity was
50 detected. Pyruvate accumulation was detected after most of the herbicide treatments.
51 These results demonstrate that both ABIH induce the less-efficient ATP-producing
52 pathways, namely fermentation and alternative respiration, **probably** by increasing the
53 key metabolite pyruvate. The plant response was similar not only for the two ABIH but
54 also after foliar or residual applications.

55 INTRODUCTION

56 The first commercialized herbicide that specifically inhibits the biosynthesis of
57 amino acids was glyphosate (GLP), which was developed in the early 1970s and is still
58 of great agronomic and commercial importance. The inhibition of plant amino acid
59 biosynthesis has become a major target of herbicide development, as only plants and
60 microorganisms can synthesize all required amino acids themselves. Consequently, the
61 amino acid biosynthesis inhibiting herbicides (ABIH), which act upon the essential
62 amino acid synthesis pathway, are very likely not toxic to animals. Two sites of amino
63 acid biosynthesis are important targets for herbicide action and correspond to two
64 enzymes in different biosynthetic pathways: acetolactate synthase (ALS, EC 2.2.1.6;
65 also termed acetohydroxyacid synthase) in the branched-chain amino acid biosynthesis
66 pathway and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) in the
67 aromatic amino acid biosynthesis pathway (Duke 1990).

68 ALS is the first common enzyme in the biosynthesis of the branched-chain
69 amino acids (valine, leucine and isoleucine). ALS inhibitors have become one of the
70 most important herbicide groups because of their wide-spectrum weed control activity,
71 high crop selectivity, low application rates and low mammalian toxicity (Zhou *et al.*
72 2007). More than 40 structurally different active ingredients have ALS as their primary
73 target. Imazamox (IMX, 2-[(RS)-4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]-5-
74 methoxymethylnicotinic acid) is one of these active ingredients that belongs to the
75 imidazolinone chemical family. On the other hand, EPSPS is an enzyme in the
76 biosynthesis of aromatic amino acids (tyrosine, phenylalanine and tryptophan) and is
77 the target for the herbicide glyphosate (Steinrucken & Amrhein 1980). GLP (*N*-
78 (phosphonomethyl) glycine) is a wide-spectrum, non-selective postemergence
79 herbicide that is currently the most popular herbicide. Biotechnology has increased the

80 importance of GLP in weed management, as GLP-tolerant crops (maize, soybean,
81 cotton, and canola) have been commercialized (Schmid & Amrhein 1999; Tan *et al.*
82 2006).

83 Although both ALS and EPSPS are nuclear-encoded enzymes, their catalytic site
84 is located in the plastid (Schmid & Amrhein 1999; Singh 1999). Both activities are
85 well-established herbicide target sites, but it is not fully understood how plants actually
86 die after the inhibition of ALS or EPSPS, and the sequence of events from herbicide
87 application to plant death is still being debated.

88 Both types of herbicides produce plant growth arrest and a slow death in the
89 treated plants (Gruys & Sikorski 1999; Wittenbach & Abell 1999). Many important
90 effects following treatments with one or the other ABIH have been described.
91 Interestingly, they have been shown to provoke some similar physiological effects in
92 plants: a general increase in total free amino acid content (Shaner & Reider 1986; Wang
93 2001) with a transient decrease in the proportion of the amino acids whose pathways are
94 specifically inhibited, quinate and carbohydrate accumulation (Orcaray *et al.* 2010) and
95 the induction of fermentation and the alternative oxidase (AOX) pathway (Gaston *et al.*
96 2003; Zabalza *et al.* 2005; Zulet *et al.* 2015). The increase in total free amino acid
97 content is a rapid response common to both types of herbicides and may be the key of
98 the common response that has been associated to a plant proteolysis response (Zulet *et*
99 *al.* 2013).

100 ALS inhibitors and GLP have been reported to alter the carbon metabolism of
101 plants that are treated through the root system. An accumulation of carbohydrates has
102 been described in both the leaves and roots of treated plants (Zabalza *et al.* 2004;
103 Orcaray *et al.* 2012). Carbon consumption is diverted to the low-efficiency fermentative
104 and alternative respiratory pathways in the ABIH-treated roots (Gaston *et al.* 2002,

105 2003; Zabalza *et al.* 2005; Orcaray *et al.* 2012). This impairment indicates that the
106 effect of these herbicides on the primary metabolism has broader physiological
107 consequences than solely the lack of certain amino acids. However, all of these studies
108 reporting an altered carbon metabolism were performed with herbicides that were
109 applied to the root system. It is unclear if this effect is a consequence of amino acid
110 biosynthesis inhibition or a general stress response of the root system. There have been
111 no exhaustive studies on the effect of sprayed herbicides on these parameters. The way
112 in which the herbicide is applied (sprayed on the leaves or supplied to the roots) may
113 affect the physiological response of the treated plants. Additionally, it is unknown
114 whether the induction of fermentation and alternative respiration in roots following ALS
115 or EPSPS inhibition is related to any change in the pyruvate content. Pyruvate is the
116 substrate of pyruvate decarboxylase (PDC; EC 4.1.1.1), the first enzyme in ethanol
117 fermentation, and it has been reported to have a significant stimulating effect on the
118 activity of alternative oxidase (Millar *et al.* 1993; Vanlerberghe *et al.* 1995). Based on
119 these results, we may expect to observe changes in the concentration of this key
120 metabolite in the ABIH-treated plants; however, no in-depth studies have monitored the
121 possible effects of these herbicides on pyruvate content, to the authors' knowledge.

122 The objectives of this work were to first ascertain whether there is a general
123 pattern of carbon allocation and utilization in the plant following IMX or GLP
124 treatment, independent of the site of application (sprayed on the leaves or supplied to
125 the nutrient solution); and second, to study if the changes in the carbon utilization
126 pathway were related to the changes in the availability of the key metabolite pyruvate.
127 For these purposes, the carbohydrate content in the leaves and roots, ethanol
128 fermentation, alternative respiration and the pyruvate content in roots were studied in

129 pea plants treated with IMX or GLP that was sprayed onto the foliage or supplied to the
130 nutrient solution.

