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

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Keywords
Imazamox, glyphosate, fermentation, alternative respiratory pathway, acetolactate synthase inhibitor, imidazolinone, 5-enolpyruvylshikimate-3-phosphate synthase
Abbreviations

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

ABSTRACT

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

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

ALS is the first common enzyme in the biosynthesis of the branched-chain amino acids (valine, leucine and isoleucine). ALS inhibitors have become one of the most important herbicide groups because of their wide-spectrum weed control activity, high crop selectivity, low application rates and low mammalian toxicity (Zhou et al. 2007). More than 40 structurally different active ingredients have ALS as their primary target. Imazamox (IMX, 2-[(RS)-4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]-5-methoxymethylnicotinic acid) is one of these active ingredients that belongs to the imidazolinone chemical family. On the other hand, EPSPS is an enzyme in the biosynthesis of aromatic amino acids (tyrosine, phenylalanine and tryptophan) and is the target for the herbicide glyphosate (Steinrucken & Amrhein 1980). GLP (N-(phosphonomethyl) glycine) is a wide-spectrum, non-selective postemergent herbicide that is currently the most popular herbicide. Biotechnology has increased the
importance of GLP in weed management, as GLP-tolerant crops (maize, soybean, cotton, and canola) have been commercialized (Schmid & Amrhein 1999; Tan et al.
2006).

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

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

ALS inhibitors and GLP have been reported to alter the carbon metabolism of plants that are treated through the root system. An accumulation of carbohydrates has been described in both the leaves and roots of treated plants (Zabalza et al. 2004; Orcaray et al. 2012). Carbon consumption is diverted to the low-efficiency fermentative and alternative respiratory pathways in the ABIH-treated roots (Gaston et al. 2002,
This impairment indicates that the effect of these herbicides on the primary metabolism has broader physiological consequences than solely the lack of certain amino acids. However, all of these studies reporting an altered carbon metabolism were performed with herbicides that were applied to the root system. It is unclear if this effect is a consequence of amino acid biosynthesis inhibition or a general stress response of the root system. There have been no exhaustive studies on the effect of sprayed herbicides on these parameters. The way in which the herbicide is applied (sprayed on the leaves or supplied to the roots) may affect the physiological response of the treated plants. Additionally, it is unknown whether the induction of fermentation and alternative respiration in roots following ALS or EPSPS inhibition is related to any change in the pyruvate content. Pyruvate is the substrate of pyruvate decarboxylase (PDC; EC 4.1.1.1), the first enzyme in ethanol fermentation, and it has been reported to have a significant stimulating effect on the activity of alternative oxidase (Millar et al. 1993; Vanlerberghe et al. 1995). Based on these results, we may expect to observe changes in the concentration of this key metabolite in the ABIH-treated plants; however, no in-depth studies have monitored the possible effects of these herbicides on pyruvate content, to the authors’ knowledge.

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

MATERIAL AND METHODS

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 ((Rigaud & Puppo 1974), supplemented with 10 mM KNO₃) and placed in a growth chamber. (Zabalza et al. 2005). Nutrient solution (2.7 l tank⁻¹) was aerated continuously (700 ml tank⁻¹ min⁻¹) and renewed every 3 days. At 12 days of age, the plants were divided into two groups, one to assess the nutrient solution treatments, and the other one to assess the spray treatments to the leaves. In each group, one-third of the plants was not treated and served as the control treatment, and the other two-thirds were treated with GLP or IMX. The experiment was repeated twice, and in each experiment both groups were evaluated.

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

The root and shoot lengths were measured, and gas exchange measurements were conducted at 0, 1, 3 and 7 days after treatment. The leaf and root samples were
taken 4 h after the beginning of the photoperiod at 0, 1, 3 and 7 days after herbicide treatment for analytical determinations. The plant material was immediately frozen in liquid nitrogen and stored at –80 ºC. Some material was dried for 48 hours at 75-80ºC to obtain the fresh weight/dry weight ratio.

Gas exchange measurements

The net CO₂ assimilation rate was measured in intact plants in the youngest fully expanded leaf using a portable ADC-LCi system (ADC BioScientific Ltd., Herts England). The leaf area was determined using an Li-3000 system (Li-Cor, Lincoln, Nebraska, USA).

