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# Alterations induced by imazamox on acetohydroxyacid synthase activity of common bean (*Phaseolus vulgaris*) depend on leaf position

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ABSTRACT

Imazamox is a selective herbicide applied in a wide spectrum of crops which belongs to the imidazolinone family, which mode of action is related to the inhibition of acetohydroxyacid synthase activity. The effect of this herbicide on plant growth, amino acids balance as well as the rate of inhibition of acetohydroxyacid synthase activity in different organs of common bean was studied. Our results indicated that total plant dry weight was not significantly affected two and seven days after herbicide application. Acetohydroxyacid synthase activity decreased in young tissues in response to imazamox treatments, whereas in mature organs the herbicide reversed the natural decrease of activity linked to ripeness. In shoot apical meristem and fully expanded leaves isoleucine content increased, while valine and leucine decreased less than expected considering the decline of acetohydroxyacid synthase activity, at less in young tissues. The results suggest that the herbicide is transported primarily to the growing areas where exerts its action by inhibiting the acetohydroxyacid synthase. Results found here allow to progress in the knowledge about legumes responses to imazamox herbicide.



## 1. Introduction

Legumes, the second most widespread crop in world agriculture after cereals, represent an important source of protein for animal and human diet [1]. They establish symbiosis with soil *Rhizobium* which allows them to be largely independent of nitrogen external fertilizers [2]. In addition, legumes are able to tolerate and adapt to different abiotic stresses [3,4] and have high potential in phytoremediation processes and to recover marginal soils [5]. Therefore, they are used to immobilize heavy metals [6,7], and to degrade organic pollutants, such as oil [8], polychlorinated biphenyls [9] or pesticides like triazines, phenylureas, carbamates and pyrethroids [10]. In particular *Phaseolus vulgaris* has been shown to have potential to extract heavy metals from soil [11] and water [12].

Imazamox (2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-(methoxymethyl)-3-pyridinecarboxylic acid) is a selective herbicide against annual and perennial grasses and broad-leafed weeds applied either pre- or post-emergence in a wide spectrum of crops [13]. This herbicide, which belongs to the chemical family of the imidazolinones, is relatively persistent in soil with half-life ranging from 20 to 90 days depending on initial concentration, temperature, soil moisture and soil type [14]. Its presence in soil often resulted in carryover effects on sensitive rotational crops [15,16] and provokes a decrease in the microbial biomass content up to 25% [14]. Moreover imazamox shows high potential for leaching, which can cause problems of aquifer contamination [17].

The primary mode of action of imazamox involves the inhibition of acetohydroxyacid synthase activity (AHAS, EC 4.1.3.18), also referred to acetolactate synthase. This enzyme catalyzes the first common step in the biosynthetic pathway leading to the essentials branched-chain amino acids (BCAA) valine, leucine and isoleucine. The exact consequences of AHAS inhibition by imidazolinones in sensitive plants, as well as the processes that eventually lead to plant death, remain unclear. One of the first effects is growth arrest and the meristematic regions of the plant are targeted with symptoms of foliar chlorosis and necrosis [18]. Also changes in the free amino acids pool and total protein level have been reported [19] as well as variations of AHAS activity among different parts of plants and in different growth stages [20–22]. Precise consequences of AHAS inhibition may vary across plant species or families, and the knowledge about the complex regulation of this pathway in plants is far from complete [23].

The aim of the present work was to study imazamox effects on AHAS activity in different common bean tissues and its relationship with changes in the free amino acids pool. To our knowledge, no quantitative or qualitative studies about distribution of BCAA in different organs of common bean in response to imazamox treatment have been reported before.

*Abbreviations:* AHAS, acetohydroxyacid synthase; BCAA, branched-chain amino acids; PVPP, polyvinyl-polypyrrolidone.

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## 2. Materials and methods

### 2.1. Plant material and growth conditions

Seeds of common bean (*P. vulgaris* cv. Coco Blanc) were surface sterilized by immersion in 5% NaClO for 3 min, rinsed three times with sterile water, and germinated in moist sterile vermiculite at 28 °C for 48 h. The young seedlings were sown in a modified Leonard jars [24] with nutrient solution containing: 10 mM $NaNO_3$, 2 mM $NaH_2PO_4$, 5 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 2 mM $Na_2SO_4$, 2 μM $MnCl_2$, 0.75 μM $ZnCl_2$, 0.25 μM $CuCl_2$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 5 μM Fe-EDDHA and 50 μM $H_3BO_3$. Plants were grown in a controlled environmental chamber with a 16/8 h light–dark cycle, 23/18 °C day-night temperature, relative humidity 55/65% and photosynthetic photon flux density (400–700 nm) of 450 μmol $m^{-2}$ $s^{-1}$ supplied by combined fluorescent and incandescent lamps.

### 2.2. Treatments and harvest

When plants were 21 days old (vegetative growth) they were treated with two imazamox concentrations (100 and 250 μM) added to the growth medium. This herbicide is marketed by BASF by the trade name Pulsar 40 and contains an imazamox concentration of 40 g $l^{-1}$ (BASF The Chemical Company, Spain). Fresh nutrient solution replaced the old one every three days, and the pH was kept at 5.8 ± 0.1. Plants were harvested at 0, 2 and 7 days after herbicide treatments. Six plants were included per treatment and harvest. Samples of the shoot apical meristem and leaves (separated in expanding leaf and leaf full expanded) were pooled and stored at −80 °C for the enzyme assays and analytical determinations. The fresh weight of roots, stems, and leaves were recorded, and then all organs were dried at 70 °C for 48 h and weighted again.