131

132 MATERIAL AND METHODS

133 Plant material and treatment application

134 Seeds of pea *Pisum sativum* L. cv. Snap Sugar Boys surface sterilized, were grown in
135 vermiculite for 3 days at 26 °C in darkness, prior to transfer to hydroponic tanks filled
136 with nutrient solution ((Rigaud & Puppo 1974), supplemented with 10 mM KNO₃) and
137 placed in a growth chamber. (Zabalza et al. 2005). Nutrient solution (2.7 l tank⁻¹) was
138 aerated continuously (700 ml tank⁻¹ min⁻¹) and renewed every 3 days.

139 At 12 days of age, the plants were divided into two groups, one to assess the nutrient
140 solution treatments, and the other one to assess the spray treatments to the leaves. In
141 each group, one-third of the plants was not treated and served as the control treatment,
142 and the other two-thirds were treated with GLP or IMX. The experiment was repeated
143 twice, and in each experiment both groups were evaluated.

144 The herbicide (IMX and GLP) concentrations necessary to induce similar effects (in
145 intensity and speed) in peas after application to the roots or to leaves were determined in
146 preliminary studies. The herbicide concentrations in the nutrient solution were
147 maintained constant throughout the experiment: IMX 5 mg active ingredient l⁻¹ (16
148 µM), and GLP 53 mg active ingredient l⁻¹ (234 µM). The herbicides applied to the
149 leaves were sprayed with a mechanical sprayer. IMX was sprayed on the plants at a
150 concentration of 375 mg active ingredient l⁻¹ (1.23 mM), and GLP was sprayed at 875
151 mg active ingredient l⁻¹ (3.86 mM).

152 The root and shoot lengths were measured, and gas exchange measurements
153 were conducted at 0, 1, 3 and 7 days after treatment. The leaf and root samples were

154 taken 4 h after the beginning of the photoperiod at 0, 1, 3 and 7 days after herbicide
155 treatment for analytical determinations. The plant material was immediately frozen in
156 liquid nitrogen and stored at -80°C . Some material was dried for 48 hours at $75-80^{\circ}\text{C}$ to
157 obtain the fresh weight/dry weight ratio.

158

159 **Gas exchange measurements**

160 The net CO_2 assimilation rate was measured in intact plants in the youngest fully
161 expanded leaf using a portable ADC-LCi system (ADC BioScientific Ltd., Herts
162 England). The leaf area was determined using an Li-3000 system (Li-Cor, Lincoln,
163 Nebraska, USA).

164

165 **Carbohydrate determination**

166 The glucose, fructose and sucrose concentrations were determined in ethanol-
167 soluble extracts, and the ethanol-insoluble residue was extracted for starch analysis.
168 Starch and soluble sugar concentrations were determined using high-performance
169 capillary electrophoresis as previously described (Zabalza *et al.* 2004).

170

171 **Pyruvate determination**

172 Ground root samples (0.1 g) were homogenized in 1 M HCl. The extracts were
173 centrifuged at 20,000 g for 30 min. The supernatants were used for pyruvate
174 determination after filtration by two ion exchange cartridges (OnGuard II Ag, OnGuard
175 II H, Dionex Corporation, Sunnyvale, CA, USA). The pyruvate levels were analysed by
176 ion chromatography in a DX-500 system (Dionex Corporation, Sunnyvale, CA, USA)
177 by gradient separation with Dionex Ion pack AG11+AS11 columns (0.2 mM NaOH for

178 5 min, up to 25 mM NaOH in 10 min, and 35 mM NaOH for 10 min, at a flux of 1 ml
179 min⁻¹).

180

181 **Respiration and fermentation measurements**

182 Respiratory oxygen consumption was measured using Clark-type electrodes
183 (Rank Brothers, Bottisha, UK) in small (5-10 mm) root pieces as previously described
184 (Zabalza *et al.* 2009). Total respiration was measured as O₂ uptake in the absence of any
185 inhibitor. To measure the capacities of the cytochrome oxidase and AOX pathways,
186 different inhibitors were directly added to the cuvette. The capacity of cytochrome
187 oxidase was determined as the KCN-sensitive O₂ uptake in the presence of SHAM, and
188 the capacity of AOX was determined as the SHAM-sensitive O₂ uptake in the presence
189 of KCN.

190 The PDC and alcohol dehydrogenase (ADH) activities were assayed in the
191 desalted extract as previously described (Gaston *et al.* 2002). The protein blots were
192 produced according to standard techniques as previously described (Zabalza *et al.*
193 2009).

194

195 **Statistics**

196 Each mean value was calculated using samples from different individual plants
197 from **both experiments** as replicates. Both types of herbicide application were applied in
198 independent **groups in the two experiments**, and statistical studies were performed for
199 each type of treatment. One-way ANOVA for each day was used to determine the
200 significance of the differences. The means were separated using the least significant
201 difference ($p < 0.05$). The significant differences between each treatment and the
202 control plants (untreated plants) are highlighted in the figures with a different symbol

203 for each treatment. A previously described transformation to arcsine $\sqrt{x}/100$ was used
204 when the values analysed were percentages.