Carbohydrate determination

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

Pyruvate determination

Ground root samples (0.1 g) were homogenized in 1 M HCl. The extracts were centrifuged at 20,000 g for 30 min. The supernatants were used for pyruvate determination after filtration by two ion exchange cartridges (OnGuard II Ag, OnGuard II H, Dionex Corporation, Sunnyvale, CA, USA). The pyruvate levels were analysed by ion chromatography in a DX-500 system (Dionex Corporation, Sunnyvale, CA, USA) by gradient separation with Dionex Ion pack AG11+AS11 columns (0.2 mM NaOH for
5 min, up to 25 mM NaOH in 10 min, and 35 mM NaOH for 10 min, at a flux of 1 ml min\(^{-1}\).  

Respiration and fermentation measurements  

Respiratory oxygen consumption was measured using Clark-type electrodes (Rank Brothers, Bottisha, UK) in small (5-10 mm) root pieces as previously described (Zabalza et al. 2009). Total respiration was measured as \(\text{O}_2\) uptake in the absence of any inhibitor. To measure the capacities of the cytochrome oxidase and AOX pathways, different inhibitors were directly added to the cuvette. The capacity of cytochrome oxidase was determined as the KCN-sensitive \(\text{O}_2\) uptake in the presence of SHAM, and the capacity of AOX was determined as the SHAM-sensitive \(\text{O}_2\) uptake in the presence of KCN.

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

Statistics  

Each mean value was calculated using samples from different individual plants from both experiments as replicates. Both types of herbicide application were applied in independent groups in the two experiments, and statistical studies were performed for each type of treatment. One-way ANOVA for each day was used to determine the significance of the differences. The means were separated using the least significant difference (\(p < 0.05\)). The significant differences between each treatment and the control plants (untreated plants) are highlighted in the figures with a different symbol.
for each treatment. A previously described transformation to arcsine $\sqrt{x/100}$ was used when the values analysed were percentages.

**RESULTS AND DISCUSSION**

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

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

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

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

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

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

The simultaneous determination of the carbohydrate content in the leaves and the roots facilitates the evaluation of the herbicide effect on phloem transport and indicates a general effect on carbon allocation within the plant for both types of
herbicides and both sites of application. The accumulation of carbohydrates in the roots occurred prior to the accumulation in the leaves, suggesting that sucrose is transported from the leaves to the roots at a higher rate than it is utilized in the sinks. Under these circumstances, the sugar gradient required for long-distance transport is abolished; thus, phloem transport is inhibited, showing that the carbohydrate accumulation in the leaves of the treated plants reflects a reduction in sink strength, as was proposed for the ALS inhibitors (Zabalza et al. 2004).

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

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

induction of AOX and fermentation

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

Total respiratory $O_2$ uptake was not significantly affected by any of the tested treatments (Fig. 5). A transient decrease in the root respiratory rate was only detected
after 3 days of spraying GLP onto the leaves (Fig. 5d). Previous studies also showed that GLP did not affect the respiration rate (Aubert *et al.* 1997; Orcaray *et al.* 2012). However, other ALS inhibitors have been reported to increase the respiratory rate in the short term when supplied to the nutrient solution (Gaston *et al.* 2003; Zabalza *et al.* 2011). Although the total O\textsubscript{2} uptake was not affected, the capacities of the cytochrome and AOX respiration pathways were studied. The capacity of the cytochrome pathway was not affected by ABIH, but the capacity of the AOX pathway increased in the treated plants (Fig. 5c, f). The pattern of increase was similar for both GLP and IMX application methods. While IMX only induced the capacity of the AOX pathway at the end of the study, GLP induced it beginning at day 3. Previous studies have shown an induction of the AOX pathway after ALS inhibition (Aubert *et al.* 1997; Gaston *et al.* 2003; Zulet *et al.* 2015). Although other studies (Zhu *et al.* 2008; Zulet *et al.* 2015) revealed an increased level of AOX after GLP treatment by gene expression profiling, to our knowledge, this is the first study where an increase in the alternative pathway capacity after GLP treatment has been shown by polarographic methods. Although the physiological role of this pathway remains to be clarified, several potential functions of AOX have been identified. AOX can help alleviate the effects of several stresses by avoiding the over-reduction of the electron transfer and helping to reduce the electron flow through the cytochrome oxidase pathway (Siedow & Umbach 1995). Therefore, AOX induction has been related to stresses where the cytochrome pathway is restricted (Vanlerberghe & McIntosh 1997; Parsons *et al.* 1999; Amor *et al.* 2000; Vanlerberghe *et al.* 2002).