### 2.3. AHAS extraction and quantification

Leaves, cut into small pieces, were ground in a mortar (0.4 g 3 $ml^{-1}$ buffer) with 33% (w/w) polyvinyl-polypyrrolidone (PVPP) and 50 mM K-phosphate buffer (pH 7) containing 5 mM sodium pyruvate, 5 mM $MgCl_2$, 5 mM EDTA, 100 mM FAD, 0.5 mM thiamine pyrophosphate and 10% glycerol (v/v). The homogenate was centrifuged at 25,000*g* for 20 min at 4 °C and the supernatant was used to determine the AHAS activity (EC 2.2.1.6) according to the method proposed by Singh et al. [25] based on the decarboxylation of acetolactate to acetoin. For this purpose, 0.3 ml of 50 mM phosphate K buffer (pH 7) containing 10 mM $MgCl_2$, 100 mM sodium pyruvate, 10 pM FAD and 1 mM thiamine pyrophosphate were added to 0.2 ml of enzyme extract and incubated at 37 °C for 60 min, then the enzymatic reaction was stopped by adding 50 μl of 3 M $H_2SO_4$. The new mixture was incubated at 60 °C for 15 min so that the acetolactate is decarboxylated to acetoin. 0.5 ml 0.6% creatine in water and 0.5 ml of 6% α-naphthol in 2.5 M NaOH were added to each tube and incubated at 37 °C for 30 min. Finally tubes were centrifuged at 5000*g* for 10 min and the amount of acetoin in the supernatant was determined spectrophotometrically at 525 nm [26], using as standard an acetoin standard curve (2–20 mg $ml^{-1}$). Also, protein was measured by the method of Lowry et al. [27] as modified by Markwell et al. [28] using the Folin-Cicolteau phenol reagent. The activity was expressed as μg of acetolactate $mg^{-1}$ prot. $h^{-1}$.

### 2.4. Free amino acid determination

Free amino acids were extracted by the method of Irigoyen et al. [29], then the organic phase was dried using an SPD 111 V speedvac concentrator (Thermo FisherScientific, USA). Amino Acids were derivatizated with Waters® AccQ-Tag™ Reagent Kit, according to the manufacturer's protocol. Subsequently, an aliquot of the reaction mixture was used for HPLC injection and the Waters AccQ-Tag method was applied. Chromatographic separation was carried out in a Waters AccQ-Tag amino acid analysis Nova-Pak™ column (3.9 mm × 150 mm, 4 μm) fitted with a Nova-Pak™ $C_{18}$ Sentry™. The column was thermostated at 37 °C, the flow rate was 1.0 ml $min^{-1}$ and the injection volume was 5 μl. Mobile phase A consisted of AccQ-Tag eluent A (100 ml AccQ-Tag A concentrate + 1 l Milli-Q water) and mobile phases B and C were acetonitrile and Milli-Q water, respectively. Derivatized amino acids were detected in a 470 scanning fluorescence detector (Waters) set for an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Total free amino acid content was calculated adding values of each amino acid result of the HPLC analysis. This data was used as reference to estimate the percentage of branched-chain amino acids.

### 2.5. Statistical analysis

The experimental layout was a randomized complete block design. The growth values were means of six replicates per treatment. Four replicates were performed for the enzyme activity assay, and three replicates for free amino acids content. The standard error (SE) was estimated and all results were subjected to a multifactor analysis of variance (Fisher's test) with a least significant difference (LSD) test between means ($P \leqslant 0.05$). The software used for the statistical analysis was SPSS 16.0 (SPSS Inc., Chicago, USA, 2007).

## 3. Results and discussion

The effect of 100 and 250 mM imazamox treatments on the growth of *P. vulgaris* was followed at 2 and 7 days after herbicide treatment. Total plant dry weight did not significantly change between treatments (Fig. 1); however in herbicide-treated plants were observed initial symptoms of deterioration, such as chlorosis and apical necrosis of some leaves. These negative effects have been described as typical primary symptoms of plants treated with sulfonylureas and imidazolinones herbicides [18]. Other experiments performed with common bean and imazamox showed a decrease in plant biomass with longer treatment (data not shown).

The acetohydroxyacid synthase activity was measured in shoot apical meristems and leaves of different age throughout the exper-

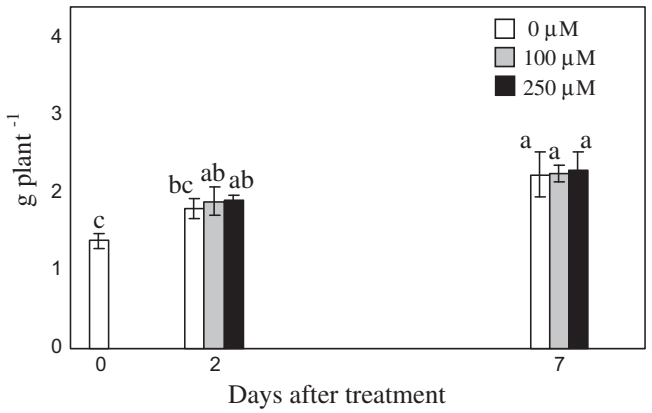


**Fig. 1.** Total plant dry weight of common bean treated with different concentrations of imazamox (0, 100 and 250 μM) in the nutrient solution. Results (±S.E.) are the mean of six values. Means followed by the same letter do not differ ($P \leqslant 0.05$) using the LSD test.