205

206 **RESULTS AND DISCUSSION**

207 **Effects of the herbicide treatments on growth and photosynthesis.**

208 Preliminary studies were conducted to determine the GLP and IMX herbicide
209 concentrations to be sprayed, with similar effects on growth to those described after
210 supplying 53 mg l⁻¹ GLP (Orcaray *et al.* 2012) and 5 mg l⁻¹ IMX (Zulet *et al.* 2013) to
211 the nutrient solution. The effects of the four treatments studied are shown in Fig. 1. In
212 all of the studied treatments, an arrest of shoot elongation was observed, which was
213 significant by the seventh day (Fig. 1a, c). Root growth arrest was more evident and was
214 significant after 3 days when herbicides were sprayed to the leaves (Fig. 1b, d). In all
215 cases, the plants died after approximately 20 days. The concentrations used were
216 suitable to compare the initial toxic consequences of IMX and GLP because the
217 measurements performed in this study were performed in the initial phase of the
218 treatment (up to one week). This allowed us to evaluate the effects when plant viability
219 was not compromised and over a short time period to avoid secondary reactions from
220 these treatments that would be difficult to explain.

221 Both ABIH have been reported to arrest the growth of the treated plants, which
222 is followed by a slow death (Gruys & Sikorski 1999; Wittenbach & Abell 1999). GLP-
223 treated plants may not show physical symptoms of the treatment for 7-10 days. When
224 they appear, the GLP symptoms include growth inhibition, chlorosis, necrosis and
225 subsequent plant death (Gruys & Sikorski 1999). For the ALS inhibitors, physical
226 symptoms may also take days to develop and include chlorosis and necrosis in young
227 meristemic regions of both shoots and roots, in addition to growth inhibition. Plant

228 death may occur at up to two months, when weed growth is slow (Wittenbach & Abell
229 1999).

230 A decline in the net photosynthesis was observed in the ABIH-treated plants.
231 When the herbicides were supplied to the nutrient solution, the net photosynthetic
232 decline was significant after 1 day for GLP and after 7 days for IMX (Fig. 2a). The
233 decrease in photosynthesis was significant after 3 and 7 days when IMX or GLP was
234 sprayed on the leaves (Fig. 2b). Thus, the net photosynthesis was similarly affected by
235 the four treatments at the end of the study period (7 days).

236

237 **Effects of the herbicide treatments on carbon allocation: carbohydrate** 238 **accumulation in leaves and roots**

239 The carbohydrate, starch and total soluble sugar (sum of glucose, fructose and
240 sucrose) concentrations in the leaves are shown in Fig 3. In general, all of the applied
241 treatments resulted in carbohydrate accumulation in the leaves in the form of total
242 soluble sugars (Fig 3a, c). However, starch accumulation in the leaves was only detected
243 when IMX was supplied to the nutrient solution and was not detected after the other
244 treatments (Fig. 3b, d). Similar to the leaves, carbohydrate accumulation was found in
245 the roots of the treated plants (Fig. 4). In the four studied treatments, sucrose was
246 accumulated in roots from the first day of the treatment (Fig. 4 a, c). Starch
247 accumulation in roots was consistently induced after IMX treatments (both supplied to
248 the nutrient solution and sprayed to the leaves) throughout the experiment, while the
249 GLP treatments had no effect on this parameter (Fig. 4 b, d).

250 The simultaneous determination of the carbohydrate content in the leaves and
251 the roots facilitates the evaluation of the herbicide effect on phloem transport and
252 indicates a general effect on carbon allocation within the plant for both types of

253 herbicides and both sites of application. The accumulation of carbohydrates in the roots
254 occurred prior to the accumulation in the leaves, suggesting that sucrose is transported
255 from the leaves to the roots at a higher rate than it is utilized in the sinks. Under these
256 circumstances, the sugar gradient required for long-distance transport is abolished; thus,
257 phloem transport is inhibited, showing that the carbohydrate accumulation in the leaves
258 of the treated plants reflects a reduction in sink strength, as was proposed for the ALS
259 inhibitors (Zabalza *et al.* 2004).

260 It is noticeable that while photosynthesis declined by day 3 in almost all
261 treatments (Fig. 2), the carbon assimilation rates allowed carbon to accumulate in the
262 leaves and roots throughout the study. Indeed, the sugars accumulated in leaves (Fig. 3)
263 and roots (Fig. 4) of treated plants did not tend to decrease, and sugar accumulation was
264 even enhanced over time, demonstrating a lack of utilization. The growth arrest detected
265 after IMX or GLP treatment was not due to a lack of respiratory substrates, and it
266 suggests that metabolism was impaired, which does not facilitate the utilization of the
267 available carbohydrates at the expected rates.

268

269 **Effects of the herbicide treatments on carbon utilization by the roots:**

270 **induction of AOX and fermentation**

271 To evaluate a common effect of both types of inhibitors on the carbon utilization
272 by the roots, the main respiratory parameters and ethanol fermentation were monitored,
273 as both parameters have been reported to be affected by IMX and GLP when supplied to
274 the nutrient solution (Gaston *et al.* 2002, 2003; Zabalza *et al.* 2005, 2011; Orcaray *et al.*
275 2012).

276 Total respiratory O₂ uptake was not significantly affected by any of the tested
277 treatments (Fig. 5). A transient decrease in the root respiratory rate was only detected

278 after 3 days of spraying GLP onto the leaves (Fig. 5d). Previous studies also showed
279 that GLP did not affect the respiration rate (Aubert *et al.* 1997; Orcaray *et al.* 2012).
280 However, other ALS inhibitors have been reported to increase the respiratory rate in the
281 short term when supplied to the nutrient solution (Gaston *et al.* 2003; Zabalza *et al.*
282 2011). Although the total O₂ uptake was not affected, the capacities of the cytochrome
283 and AOX respiration pathways were studied. The capacity of the cytochrome pathway
284 was not affected by ABIH, but the capacity of the AOX pathway increased in the treated
285 plants (Fig. 5c, f). The pattern of increase was similar for both GLP and IMX
286 application methods. While IMX only induced the capacity of the AOX pathway at the
287 end of the study, GLP induced it beginning at day 3. Previous studies have shown an
288 induction of the AOX pathway after ALS inhibition (Aubert *et al.* 1997; Gaston *et al.*
289 2003; Zulet *et al.* 2015). Although other studies (Zhu *et al.* 2008; Zulet *et al.* 2015)
290 revealed an increased level of AOX after GLP treatment by gene expression profiling,
291 to our knowledge, this is the first study where an increase in the alternative pathway
292 capacity after GLP treatment has been shown by polarographic methods. Although the
293 physiological role of this pathway remains to be clarified, several potential functions of
294 AOX have been identified. AOX can help alleviate the effects of several stresses by
295 avoiding the over-reduction of the electron transfer and helping to reduce the electron
296 flow through the cytochrome oxidase pathway (Siedow & Umbach 1995). Therefore,
297 AOX induction has been related to stresses where the cytochrome pathway is restricted
298 (Vanlerberghe & McIntosh 1997; Parsons *et al.* 1999; Amor *et al.* 2000; Vanlerberghe
299 *et al.* 2002).