The induction of aerobic fermentation in roots is a common effect of ALS and EPSPS inhibitors applied to the roots (Zabalza *et al.* 2005; Orcaray *et al.* 2012; Zulet *et al.* 2015). It remains to be determined if this effect in the roots is also detected when the
ABIH are sprayed onto the foliage. The specific activities of both PDC and ADH were increased following all of the evaluated treatments (Fig. 6). Protein blotting showed that these increases correlated with increases in the amounts of the respective proteins (Fig. 6e).

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

Pyruvate availability has been proposed to be related to the induction of fermentation under aerobic conditions after ALS inhibition. Pyruvate is a common substrate of ALS and PDC; thus, ALS inhibition would involve an increased availability of pyruvate for use by other enzymes such as PDC. Moreover, pyruvate has been described to act as an allosteric activator of AOX. Aubert et al. (1997) tested several metabolites known to be accumulated after ALS inhibition (α-oxobutyrate and α-...
aminobutyrate), but none of them activated AOX. However, pyruvate accumulation after ALS inhibition has not yet been studied in depth, and it remained to be determined whether GLP affects the concentration of this metabolite. Therefore, the pyruvate concentration was measured in the roots of pea plants that were treated with IMX or GLP either in the nutrient solution or sprayed onto the leaves.

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

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

Fermentative induction after GLP treatment was not expected to be related to increased pyruvate content, as this herbicide does not inhibit a pyruvate-consuming enzyme. However, pyruvate was accumulated in the roots after both types of GLP application. The inhibition of the shikimate pathway at the EPSPS level deregulates the carbon flow into the pathway, causing a massive carbon entrance that accumulates in compounds upstream of the EPSPS inhibition point, such as shikimate (Orcaray et al. 2010). It has been proposed that this shikimate accumulation would divert most of the phosphoenolpyruvate from the glycolytic flow to the shikimate pathway because shikimate is a potent phosphoenolpyruvate carboxylase inhibitor (De María et al. 2006). It can be proposed that the pyruvate accumulation after GLP treatment is a cross-physiological effect that is induced by increased availability of phosphoenolpyruvate that is not being consumed by phosphoenolpyruvate carboxylase.
Because pyruvate is the main substrate of ALS, we would expect that the pyruvate concentrations may be increased in plants treated with IMX supplied to the nutrient solution, as has been previously reported in soybean roots (Gaston et al. 2003). Nevertheless, it was only possible to detect pyruvate accumulation when this herbicide was sprayed onto the foliage.

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

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

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

Carbohydrate accumulation in the leaves and roots and fermentation and alternative oxidase induction in roots have been described as common physiological effects of ALS inhibitors and GLP supplied to the nutrient solution. This study is the
first to demonstrate that they are also detected when the herbicides are sprayed onto the leaves.

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

**CONCLUSIONS**

GLP- or IMX-treated plants showed impaired carbon metabolism and provided evidence of similar physiological effects of both types of herbicides. The validity of the results presented has been demonstrated using herbicides supplied to the nutrient solution and sprayed onto the foliage. Carbohydrate accumulation was detected in both the leaves and roots of treated plants. The accumulation in the roots was due to the lack of utilization of available sugars as growth was arrested, which elicited soluble carbohydrate accumulation in the leaves due to a decrease in sink strength. Under aerobic conditions, the ethanol fermentative metabolism was enhanced in the roots of treated plants. This fermentative response was not related to a change in the total respiratory rates or cytochrome respiratory capacity, but an increase in the AOX capacity was observed. Pyruvate accumulation was detected after most of the herbicide
treatments. These results suggest that the induction of the less-efficient ATP-producing pathways of fermentation and alternative respiration by ALS and EPSPS inhibitors might be related to an increase in pyruvate. This plant response was produced in the roots of the treated plants, even when the ABIH were sprayed on the leaves, showing that is a common effect after inhibiting both the biosynthesis of aromatic or branched amino acids by herbicides and is independent of the site of application.

ACKNOWLEDGEMENTS

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


Figure captions

**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 ± 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).

**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 ± 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).

**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 ± 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).

**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 ± 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).

**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 ± 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).

**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 ± 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 µg of protein.

**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 ± 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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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 ± 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).
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 ± 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)
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 ± 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).
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 ± 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 µg of protein.

254x190mm (96 x 96 DPI)
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 ± 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).