300 The induction of aerobic fermentation in roots is a common effect of ALS and
301 EPSPS inhibitors applied to the roots (Zabalza *et al.* 2005; Orcaray *et al.* 2012; Zulet *et*
302 *al.* 2015). It remains to be determined if this effect in the roots is also detected when the

303 ABIH are sprayed onto the foliage. The specific activities of both PDC and ADH were
304 increased following all of the evaluated treatments (Fig. 6). Protein blotting showed that
305 these increases correlated with increases in the amounts of the respective proteins (Fig.
306 6e).

307 Although PDC activity was significantly increased by supplying IMX to the
308 nutrient solution during the entire study, the most prominent increase was detected in
309 the first 24 h, which decreased to values similar to those of control plants at day 7 (Fig.
310 6a). Similarly, PDC increased after IMX was sprayed onto the foliage, but it was only
311 significant after 1 day of treatment (Fig. 6c). The effect of GLP on PDC and the effect
312 of the four treatments on ADH increased over time, reaching the highest differences
313 after 7 days of treatment (Fig. 6). Our results show that fermentation induction under
314 aerobic conditions is a common effect of the four treatments. It has been proposed that
315 fermentation has a general function in aerobic metabolism under stress conditions
316 (Tadege *et al.* 1999), and it has been reported recently in *Nicotiana sylvestris* plants
317 with an altered respiratory complex I (Shah *et al.* 2013). Usually, a decrease in the
318 energy state of the tissue correlates well with the fermentation induction under low-
319 oxygen conditions. However, in plants treated with ALS inhibitors or GLP, the
320 adenylate energy charge was similar to or higher than the untreated plants (Zabalza *et*
321 *al.* 2011; Orcaray *et al.* 2012).

322 Pyruvate availability has been proposed to be related to the induction of
323 fermentation under aerobic conditions after ALS inhibition. Pyruvate is a common
324 substrate of ALS and PDC; thus, ALS inhibition would involve an increased availability
325 of pyruvate for use by other enzymes such as PDC. Moreover, pyruvate has been
326 described to act as an allosteric activator of AOX. Aubert *et al.* (1997) tested several
327 metabolites known to be accumulated after ALS inhibition (α -oxobutyrate and α -

328 aminobutyrate), but none of them activated AOX. However, pyruvate accumulation
329 after ALS inhibition has not yet been studied in depth, and it remained to be determined
330 whether GLP affects the concentration of this metabolite. Therefore, the pyruvate
331 concentration was measured in the roots of pea plants that were treated with IMX or
332 GLP either in the nutrient solution or sprayed onto the leaves.

333

334 ***Herbicide-induced pyruvate accumulation in the roots***

335 With the exception of IMX applied to the nutrient solution, the supply of
336 herbicides caused an increase in the root pyruvate concentration (Fig. 7). When IMX
337 was sprayed on the leaves, there was a significant increase in the pyruvate level at day 3
338 and day 7. The pattern of this increase was very similar in both GLP treatments and
339 increased from day 3 to day 7.

340 Fermentative induction after GLP treatment was not expected to be related to
341 increased pyruvate content, as this herbicide does not inhibit a pyruvate-consuming
342 enzyme. However, pyruvate was accumulated in the roots after both types of GLP
343 application. The inhibition of the shikimate pathway at the EPSPS level deregulates the
344 carbon flow into the pathway, causing a massive carbon entrance that accumulates in
345 compounds upstream of the EPSPS inhibition point, such as shikimate (Orcaray *et al.*
346 2010). It has been proposed that this shikimate accumulation would divert most of the
347 phosphoenolpyruvate from the glycolytic flow to the shikimate pathway because
348 shikimate is a potent phosphoenolpyruvate carboxylase inhibitor (De María *et al.* 2006).
349 It can be proposed that the pyruvate accumulation after GLP treatment is a cross-
350 physiological effect that is induced by increased availability of phosphoenolpyruvate
351 that is not being consumed by phosphoenolpyruvate carboxylase.

352 Because pyruvate is the main substrate of ALS, we would expect that the
353 pyruvate concentrations may be increased in plants treated with IMX supplied to the
354 nutrient solution, as has been previously reported in soybean roots (Gaston *et al.* 2003).
355 Nevertheless, it was only possible to detect pyruvate accumulation when this herbicide
356 was sprayed onto the foliage.

357 The different effects of the two herbicides on the pyruvate concentrations may
358 be related to the different patterns detected for PDC, which is an enzyme that uses
359 pyruvate as a substrate (Fig. 6a). The highest PDC induction was detected only in the
360 case of IMX supplied to the nutrient solution as soon as with 1 day of treatment,
361 suggesting that this peak activity may be related to a higher pyruvate consumption that
362 would result in the subsequent prevention of pyruvate accumulation.

363 In all treatments, our results show that the pyruvate accumulation in the roots
364 (Fig. 7) was related to a concomitant increase in the alternative respiratory capacity
365 (Fig. 5c, f). Other studies have shown that increases in the AOX pathway (both in AOX
366 protein levels and activation state) are related to increases in the pyruvate content
367 (Gaston *et al.* 2003; Oliver *et al.* 2008; Dinakar *et al.* 2010).

368 Pyruvate is a key metabolite affecting multiple biosynthetic and catabolic
369 cellular pathways so pyruvate accumulation can be due to multiple causes. Although no
370 conclusive cause-effect relationship can be drawn, a clear relation between pyruvate
371 accumulation and fermentation and alternative oxidase induction can be proposed.
372 What remains to be elucidated if pyruvate accumulation is the only cause or a player in
373 a cascade of signals after herbicide treatment.

374 Carbohydrate accumulation in the leaves and roots and fermentation and
375 alternative oxidase induction in roots have been described as common physiological
376 effects of ALS inhibitors and GLP supplied to the nutrient solution. This study is the

377 first to demonstrate that they are also detected when the herbicides are sprayed onto the
378 leaves.

379 Carbohydrate accumulation following ALS and EPSPS inhibition is most likely
380 related to the observed growth inhibition and to one of the phytotoxic imbalances in the
381 carbon/nitrogen metabolism that is induced by these herbicides. The produced ATP is
382 not being consumed at the expected rate, and thus, the energy charge never becomes
383 limiting. In addition, pyruvate accumulates after the inhibition of other pyruvate-
384 consuming enzymes (ALS) or as a cross-effect of the altered carbon flow after EPSPS
385 inhibition. As a consequence of these two physiological effects, the plants treated with
386 herbicides that inhibit aromatic or branched-chain amino acid biosynthesis activated the
387 less-efficient ATP-producing pathways, that is, alternative respiration and ethanol
388 fermentation.

389 .

390 **CONCLUSIONS**

391 GLP- or IMX-treated plants showed impaired carbon metabolism and provided
392 evidence of similar physiological effects of both types of herbicides. The validity of the
393 results presented has been demonstrated using herbicides supplied to the nutrient
394 solution and sprayed onto the foliage. Carbohydrate accumulation was detected in both
395 the leaves and roots of treated plants. The accumulation in the roots was due to the lack
396 of utilization of available sugars as growth was arrested, which elicited soluble
397 carbohydrate accumulation in the leaves due to a decrease in sink strength. Under
398 aerobic conditions, the ethanol fermentative metabolism was enhanced in the roots of
399 treated plants. This fermentative response was not related to a change in the total
400 respiratory rates or cytochrome respiratory capacity, but an increase in the AOX
401 capacity was observed. Pyruvate accumulation was detected after most of the herbicide

402 treatments. These results suggest that the induction of the less-efficient ATP-producing
403 pathways of fermentation and alternative respiration by ALS and EPSPS inhibitors
404 might be related to an increase in pyruvate. This plant response was produced in the
405 roots of the treated plants, even when the ABIH were sprayed on the leaves, showing
406 that is a common effect after inhibiting both the biosynthesis of aromatic or branched
407 amino acids by herbicides and is independent of the site of application.

408

409 **ACKNOWLEDGEMENTS**

410 We thank Gustavo Garijo for technical assistance. A. Zulet and M. Gil-Monreal
411 received funding from fellowships through the Ministerio de Educación and
412 Universidad Pública de Navarra, respectively. This work was financially supported by a
413 grant from the Ministerio Español de Economía y Competitividad (AGL-2013-4067R).

414

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Figure captions

415 **Fig. 1.** The shoot (a, c) and root (b, d) lengths of the control and imazamox- or
416 glyphosate-treated pea plants. The herbicides were applied to the nutrient solution (a, b)
417 or sprayed onto the leaves (c, d). Each value is the mean \pm standard error (n=10). The
418 circles and triangles on top of each box indicate a significant difference between the
419 control and imazamox-treated (circle) or glyphosate-treated (triangle) plants on a given
420 day ($p \leq 0.05$).

421 **Fig. 2.** Net photosynthesis of the control and imazamox- or glyphosate-treated pea
422 plants. The herbicides were applied to the nutrient solution (a) or sprayed onto the
423 leaves (b). Each value is the mean \pm standard error (n=4). The circles and triangles on
424 top of each box indicate a significant difference between the control and imazamox-
425 treated (circle) or glyphosate-treated (triangle) plants on a given day ($p \leq 0.05$).

426 **Fig. 3.** The total soluble sugars (a, c) and starch concentrations (b, d) in the leaves of the
427 control and imazamox- or glyphosate-treated pea plants. The herbicides were applied to
428 the nutrient solution (a, b) or sprayed onto the leaves (c, d). Each value is the mean \pm
429 standard error (n=4). The circles and triangles on top of each box indicate a significant
430 difference between the control and imazamox-treated (circle) or glyphosate-treated
431 (triangle) plants on a given day ($p \leq 0.05$).

432 **Fig. 4.** The sucrose (a, c) and starch concentrations (b, d) in the roots of the control and
433 imazamox- or glyphosate-treated pea plants. The herbicides were applied to the nutrient
434 solution (a, b) or sprayed onto the leaves (c, d). Each value is the mean \pm standard error
435 (n=4). The circles and triangles on top of each box indicate a significant difference

436 between the control and imazamox-treated (circle) or glyphosate-treated (triangle)
437 plants on a given day ($p \leq 0.05$).

438 **Fig. 5.** The total respiratory rate (a, d), capacity of cytochrome pathway (b, e) and
439 capacity of alternative pathway (c, f) in the roots of the control and imazamox- or
440 glyphosate-treated pea plants. The herbicides were applied to the nutrient solution (a, b,
441 c) or sprayed onto the leaves (d, e, f). Each value is the mean \pm standard error (n=3).
442 The circles and triangles on top of each box indicate a significant difference between the
443 control and imazamox-treated (circle) or glyphosate-treated (triangle) plants on a given
444 day ($p \leq 0.05$).

445 **Fig. 6.** Pyruvate decarboxylase (PDC) (a, c) and alcohol dehydrogenase (ADH) (b, d)
446 activities in the roots of the control or imazamox (IMX)- or glyphosate (GLP) -treated
447 pea plants. The herbicides were applied to the nutrient solution (a, b) or sprayed onto
448 the leaves (c, d). Each value is the mean \pm standard error (n=4). The circles and
449 triangles on top of each box indicate a significant difference between the control and
450 imazamox-treated (circle) or glyphosate-treated (triangle) plants on a given day ($p \leq$
451 0.05). (e) Protein blots of PDC and ADH in the roots of control (C) pea plants or those
452 treated with GLP or IMX for 1, 3 or 7 days. Each lane contains 25 μ g of protein.

453 **Fig. 7.** Pyruvate content of the control and imazamox- or glyphosate-treated pea plants.
454 The herbicides were applied to the nutrient solution (a) or sprayed onto the leaves (b).
455 Each value is the mean \pm standard error (n=4). The circles and triangles on top of each
456 box indicate a significant difference between the control and imazamox-treated (circle)
457 or glyphosate-treated (triangle) plants on a given day ($p \leq 0.05$).

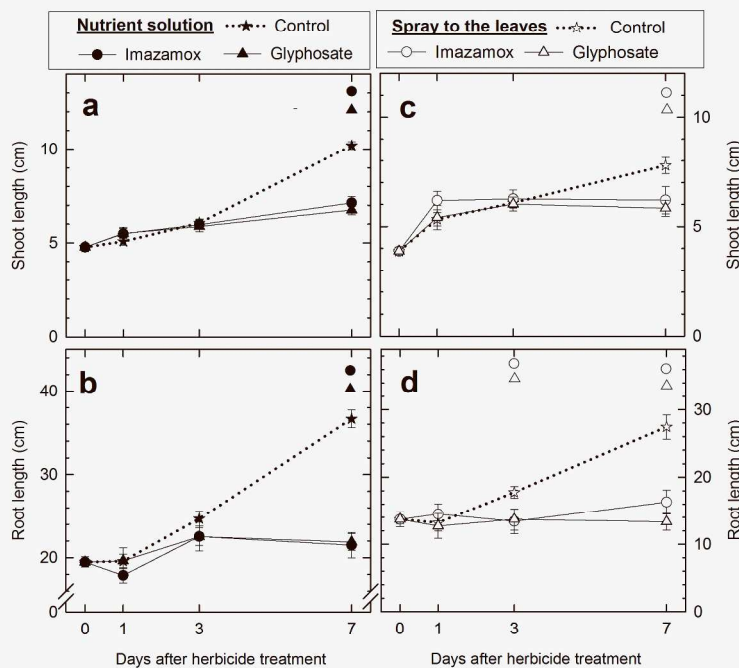


Figure 1

Fig. 1. The shoot (a, c) and root (b, d) lengths of the control and imazamox- or glyphosate-treated pea plants. The herbicides were applied to the nutrient solution (a, b) or sprayed onto the leaves (c, d). Each value is the mean \pm standard error (n=10). The circles and triangles on top of each box indicate a significant difference between the control and imazamox-treated (circle) or glyphosate-treated (triangle) plants on a given day ($p < 0.05$).
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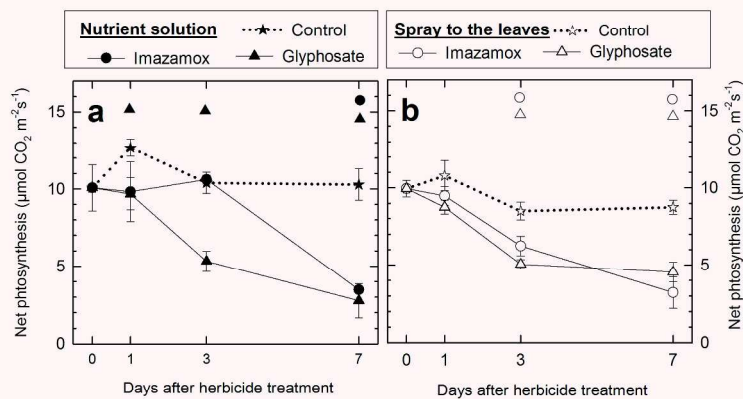


Figure 2

Fig. 2. Net photosynthesis of the control and imazamox- or glyphosate-treated pea plants. The herbicides were applied to the nutrient solution (a) or sprayed onto the leaves (b). Each value is the mean \pm standard error (n=4). The circles and triangles on top of each box indicate a significant difference between the control and imazamox-treated (circle) or glyphosate-treated (triangle) plants on a given day ($p < 0.05$).

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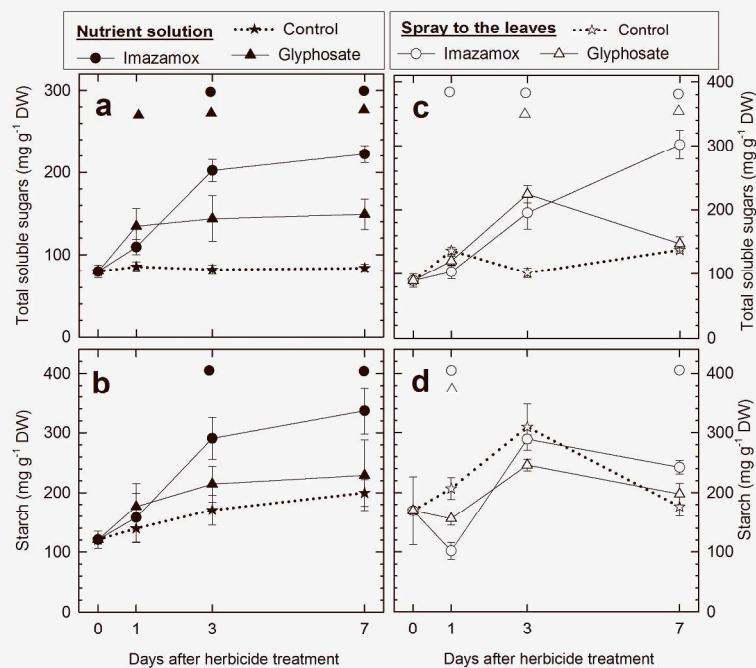


Figure 3

Fig. 3. The total soluble sugars (a, c) and starch concentrations (b, d) in the leaves of the control and imazamox- or glyphosate-treated pea plants. The herbicides were applied to the nutrient solution (a, b) or sprayed onto the leaves (c, d). Each value is the mean \pm standard error (n=4). The circles and triangles on top of each box indicate a significant difference between the control and imazamox-treated (circle) or glyphosate-treated (triangle) plants on a given day ($p < 0.05$).
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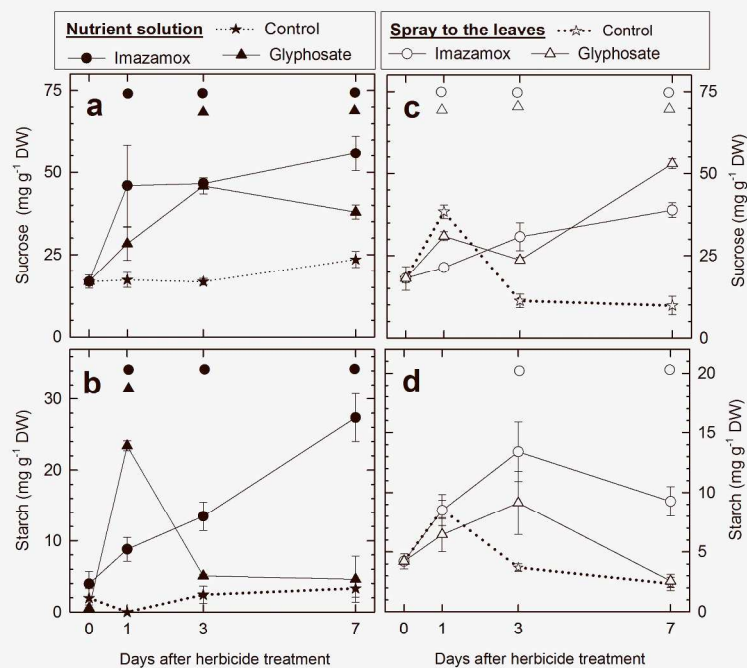


Figure 4

Fig. 4. The sucrose (a, c) and starch concentrations (b, d) in the roots of the control and imazamox- or glyphosate-treated pea plants. The herbicides were applied to the nutrient solution (a, b) or sprayed onto the leaves (c, d). Each value is the mean \pm standard error (n=4). The circles and triangles on top of each box indicate a significant difference between the control and imazamox-treated (circle) or glyphosate-treated (triangle) plants on a given day ($p < 0.05$).

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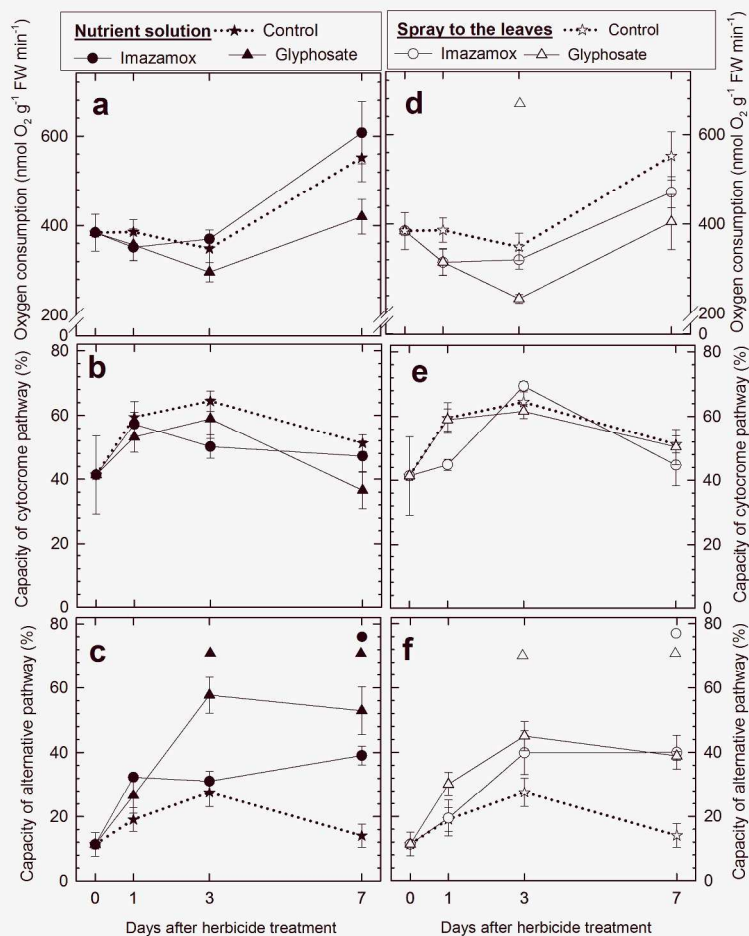


Figure 5

Fig. 5. The total respiratory rate (a, d), capacity of cytochrome pathway (b, e) and capacity of alternative pathway (c, f) in the roots of the control and imazamox- or glyphosate-treated pea plants. The herbicides were applied to the nutrient solution (a, b, c) or sprayed onto the leaves (d, e, f). Each value is the mean \pm standard error (n=3). The circles and triangles on top of each box indicate a significant difference between the control and imazamox-treated (circle) or glyphosate-treated (triangle) plants on a given day ($p < 0.05$).
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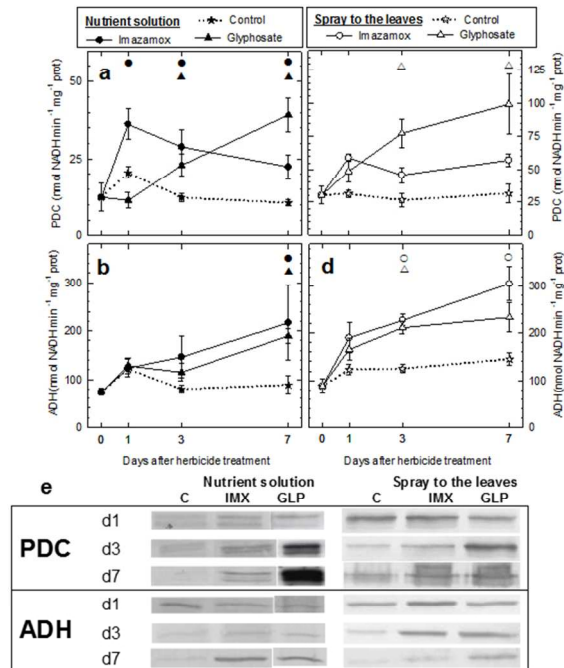


Figure 6

Fig. 6. Pyruvate decarboxylase (PDC) (a, c) and alcohol dehydrogenase (ADH) (b, d) activities in the roots of the control or imazamox (IMX)- or glyphosate (GLP) -treated pea plants. The herbicides were applied to the nutrient solution (a, b) or sprayed onto the leaves (c, d). Each value is the mean \pm standard error ($n=4$).

The circles and triangles on top of each box indicate a significant difference between the control and imazamox-treated (circle) or glyphosate-treated (triangle) plants on a given day ($p < 0.05$). (e) Protein blots of PDC and ADH in the roots of control (C) pea plants or those treated with GLP or IMX for 1, 3 or 7 days. Each lane contains 25 μ g of protein.

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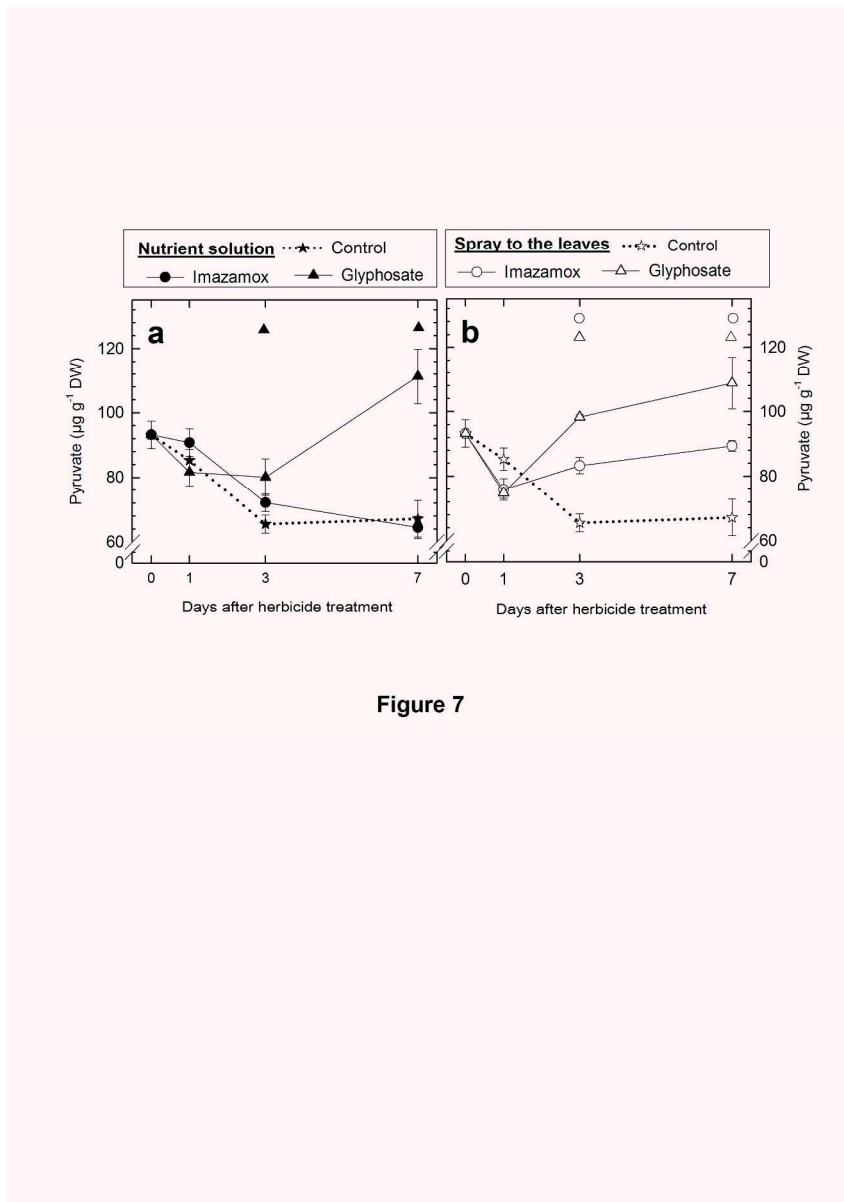


Figure 7

Fig. 7. Pyruvate content of the control and imazamox- or glyphosate-treated pea plants. The herbicides were applied to the nutrient solution (a) or sprayed onto the leaves (b). Each value is the mean \pm standard error (n=4). The circles and triangles on top of each box indicate a significant difference between the control and imazamox-treated (circle) or glyphosate-treated (triangle) plants on a given day ($p < 0.05$).

209x296mm (300 x 300 DPI)